The risk of Lyme borreliosis infection following tick bite in Pristina region, Kosovo

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DISSERTATION

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This dissertation was completed at the Clinic of Infectious Diseases, University Clinical Center of Kosovo, and the University of Zagreb School of Medicine.

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Dedidication

To my father, who would be very happy and proud.

To my beloved mother and sisters.

To my lovely husband Ardian and my two children Adris and Bena.

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List of abbreviations

a.s.l.	Above sea level
ACA	Acrodermatitis chronica atrophicans
Bb	*Borrelia burgdorferi*
bid	Two times a day
BL	Borrelial lymphocytoma
BSK medium	Barbour-Stoenner-Kelly medium
CCHF	Crime-Congo hemorrhagic fever
CDC	Centers for Disease Control and Prevention
CI	Confidential interval
CMV	Cytomegalovirus
CNS	Central nervous system
CRASPs	Complement regulator-acquiring surface proteins
CSF	Cerebrospinal fluid
DbpA and DbpB	Decorin-binding proteins (A and B)
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
ECG	Electrocardiography
ELISA	Enzyme-linked immunosorbent assay
EM	Erythema migrans
ESCMID	European Society for Clinical Microbiology and Infectious Diseases
EUCALB	European Union Concerted Action on Lyme Borreliosis
I.ricinus	Ixodes ricinus
IDSA	Infectious Diseases Society of America
IFA	Immunofluorescence assay
IFNγ	Interferon gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M
kDa	Kilodalton
LB	Lyme borreliosis
LBC	Lymphadenosis benigna cutis
LNB	Lyme neuroborreliosis

LPS	Lipopolysaccharide
LUAT	Lyme urine antigen test
m	Meter
mg	Milligram
mRNA	Messenger ribonucleic acid
MTTT	Modified two-tiered test
NB	Neuroborreliosis
Osp	Outer surface proteins
PCR	Polymerase chain reaction
PN	Persistent negative
PO	Per os/oral
PR	PR interval ECG
Salp 15	Salivary gland protein
SD	Standard deviation
STTT	Standard two-tiered test
tid	Three times a day
UK	United Kingdom
USA	United States of America
VlsE	Variable major protein-like sequence E
WB	Western-blot
WCS	Whole-cell sonicate
WHO	World Health Organization

1. Introduction

Arthropod-borne diseases are increasingly a public health concern because of the range expansions of both vectors and pathogens (1–3). Lyme borreliosis (LB) is the most common tick-borne human disease in temperate regions in the northern hemisphere (4–9).

LB was first described as a new entity in the USA in the late 1970s, but the majority of its individual manifestations had been documented many decades earlier in Europe (8,10–12). *Borrelia burgdorferi* (Bb) was discovered by the Swiss American scientist Willy Burgdorfer and co-workers at the Rocky Mountain Laboratories, Hamilton, USA, in 1982 (8,13–15).

The disease is transmitted by ticks belonging to the Ixodidae family in Europe by *Ixodes ricinus* (3–5,8,11,16), in the United States, primarily by *Ixodes scapularis*—the deer tick (3). Reservoirs of infection comprise many wild and domestic animals (10,16,17). Ticks of the *Ixodes ricinus* complex have larval, nymphal, and adult stages, and they require a blood meal at each stage (3). Ixodes ticks need up to 17-48 hours to transmit Bb to humans and animals (2,13,18,19).

LB is caused by the spirochete *Borrelia burgdorferi* sensu lato and is a potentially chronic multisystem disease (1,2,4,8,20–23). At least five species of *Borrelia burgdorferi – Borrelia burgdorferi* sensu stricto (*B. burgdorferi* in the strict sense), *Borrelia garinii*, *Borrelia afzelii*, *Borrelia bavariensis* and *Borrelia spielmani* – are pathogenic for humans in Europe (2,5,10,11,22). *B. burgdorferi*, *B. garinii* and *B. afzelii* are the main important etiologic agents in Eurasian LB, whereas in the United States *B. burgdorferi* sensu stricto is the main etiologic agent of LB (2,4,8,11,18,21,24,25).

Bb can attack various organs/systems such as skin, joints, nervous system, heart, and eyes (3,4,8,10,11,13,23,26,27). LB clinical manifestations can vary from asymptomatic (16,19,25,28–30) through mildly symptomatic to severe forms with significant organ injury (11), resulting even in death in severe Lyme carditis cases (31). Erythema migrans (EM), which appears early in the course of the disease, is the most common clinical manifestation (2–5,8,10,32). Early disseminated infection is usually manifested by neurological or musculoskeletal symptoms, and late disseminated infection in untreated patients is manifested as acrodermatitis chronica atrophicans (ACA), chronic neuroborreliosis (NB), or Lyme arthritis (2–5,8,32–34).

Serology is the most common laboratory aid in diagnosis. All stages of the disease can usually be treatable by appropriate antibiotic therapy (35,35–37).

The spread of LB depends on geographical, environmental, and climate factors (18,21,23,38). There are 65,000-85,000 estimated annual LB cases in Europe (1). The highest incidences of LB have been reported from Austria, Germany, Slovenia, and Sweden (5,8,11,21,25,28).

Kosovo is a small country (10887 km2) in South-Eastern Europe, with a population of approximately 1.8 million. Tick-borne diseases present a serious threat to human health in South-Eastern Europe, including Kosovo. Crimean–Congo hemorrhagic fever (CCHF) is a well-known emerging and life-threatening disease in this area (39,40). However, little is known about the prevalence and clinical significance of LB in Kosovo. To our knowledge, very few cases have been recognized, and no clinical and seroprevalence studies have been published yet. On the other side, the presence of Ixodidae ticks, which are the main vectors of Lyme borreliosis in Europe, has been confirmed in Kosovo's natural environment as well (40). Those facts encouraged us to perform a prospective study with the main objective to define the risk of developing Lyme borreliosis after a tick bite in Kosovo.

1.1. History of Lyme disease

A condition now known as ‘Lyme disease’ (or Lyme borreliosis), introduced in medical literature after Steere and coworkers from Yale University in the late 1970s investigated an arthritis epidemic among young children who were thought to have juvenile rheumatoid arthritis in the community of Old Lyme, Connecticut, USA, has a very long European history (3,41–43).

The first documented case of Lyme disease was found by polymerase chain reaction (PCR) of DNA from a 5300-year-old deceased human recovered from a frozen glacier in the Italian Alps (36,44).

The cutaneous manifestations, which are the most common forms of the disease, had already been described at the end of the 19th century and the beginning of the 20th century by physicians like Alfred Buchwald, who described a chronic skin disorder that was later named acrodermatitis chronica atrophicans (ACA) by Herxheimer and Hartmann (36,42,43,45).

In 1910, Arvid Afzelius presented a Swedish patient with a slowly expanding skin lesion, which he named erythema migrans (EM), suggesting a possible connection of skin lesion with previous tick bites (36). A few years later, Austrian dermatologist Benjamin Lipschütz observed a similar lesion in a 29-year-old woman, which migrated over months from the knee to the back and the neck. Lipschütz speculated on infection possibly transmitted by the tick bite and suggested microscopic/bacteriologic investigations of the intestinal tract and the salivary gland

secretions of the tick. However, at that time, no one followed his suggestions (43). Another skin manifestation resembling that of today's borrelial lymphocytoma was first described in 1911 by Burckhardt. Extended description and definition of solitary lymphocytoma benigna cutis were given by Bäferstedt, based on about 150 cases (43,45,46).

Two French physicians, Garin and Bujadoux, reported the first case of neuroborreliosis (36,43). In 1922, they reported a patient who developed erythema chronicum migrans followed by painful meningoradiculitis. The patient was bitten by a tick shortly before the symptoms began, and he had a positive Bordet-Wasserman test, which was used at that time for diagnosing syphilis. However, they agreed that although the test was positive for syphilis, this patient had another disease that was probably tick-borne caused by a spirochete different from *Treponema pallidum*, and that can cause both cutaneous and neurological symptoms (42). A relationship between tick bite, EM, and disorders of the nervous system was first considered by Hellerström (1930). Bannwarth from Munich analyzed several patients with chronic lymphocytic meningitis accompanied by the clinical syndrome of neuralgia (intense radicular pain) and cranial neuritis (facial palsy), thought that the conditions were due to a rheumatic, allergic cause, missing the link with a tick bite. Despite this, Bannwarth's name is now synonymous with meningo-polyradiculoneuritis, the typical clinical manifestation of neuroborreliosis (43,45,46).

It was not until the early 1980s when Willy Burgdorfer - a medical entomologist working at the Rocky Mountain Laboratories in Hamilton, Montana - isolated and cultivated the causative agent *B. burgdoreferi*, a bacterium belonging to the family of Spirochaetaceae, from the midgut of Ixodes ticks, sent to him from Shelter Island, New York - a place known to have endemic LB (36,37,47). Unable to find the Rocky Mountain spotted fever agent in *Dermacentor variabilis* ticks, the researchers investigated other ticks such as *I. scapularis* (dammini), which were there in abundance and that might carry the agent. No rickettsiae were found in the midguts of many dissected ticks, yet poorly stained and relatively long, irregularly coiled spirochaetes were observed (35,46). The bacteria were isolated and cultivated in a modified Kelly's medium, which is now known as Barbour-Stoenner Kelly (BSK) medium, and the organisms were linked with LB after they were observed to react with sera of LB patients in immunofluorescence assays (35,43,45).

Soon after the successful isolation of a new borrelia from hard ticks and LB patients in the USA, researchers in Europe detected the organism in human clinical specimens, such as blood, skin, and cerebrospinal fluid of few Lyme disease patients in Sweden, Austria, Switzerland, and

Germany. Using the methods available at that time, the spirochaetes appeared to be the same as the US isolates (42,43).

1.2. Vector of *B. burgdorferi*

The vectors of *B. burgdorferi s.l.* are hard ticks of the genus Ixodes (ixodid tick species) that are part of the *Ixodes ricinus*/*persulcatus* complex. The occurrence of Lyme borreliosis globally is closely tied with the geographic distribution of ticks belonging to this complex what is the main reason why the disease is mainly registered in the northern hemisphere (8,36,48,49). There are no confirmed cases of LB in the southern hemisphere apart from cases imported from Europe. However, *B. garinii* DNA has been amplified from the seabird tick *Ixodes uriae* found in the southern hemisphere (50).

The primary vector throughout the west and central Europe is the sheep tick *Ixodes ricinus*, while in Asia, the main vector is the taiga tick, *Ixodes persulcatus*. In Northeastern and Midwestern United States, the deer tick, *Ixodes scapularis* (*Ixodes dammini*) is the vector, and *Ixodes pacificus* is the vector on the West Coast and Canada (8,36,48,51).

Some other European tick species, such as the hedgehog tick, *Ixodes hexagonus*, even thought to be a vector competent for *B. burgdorferi,* only contribute to the natural circulation of the spirochaetes and is rarely if ever involved in transmission to humans (49,52,53).

Vector competence for *B. burgdorferi s.l.* indicates that a tick species must feed on infected vertebrates. It must also be able to receive the pathogen during the blood meal, be involved in the pathogen's natural maintenance cycle by maintaining it through one or more life stages (transstadial passage), and transmit a given pathogen to other hosts when feeding again. Otherwise, it is a non-vector tick (49). Vector competence for *B. burgdorferi s.l.* has been experimentally confirmed for 12 tick species, including six of the 14 members of the *I.ricinus* complex (*I. affinis, I. jellisoni, I. pacificus, I. persulcatus, I. ricinus, I. scapularis*) and six other *Ixodes* spp. (*I. angustus, I. dentatus, I. hexagonus, I. minor, I. muris, I. spinipalpis*) (8,49).

1.2.1. Biology of *Ixodes ricinus*

Ixodes ricinus has an extensive geographical distribution throughout Europe (52,54). *I. ricinus* survives in biotopes that offer a relatively high humidity of 80% and more (52,53) when atmospheric water vapor can be actively taken up by the tick through the secretion and then ingestion of hygroscopic fluids so that they can avoid fatal desiccation of the free-living stage (51,54,55). In continental Europe, *I. ricinus* is mainly present in sheltered woodlands with

deciduous forests with moist soil and shrubs, areas with high rainfall, and with good vegetation cover (49,52,53).

The duration of the life cycle depends not only on climatic factors but also on seasonal questing time, host density, and availability (48,54). The length of a tick's life cycle varies between 2 and 6 years (36,48,51–53).

All ixodid ticks, *I. pacificus*, *I. persulcatus*, *I. ricinus*, and *I. scapularis* undergo four developmental stages: egg, larva, nymph, and adult (36,49,52). Ixodes ticks have three life stages that require blood meal: larvae, nymphs, and adults feeding once during every developmental stage (36,52). At any stage, ticks can be infected with *B. burgdorferi* (3,48,49). Each of these three motile life stages of hard ticks must get on a host, feed (this lasts for several consecutive days), fall off, and then transform into the next stage (36). If no blood-providing host is available, the ticks will perish (47).

Adult male *I. ricinus* and *I. persulcatus* are considered facultative blood feeders and usually do not require a blood meal to fertilize females. However, males may repeatedly take sporadic small blood meals and can remain on a host for weeks to months in search of females. While in the active developmental stages of larva, nymph, and adult female each take a single large blood meal. Each blood meal is followed by the development phase, hence after feeding for a few days (about 3 days for larvae, 5 for nymphs, and 7 days for adult females), the ticks drop off their host and locate on or near the soil surface and take several months to develop into their next developmental stage, except for the females that will lay eggs (about 2000 eggs) after their blood meal and then die (37,48,49,54). *Borrelia burgdorferi s.l.* is taken up with the blood when the tick, usually a larva or a nymph, feeds on an infected host. The spirochaete locates in the midgut of the tick and is transmitted by the next tick stage when it feeds on another vertebrate host, which can include humans (36). The adult female is usually a dead-end for *B. burgdorferi s.l.* because transovarial transmission is rare (37,49,54,55).

1.2.2. Vector and animal hosts

LB is categorized as a zoonotic disease in humans because the lifestyle of *B. burgdorferi s.l.* is obligate parasitic, with no free-living style stages known, and the infection with *B. burgdorferi s.l.* is maintained in nature by animals (a large variety of mammalian and avian hosts) and hard ticks as a vector; humans are infected accidentally (43).

LB can be considered as a class III-zoonosis, where transmission occurs between one tick vector, *I. ricinus*, and multiple vertebrate reservoir hosts. Whereas clinical manifestation

appears only in dead-end hosts, such as humans who are not involved in the transmission cycle of *B. burgdorferi s.l.* in nature (55).

The animal reservoirs of *B. burgdorferi s.l.* are numerous, and the majority are small wild mammals and birds (43,52,53). More than 300 vertebrate species are potential hosts for *I. ricinus* (54). Different host species vary in their ability to acquire *B. burgdorferi s.l.* from infected ticks (52). There must be an encounter with the infected tick, take up a critical number of infectious agents during tick bite, permit the multiplying of the agent and develop a prolonged or even persistent spirochaete infection or to survive for some time in at least certain parts of its body; to serve as hosts for the vector's life stages and to subsequently transmit it to other, uninfected ticks, that is called reservoir competence (49,52,55).

The maintenance of *B. burgdorferi s.l.* depends on small and medium-sized mammals, birds, and reptiles, acquiring the infection from nymphs that are active in spring and subsequently transmitting it to larvae that are active in the summer of the same year, so that reservoir hosts are first infected by nymphs and then transmit to larvae (8,52,54,55).

The abundance of reservoir hosts in a particular habitat is the most important and determining factor of infected tick populations (53). Important competent reservoirs of *B. burgdorferi s.l.* in Europe are rodents such as Apodemus mice and voles, which usually show a critical host density in wooded areas, their infestation by larval ticks is high, and their infectivity to feeding ticks is high as well with sustaining borrelia infection over winter months and even as long as a rodent lifetime; insectivores such as hedgehogs that in an urban or suburban area can be more efficient reservoirs along with rats (*Rattus rattus*, *Rattus norvegicus*); hares which appear to more efficiently transmit *Borrelia* to ticks than rabbits do; and several bird species like passerine (blackbirds) and game birds (pheasants), including migratory birds (51–53).

Larger rodents like squirrels and dormice are also involved as reservoirs of *Borrelia* (53).

The most thought-provoking point about the ecology of LB in Europe is the specific host associations of *B. burgdorferi s.l.* In continental Europe, *B. afzelii* is associated with small rodents (mainly Apodemus mice, voles, rats, and shrews), *B. burgdorferi s.s.* is associated with squirrels, while *B. garinii* and *B. valaisiana* with birds. Whereas, *B. spielmanii* was shown to be maintained by the garden dormice (*E. quercinus*), and the reservoir hosts of *B. lusitaniae* have been identified to be lizards (49,53).

These animals do not seem to show signs of disease as a result of infection with *B. burgdorferi s.l.* Anti-borrelial antibodies have been detected in a wide range of domestic animal species, and most data on LB in domestic animals relate to dogs and horses, with isolated reports of

infection in cattle, sheep, and cats. They may show similar manifestations to those that occur in humans (43). However, most infections are probably subclinical and self-limiting, there are no pathognomic symptoms such as EM, and there are difficulties in laboratory diagnosis because of little quality assurance applied for animal LB (43).

The persistence of the borreliae in endemic areas requires the involvement of reservoir hosts. Among these hosts, some act as blood meal sources and as reservoir hosts for pathogens, others as blood meal sources only (54,56). If immature ticks feed on hosts that are refractory to infection with the spirochete, the prevalence of the infection in an area will decrease. However, if there are plentiful hosts that could be efficiently infected with *B. burgdorferi* establishing persistent spirochetemia, the prevalence of the infection will increase (47,56).

Systemic infection of vertebrates, followed by a relatively long duration of infectivity for ticks, is the main route of transmission of *B. burgdorferi* s.l. from reservoir hosts to ticks (52,55).

The encounter between ticks and their hosts, including humans, is based mainly on the active part of the host where the tick is immobile on vegetation waiting for a host that is moving (52,54).

To find a host, *I. ricinus* climbs onto low vegetation and stays immobile at the tip of the vegetation where it quests for a host using the "lie in wait for" strategy and rarely moves more than a few meters while questing (49,52,54). Hosts are detected by vibrations caused by animal movements, odors, body heat, and shadows (49,54). When hosts pass close, ticks respond to mechanical and chemical stimuli produced by hosts, including humans, and grab them (49,54). The feeding duration and success rate depend on host species, attachment sites, and host immune response (49,52). During questing periods, *I. ricinus* often experiences desiccating conditions. The active questing is interrupted by periods when ticks seek out humid microhabitats to regain lost body water, implying that they do not have unlimited time to find their hosts.

Moreover, their survival is limited by the amount of energy they gain from blood meals and their ability to maintain water content in a desiccating atmosphere (52,53). Once the encounter has taken place, the tick will move on its host to look for an adequate place to attach to the skin. The duration of attachment varies from 3 to 10 days, depending on the developmental stage (52). Before the blood meal, in unfed ticks, spirochetes are located in the tick midgut. When unfed *I. ricinus* attaches to a vertebrate host, *Borrelia* transmission does not occur at the beginning of the blood uptake. However, later on, the uptake of blood seems to trigger spirochetes to migrate from the tick midgut to the salivary glands (49,55,57).

The importance of different tick stages varies as a bridging vector that depends on how commonly they attach to humans, how frequently they are infected with *spirochaetes* and how likely they are to be detected and removed before spirochaetes are transmitted. The nymphal stage is considered the primary bridging vector for *I. scapularis* in the eastern USA and *I. ricinus* in Europe, whereas in the larval stage, they are not aggressive human biters. Additionally, the prevalence of infection with *B. burgdorferi* is usually low in unfed larvae (36,49,53).

1.2.3. Duration of Tick Attachment (Transmission Timing)

One of the frequently asked questions is: What is the minimum feeding period required for an infected tick to transmit borreliae that eventually cause disease in humans? There is a difference between North America and Europe in the duration of tick attachment required for transmission of borreliae into the human host. Most North American epidemiological studies show that transmission of borreliae to humans can occur at least 48 hours after attachment because borreliae are present in the midgut of infected unfed ticks, and they migrate to the salivary gland after 24-48hours (53). In Europe, however, it has been shown that the risk of transmission is high even in situations when tick attachment lasts less than 24 hours (18,49,53,54).

1.3. Epidemiology of Lyme Borreliosis

According to the World Health Organization estimates, the vector-borne disease accounts for 17% of the human global infectious disease burden (58,59).

Lyme borreliosis now represents the most common tick-borne human disease occurring in the northern hemisphere (60). LB occurs widely through North America, Europe, and Asia (3).

The worldwide geographical distribution of LB is related to the known distribution of the ixodid vectors (46). LB is widely distributed throughout Europe, matching with the distribution of *Ixodes ricinus* and *Ixodes persulcatus* ticks (46). The distribution of *I. ricinus* overlaps with the distribution of *I. persulcatus* in the coastal regions east of the Baltic Sea and further south along that longitude into middle Europe, from where the range of I. persulcatus extends to the Pacific Ocean (46,49,52).

LB in North America occurs in the temperate climate zone. Although the North American vectors, black-legged ticks of the genus Ixodes, are widely distributed, only a few regions are considered endemic for LB (46). The disease occurs from the Mexican border in the south to the southern Canadian provinces in the north (3,35,60). Parts of North Africa and Northern Asia

(Russian Siberia and the Far East, Sakhalin, Japan, China, and Korea) are endemic areas, as well (3,35,49,60).

The disease in Europe is reported from Austria, Slovenia, Sweden, Germany, Czech Republic, Bulgaria, Switzerland, France, Slovakia, Belgium, Denmark, Croatia, Hungary, Italy, Luxemburg, Netherlands, Baltic States, and the United Kingdom (3,49,52,60,61).

A national surveillance case definition was adopted in the USA in 1990 to establish a uniform and specific diagnostic criteria for surveillance and was revised most recently in 2011 (8,12,18,60,62).

CDC Lyme Disease (*Borrelia burgdorferi*) 2017 Case Definition defines a confirmed case as either: 1. a person with erythema migrans (EM) with possible exposure to tick habitat in an area where LB is endemic or who has laboratory evidence of infection and a known exposure in a low incidence state, or, 2. a person with at least one other defined clinical manifestation of LB that has laboratory evidence of infection (47). There are heterogeneous surveillance systems within Europe that complicate the direct comparison of the incidence and trends between countries. Some other issues like under- or over-reporting, differences in case definitions, diagnostic obstacles, and different laboratory methods, all might have an influence on reported data (52,60,63). Despite these conditions, it appears that both disease incidence and antibody prevalence are higher in the central and eastern parts of Europe than in the western parts. Disease incidence increases from west to east and decreases from south to north in Scandinavia and from north to south in Italy, Spain, Portugal, and Greece (46,64).

The highest incidences of LB in Europe are found in Austria, Czech Republic, Germany, Slovenia, the Baltic States, Sweden, and Central Europe (52).

However, the incidence of LB has been increasing across the globe, with the number of reported cases in Europe rising from the early 1990s (e.g., the Czech Republic, Estonia, Lithuania). Such an increase is related not just to improved awareness and diagnostics, increased tick density, and burden of tick disease, but also climate changes that contribute to the broadening of ticks' areas (46,52,65). The Fourth Assessment Report of the Inter-Governmental Panel on Climate Change reports that in northern temperate Europe, temperature increases of 1.5–2.5°C – that may occur over the next few decades as a result of global warming – can affect the tick survival, development, and reproduction of vector *I. ricinus* as the main vector of *B. burgdorferi* in Europe. Moreover, the effects on the numbers, migration patterns, and diversity of hosts will influence the tick abundance and distribution (52,53).

Seasonal climatic conditions affect the latitude and altitude distribution of ticks in Europe. During recent years, studies from the Czech Republic and Sweden show changes in vector abundance, as well as changes in latitudinal or altitudinal distribution of ticks (during the same time period), and ticks are now found in abundance up to 1100m above sea level (a.s.l.) in the Czech Republic and Austria, up to 1300m a.s.l. in the Italian Alps and up to latitude 65°N in Sweden (52,53).

Three principal factors govern the epidemiology of LB in humans: the tick population density, the prevalence of infection with Bb among ticks, and human professional or recreational frequentation of the forest environment - degree of human-tick contact (8,12,18,52,60,62).

The prevalence of Borrelia in ticks in Europe is 13.7%, whereas the prevalence is higher in adults (18.6%) than in nymphs (10.1%) (60).

The risk of human LB infection in a specific area depends both on the number of infective ticks in active search for a blood meal and on factors influencing human exposure to ticks. Weather factors influence human recreational behavior with potential human exposure to infected tick bites in the warmer parts of the year. At the highest risk are people residing or working in endemic areas for LB (52,60).

All activities that increase human contact with ticks represent risks, particularly: recreational (leisure time) activities in forested areas, such as camping, picnicking, walking and hiking, sitting on leaves, berry and mushroom picking, clearing of bushes around the home, and gardening (52,60).

Outdoor employment and work (forestry workers, military personnel in the field, farmers, gardeners, gamekeepers, hunters, and rangers) are also at risk (60).

The risk of developing LB symptoms after a tick bite is estimated to be 2–4%. In some parts of Europe, it is even less than 1% (60,65).

1.4. Etiology of Lyme Borreliosis

The genus Borrelia is a member of the spirochaete phylum (Borrelia species), and along with Leptospira and Treponema are spirochetes (3,36,37,48,66). There are two distinct groups of the Borrelia genus: the relapsing fever Borrelia group and the *Borrelia burgdorferi sensu lato* complex that are characterized by a strict parasitic way of life and a biphasic cycle involving arthropod vectors and vertebrate hosts (35,36,57,66).

Borreliae are thin, motile, sensitive, microaerophilic bacterium, which grow best at 33˚C in a complex liquid medium called BSK (Barbour-Stoenner-Kelly). *B. burgdorferi* is a gram-

negative bacteria, and among *Borrelia* spp., it is the longest (10 to 30 µm in length) and thinnest (0.2 to 0.5 µm in width). Moreover, it presents a characteristic morphology, with inner and outer membranes surrounding periplasmic flagella (7-11 bipolar flagella) and a flexible cell wall. The flexible outer membrane does not contain lipopolysaccharide (LPS) but is rich in lipoproteins, the highly immunogenic outer-surface proteins (Osp) (8,36,48). Unlike other bacterial flagella, those of the spirochaetes are endoflagella, located in the periplasmic space where they are attached to the poles and wrapped around the cell cylinder giving the bacterium its characteristic flat wave shape and motility (37,50).

B. burgdorferi was the first spirochete in which the complete genome was sequenced (35,36,48,50,67).

There are sequences for more than 160 known and predicted membrane lipoproteins of the *B. burgdorferi* genome, including the plasmid-encoded outer surface proteins (Osp) that become expressed during different stages of its life cycle (8,36). Because *B. burgdorferi* has unusually large numbers of lipoproteins, many scientists focused on investigating the function of these lipoproteins. The best-studied Osps are OspA, followed by OspB, C, D, E, F, pG, VlsE, p66, and a few others (3,61,68). No genes for recognizable toxins have been identified (8,36). The only virulence factors of *B. burgdorferi* are surface-exposed lipoproteins that allow the spirochete to attach to mammalian cells (3,36,37,48,50,55,61).

The complete DNA sequence of 22 strains of borrelia is known (36). The *B. burgdorferi s.l.* genome is approximately 1.5 x 10^6 base pairs and is quite unusual for a bacterium since it includes several replicons (15-21 replications), a single small linear chromosome (of approximately 1 Mb), and 21 linear and circular plasmids (ranging in size from 5 to 56 kb) in which relevant Osps are encoded, together containing 1780 genes (35,36,50). The linear chromosome is (entirely) stable and presents a clonal evolution by genetic drift. The plasmids represent almost 40% of the genome. Most of the genes coding outer membrane proteins or virulence factors are variable and plasmid-encoded (e.g., OspC, dbpAB genes). Furthermore, they take part in the adaption of borrelia with either tick or host reservoirs and are those that play a role in the process of binding with host or tick ligands helping in pathogen survival and transmission and probably associated with pathogenicity in humans too (36,37,48,50,55,66).

Studies of genome diversity between American and European isolates led the investigation to the discovery of several genospecies within the nomenspecies of *B. burgdorferi sensu lato* (complex), defining the borrelial isolates that are associated with Lyme borreliosis (61,67).

Globally, the *B. burgdorferi s.l.* complex includes at least 23 genospecies and genomospecies. Among these genospecies of *B. burgdorferi s.l.* the most common pathogens associated with human infection in Europe are the genospecies *B. afzelii*, *B. garinii*, *B. bavariensis* (formerly *B. garinii* OspA type 4), and *B. burgdorferi* (*B. burgdorferi sensu stricto*). While *B. spielmanii*, *B. bissetii*, *B. valaisiana,* and *B. lusitaniae* have been identified as pathogens in single cases only (37,55,69,70).

Borrelia burgdorferi senso stricto is the etiologic agent of LB in North America. Although at least nine *B. burgdorferi* s.l. genospecies belonging to the *B. burgdorferi sensu lato* complex are characterized in North America, namely *B. americana*, *B. andersonii*, *B. bissettii*, *B. burgdorferi* sensu stricto (s.s.), *B. californiensis*, *B. carolinensis*, *B. garinii*, *B. kurtenbachii*, B. spielmanii and *B. mayonii* (3,36,37,71). Of these genospecies, *B. americana*, *B. andersonii*, *B. bissettii*, *B. burgdorferi* s.s., *B. garinii*, *B. kurtenbachii*, *B. spielmanii* and *B. mayonii* are occasionaly isolated in symptomatic patients and are considered pathogenic for humans (36,72).

Borrelia mayonii is a novel species within the *Borrelia burgdorferi sensu lato* complex that was detected from host-seeking Ixodes scapularis and was recently found to be associated with Lyme borreliosis in the Upper Midwest of the United States (37,67,73).

Borrelia miyamotoi, which is phylogenetically close to relapsing fever Borrelia, is now a known pathogen that causes a Lyme-like disease in the northern hemisphere (36). Nonpathogenic genospecies B. japonica is discovered in Japan (37).

A proposal to change the official name of the genus of the bacteria that cause LB from Borrelia to Borreliella was thought to be controversial, and that would cause only confusion (62).

1.4.1. The main pathogenic Borrelia Species for Humans

Three genospecies of *B. burgdorferi s.l.* are primarily responsible for the human LB: *B. burgdorferi s.s*, *B. afzelii* and *B. garinii* (3,36,37,46).

B. burgdorferi s.s.

B. burgdorferi s.s. is a pathogenic species that several vectors, such as *I. scapularis*, *I. pacificus*, *I. ricinus*, *I. hexagonus,* and *I. trianguliceps*, can transmit it. In North America, *B. burgdorferi s.s.* has spread over the West Coast and the eastern half of the USA (mainly in the northeast), but also some southern areas such as Florida and Texas (35,36,66). In Europe, the density of *B. burgdorferi s.s* is lower than those of the two other main pathogenic species: *B. garinii* and *B. afzelii*. In Africa, *B. burgdorferi s.s.* have been reported in Morocco. *B. burgdorferi s.s.* – like any other pathogenic *B. burgdorferi s.l.* species – is able to cause EM (74). Each pathogenic

Borrelia species has a preferential organotropism. *B. burgdorferi s.s*. have been associated with arthritis (74). Although this organotropism is optional because it can cause neurological manifestation too (35,36,41,66,74).

B.garinii

B. garinii has spread all over Europe, Asia, and North Africa. In Europe and North Africa, it is transmitted by *I. ricinus*. In Asia, the primary vector is *I. persulcatus* and, much more rarely, *I. trianguliceps* (57). The nervous system is the main target for *B. garinii* (74). The bacteria are less frequently detected in joints, and seldomly it causes ACA (35,36,46,66).

B.afzelii

B. afzelii is present in both Europe and Asia. It is particularly frequent in northern and Eastern Europe. The only known vectors of *B. afzelii* are *I. ricinus* in Europe and *I. persulcatus* in Asia (57). *B. afzelii* seems to have an organotropism for the skin since it is the etiological agent of ACA and lymphadenosis benigna cutis (35,36,46,66,74).

1.4.2. Nonpathogenic Borrelia

Other Borrelia spp. belonging to the *B. burgdorferi sensu lato* complex with unknown documented pathogenicity, and that have never been associated with disease in humans are *B. japonica*, *B. tanukii*, *B. turdi*, *B. sinica*, *B. californiensis* (66,75).

1.5. Pathogenesis

To maintain its complex enzootic cycle, *B. burgdorferi* requires survival in two environments that differ significantly - a tick host and a mammalian host. In the tick host, *B. burgdorferi* may survive with minimal nutrition for extended periods (ticks take one blood meal every 6 to 12 months) and at the extremes of ambient temperatures during winter and summer. In contrast, life in a mammalian host gives plenty of nutrients and a stable temperature. However, an effective mammalian infection requires evasion of highly sophisticated immune defenses in contrast with the rudimentary immune systems in ticks (47,76–78).

1.5.1. Transmission of *B. burgdorferi s.l.* from the tick to the host

Transmission of *B. burgdorferi s.l.* from the tick to the mammalian host is a complex process (79).

B. burgdorferi s.l. usually is inmate to the midgut of unfed infected ticks where selectively express outer surface proteins (Osps) that are not expressed in the vertebrate host (8,35,53,79).

Studies have shown that OspA and OspB are involved in adherence to and penetration of mammalian host cells, which play a crucial role in the pathogenesis of LB (36,50,66).

The spirochete expresses Osp A in the midgut of the tick, which acts as an adhesion that binds to midgut epithelial cells. Spirochetes survive in a dormant state in the nymphal tick midgut during fall, winter, and spring (8,35,80). When the tick feeds in the late spring or summer, these proteins are downregulated, and OspC is upregulated as the organism travels to the tick's salivary gland and there binds a tick salivary gland protein (salp 15), helping the initial immune evasion in infection of the mammalian host by coating the spirochete with this tick protein (8,35,80).

The first step in the transmission of *B. burgdorferi s.l.* is the attachment of the infected tick to the host. Most ticks of the genus Ixodes feed for 2–7 consecutive days depending on the developmental stage. The uptake of blood triggers *B. burgdorferi s.l.* to multiply in the tick midgut, and they start to penetrate the midgut barrier and invade the haemocoel (80). From the salivary glands, *B. burgdorferi s.l.* can be carried into the feeding lesion that the tick has created (8,35,79,80).

1.5.2. Dissemination in the host

Once *B. burgdorferi s.l.* inoculates into a host, it must disseminate in order to survive in the long term. As the first step in dissemination through the vertebrate host, *B. burgdorferi s.l.* expresses outer surface proteins (the only known virulence factors of *B. burgdorferi*) that selectively adhere to host tissue such as endothelial cells, platelets, chondrocytes, and extracellular matrix by a variety of binding mechanisms with integrins, glycosaminoglycans, fibronectin, and collagen, also playing an essential role in the colonization of tissues (skin, joints, heart) (8,35,36,80,81). Spirochaetes bind to platelets via integrin αIIbβ3 and to endothelial cells via integrins αvβ1and α5β1. *B. burgdorferi s.l.* also adheres to glycosaminoglycans, which seems to vary amongst different *B. burgdorferi s.l.* strains that could be the reason for tissue tropism and invasive differences (8,35,36,80). Dissemination from the site of inoculation into adjacent sites of the dermis is a relatively slow process, even in the vertebrate hosts that are highly reservoir-competent for a given *B. burgdorferi s.l.* strain suggesting that haematogenous spread of *B. burgdorferi s.l.* is less critical than extravascular migration. *B. burgdorferi s.l.* do not attach directly to collagen but to decorin, a collagen-associated extracellular matrix proteoglycan found in the skin and many other host tissue, by binding two adhesins of *B. burgdorferi s.l.*, the decorin-binding proteins, DbpA and DbpB, that

are expressed within the vertebrate host (8,35,36,80). Interacting with fibroblast, fibronectin, and fibrocytes *B. burgdorferi s.l.* may be the way of immune evasion. Ingested by professional phagocytes (coiling phagocytosis), *B. burgdorferi s.l.* survive within macrophages that may help in dissemination (8,35,36,79,80).

The resistance of invasive microbial pathogens to complement the system (complement-mediated killing as a part of the innate immune system that can rapidly respond to microorganisms, long before specific antibodies are generated) by using complement neutralizing proteins such as Salp, CRASPs (complement regulator-acquiring surface proteins), and by binding the complement inhibitory factor H, is essential for dissemination (8,35,36,79,80,82).

B. burgdorferi s.l. binds to the plasminogen system of the host, which plays a significant role in fibrinolysis, allowing the bacteria to avoid fibrin-based immobilization into the feeding lesion and enhance the dissemination of *B. burgdorferi s.l.* through both skin and organ invasion (83).

The antigenic variation expressed by the Vls gene locus of *B. burgdorferi s.l.* makes it possible to persist in mammals and evade the host immune response, too. The OspE family of genes undergo mutational changes during infection and are also crucial for immune evasion during LB (8,35,36,79).

Once a *B. burgdorferi s.l.* strain has disseminated from the site of inoculation, and infections tend to persist in most animals and humans during the entire lifetime if untreated. The lack of self-limitation of *B. burgdorferi s.l.* infections are remarkable as *B. burgdorferi* s.l. induces strong cellular and humoral immune responses that target a variety of Osps expressed in the host (81).

1.5.3. Transmission of B. burgdorferi s.l. from the tick to human

In humans, the bite of the infected tick is required to inject the pathogen through healthy skin (80,82).

After injection of *B. burgdorferi* by a tick and an incubation period of 3 to 30 days, the spirochete usually first multiplies locally in the skin at the site of the tick bite. In most patients, immune cells first encounter *B. burgdorferi* at this site (8,35,54,80). During the initial infection, the lipoproteins of *B. burgdorferi* stimulate robust responses of inflammatory cells and their secreted mediators - proinflammatory cytokines, chemokines, and particularly interferon gamma (IFN) - through activation of the innate and adaptive immune system, that cause acute-

phase lesions such as the classical erythema migrans lesion (35,36,54,80). The early cytokine/chemokine response is characterized by interferon-gamma-dependent Th 1 responses with elevated levels of CD4 and CD8 T-cell chemokines CXCL9 and CXCL10. The bacterium also activates proteases and other induced host cell molecules to allow for dissemination through the blood and reach other tissues that possess organotropism like skin, joints, the heart, and nervous tissue (8,35,36,80,81). The Borrelia produces no known toxins or enzymes that cause tissue damage, thus falls into the group of highly invasive, non-toxigenic pathogens (8,35,36,81).

To control and eradicate *B. burgdorferi*, both innate and adaptive cellular elements are mobilized to fight the infection (8,35,36,80). As part of the innate immune response, complement may lyse the spirochete in the skin. Chemokines released by constituent cells in the skin lead to the recruitment of neutrophils and macrophages; the latter releases potent proinflammatory cytokines. The purpose of the adaptive immune response appears to be the production of specific antibodies, which opsonize the organism - a step necessary for optimal spirochetal killing that appears to be suppressed at the beginning of the infection, and that is important for the dissemination of the borrelia (8,35,36,61,80). The earliest evidence of humoral immunity occurs several weeks after infecting tick bite, and approximately 1 week after the identification of EM, with increasing levels of IgG antibodies produced in the months succeeding untreated infection (8,35–37).

Membrane lipoproteins are mitogenic for B cells. The specific immunoglobulin M (IgM) response is often associated with polyclonal activation of B cells - including elevated total serum IgM levels, circulating immune complexes, and cryoglobulins - and it is often linked with the appearance of the late manifestation of infection, and the adaptive IgG response which develops gradually after weeks to months after infection (8,35,61,79).

Histologic examination of all affected tissues reveals infiltration of lymphocytes, macrophages, and plasma cells with some degree of vascular damage (including mild vasculitis or hypervascular occlusion). These findings suggest that spirochete may have been present in or around blood vessels (3,8,80).

In humans, it appears that the immune system can eventually eradicate the infection since almost all patients eventually resolve their symptoms even without antibiotics (76–78).

Despite the innate and adaptive immune responses, *B. burgdorferi* may sometimes survive in certain sites, such as collagen in synovial tissue, through the enclosing of the pathogen in dense tissue (3,80,84). The ability of the spirochete to downregulate the expression of a surface-

exposed protein antigen, the inhibition of a host's innate immune response, the ability to change amino acid sequences in the protein are important mechanisms of immune evasion. However, spirochetes do not seem to be able to survive indefinitely in this combat between *B. burgdorferi* survival factors and host immune responses, and there are no mechanisms that help to protect them from antibiotic therapy. It has not been seen that *B. burgdorferi* is a "hideout" in intracellular locations to evade antibiotic exposure, although it has been identified inside cultured cells *in vitro*, in histologic sections of infected tissues (3,35,84).

In the enzootic infection, *B. burgdorferi* spirochetes must survive this immune attack for only the summer months before returning to the larval ticks to begin the cycle again next year. In contrast, due to the fact that humans are not a competent reservoir, the infection of humans is a dead-end event for the spirochete (3,35,79).

1.6. Clinical manifestation

LB in humans is a multi-stage, multi-system, inflammatory disease (8,35,36,43). Three stages of LB have been described, and patients can usually be categorized as having early localized, early disseminated, or late persistent infection (8,35,36,43). However, the disease progresses very differently, depending on the individual (74). The disease itself does not necessarily develop in stages because, for example, neurological disorders or ACA may also develop without preceded by EM (37,43). Distinguishing between early and late manifestations is more suitable since the clinical picture determines both the diagnosis and the treatment (74).

1.6.1. Erythema migrans

Although they have been proposed for different purposes, the best known among several definitions of EM are the definitions of the CDC, EUCALB, and the ESCMID study group (85). EM is defined as an erythematous skin lesion that develops days to weeks (the incubation period ranges from 3 to 30 days, but is usually about 10 days) at the site of a tick bite where borreliaes were inoculated into the skin by the bite of an infected tick (8,35–37,68,80).

EM typically begins as homogenes red macula or papula to bluish-red patch with an advancing border that expands over a period of days to weeks to usually an oval or round lesion, with or without central clearing (8,35,36,68,80).

A single primary lesion that reaches at least 5 cm in size should be present in a suspicious clinical case. A lesion of <5 cm can be accepted for the diagnosis of EM only if it develops at the site of a tick bite after a symptom-free interval of at least 2 days between the bite and the

onset of the lesion; and if the lesion is enlarging. Fulfillment of all 3 requirements is needed for a clinical diagnosis (37,68,80).

EM is the most frequent manifestation of LB (36,37,68). It occurs at any age and in both genders, with a slight predomination of female patients in Europe, but not in the USA. EM has a noticeable seasonal occurrence, and it is most frequently diagnosed in late spring and early summer. However, in individual cases, it can be seen throughout the year (37,68,83).

The lesion usually appears at the site of a tick bite. In adult patients in Europe, the erythema slowly enlarges, and in cases of long duration, the lesion will fade or clear in the central part, typically by the end of the first week, and a peripheral bright red erythematous ring will further expand until treatment or spontaneous healing (68,80). EM skin lesions are typically oval or round but can also have a triangle, square, or an irregular shape (3,35,61,68,80). Their diameter may range from a few centimeters to more than a meter. How the lesion expands depends partly on the site of the initial infection, they can be located anywhere, but the thigh, groin, and axilla are a common site (3,8,35,80). The border of EM is usually well demarcated. Erythema migrans usually feed and disappear during 4 weeks with spontaneous healing. However, sometimes untreated lesions persist and expand over days to several months (erythema chronicum migrans) or reappear in the same site (erythema migrans recidivans) (3,35,61,68). In adult patients, EM is most often located on the lower extremities; in children, the upper part of the body is relatively more often involved (68).

In adult European patients, about half of them report mild local symptoms such as itching, burning, and/or mild local pain at the site of EM (43,80). Fewer than 50 % of European patients have systemic symptoms - most often, fatigue and malaise, headache, myalgia, and arthralgia (80). The proportion of patients with systemic symptoms is higher in the USA, where as many as 80% of them have simultaneous systemic complaints (61,80). Central induration and peeling occur very rarely in long-lasting EM or after antibiotic treatment (37,61,68). EM in the USA has a greater frequency of local lymphadenopathy (39% vs. 9%) and fever recalled from more than one-third of patients. Fever is an exceptional event (fewer than 5%) in European patients with EM (37,68).

European patients with EM are mostly seronegative in convalescent-phase serum samples, whereas the majority of such patients in the USA are seropositive (80). However, in both groups, routine medical laboratory tests do not reveal signs of inflammation or any other abnormalities (35,68).

Between 58% and 73% of European patients with EM can recall a previous tick bite at the site of the EM, but only one in four patients with EM in the USA (37,68).

Multiple EM is defined as the presence of 2 or more annular secondary skin lesions in a patient, usually developing within several days to weeks, and is a consequence of hematogenous dissemination of borreliae from the primary EM skin lesion (8,35,36,68). The secondary lesions are similar in their appearance to the initial EM lesion. However, they are generally smaller in size, migrate less, have a less indurated center, and very rarely are associated with local itching or pain. In the USA, they are more common (up to 50% of patients with EM) than in Europe (≤ 8%) (57). It seems that multiple EM are more frequently present in children than in adults (8,35,36,57,68).

In 18–26% of patients with multiple EM, a mild lymphocytic pleocytosis has been seen, but with no clear clinical evidence of central nervous system involvement (68).

Etiology

In North America, EM is caused by *B. burgdorferi s.s.* According to skin culture results, EM in western, central and eastern Europe is caused predominantly by *B. afzelii* (70%–90%), less frequently by *B. garinii* (10%–20%), rarely by *B. burgdorferi*, and only exceptionally by other species such as *B. bissettii*, *B. spielmanii* and as yet unidentifiable species (37,57). However, *B. garinii* dominates in north-eastern Europe (37,68).

Histologic Findings

Histological examinations of the dermis at the site of the EM lesion usually detect mild superficial perivascular lymphocytic infiltration, sometimes accompanied by histiocytes, plasma cells, and rarely neutrophils (37,68,80). The epidermis is usually not touched (37,57). The presence of T cells and increased numbers of Langerhans cells suggest that cell-mediated immune mechanisms are involved in the initial host response to *B. burgdorferi s.l.* High levels of mRNA expression of the T cell-active chemokines CXCL9 and CXCL10 and low levels of the B cell-active chemokine CXCL13 have been established in EM (68). Blood vessels may show endothelial swelling, and extravasation of erythrocytes is frequently found (3).

Diagnosis

The diagnosis of a typical EM is clinical. For atypical lesions, proof is required by the demonstration of borreliae in the skin (68). At this stage of the disease, borreliae can be cultivated from skin lesion rather than at other stages. Often there are still not present any specific antibodies, and the cellular immune response occurs first (3,35,61,68).

However, the identification of EM remains primarily a clinical diagnosis in practice. Testing of consecutive serum samples may be a diagnostic aid in selected situations of atypical rash (80).

Differential Diagnosis

When lesions are present in the inguinal or axillary region, they could be easily misdiagnosed as dermatomycosis. Erysipelas may resemble EM, but typically the onset of the skin lesion in erysipelas is accompanied by high fever, malaise, and laboratory signs of inflammation that are not present in European patients with EM. When a skin lesion appears immediately or during the first 24 hours after a tick bite, it is usually the result of a hypersensitivity reaction and not a consequence of a borrelial infection. The differential diagnoses may include reaction to another insect bite, urticaria, contact dermatitis, eczema, folliculitis, cellulitis, tinea corporis (ringworm), or granuloma annulare (3,35,68,80).

1.6.2. Borrelial lymphocytoma

Borrelial lymphocytoma (BL) was the term introduced by Weber at al. (1985) in order to specify the etiology of the condition (80). Because of its benign course, the lesion has also been called lymphadenosis benigna cutis (LBC) (43,68,80).

BL is a rare manifestation of European Lyme borreliosis. There are no reports of this skin manifestation in the USA (37,83). BL appears as a solitary bluish-red swelling up to a few centimeters in diameter and is more frequently seen in children, mainly on the ear lobe, ear helix, nipple, or scrotum. In adults, it is located mainly in the nipple region and rarely on the nose, scrotum, arm, or shoulder (37,80,83). It develops for several weeks to months after an infected tick bite (37,43,68,80).

The lesion may occur after EM, or they may appear together. BL can resolve spontaneously but sometimes can persist and grow for several months to more than a year (37,43,80).

Etiology

B. afzelii take the major part of isolates from BL tissue. *B. garinii* and *B. burgdorferi s.s.* have been isolated in some patients (57).

Histological findings

The histological picture presents as a diffuse dermal proliferation of polyclonal lymphoid cells with a predominance of B lymphocytes or a follicular lesion with germinal centers, without detectable extracutaneous involvement (43,68,80).

Diagnosis

The isolation rate of Borrelia from BL appears to be considerably lower than from EM. Clinical diagnosis of ear lobe lymphocytoma is usually possible from positive anamnesis of the tick bite, presence or history of EM, presence of other manifestations of LB, or evidence of borrelial infection by positive serology (68).

Differential Diagnosis

Difficulties in diagnosing BL in the ear lobe are usually the result of unawareness; whereas, the main differential diagnostic possibility in breast lymphocytoma is a malignancy, which requires histologic examination. Sometimes there may be difficulties in distinguishing between BL and B cell lymphoma or other pseudolymphoma (37,68).

1.6.3. Acrodermatitis chronica atrophicans

ACA is a chronic skin manifestation of the third stage of Lyme borreliosis, seen exclusively in Europe (37,68). ACA rarely, if ever, occurs in the USA (57,80,83). In contrast to EM and borrelial lymphocytoma, ACA does not disappear spontaneously (37,43,68).

Because of the long incubation period and the long duration of the skin lesions prior to diagnosis, almost no patient recalls a tick bite at the affected body site. The exceptions are patients who remember having had EM lesion in the same location several months to many years before or a previous other manifestation of Lyme borreliosis (37,68).

ACA usually begins as insidious with violaceous livid-red color changes and soft swelling on acral parts of the body, usually on the extensor part of the extremities, most commonly on the lower leg with initial unilateral involvement of one foot that gradually increases in size (8,35–37,68). Later on, the lesions may become more or less symmetrical. After months to years, the edema slowly withdraws, and gradually the skin becomes atrophic and sclerotic, with prominently visible underlying vessels because of the thin and wrinkled, ‘cigarette paper-like’ skin (3,35–37,68). ACA is more frequently diagnosed in middle-aged women than men, while in children, it occurs only exceptionally (37,68).

There is an association between ACA and peripheral polyneuropathy, while CNS involvement and cerebrospinal fluid (CSF) abnormalities are rare (35,37,43,68,80).

Etiology

ACA is observed far less frequently than EM but is more common than borrelial lymphocytoma. ACA is caused most frequently by *B. afzeli.* although *B. garinii* and *B. burgdorferi* could be the etiologic agent as well (35,37,43,68,80).

Histologic Findings

In the early edematous/infiltrative phase, histologic findings are a nonspecific perivascular lymphocytic infiltrate and plasma cell predominantly in the peripheral parts of the lesion with fibrosis and thinned epidermis. In the clinical picture, they resemble rheumatoid nodules.

In the late atrophic phase, dilated blood vessels surrounded by lymphocytes and plasma cells can be found in the superficial cutis, where the epidermis often has only a few layers of cells (37,68).

Diagnosis

The diagnosis of ACA is based on clinical, serologic, and histologic criteria. Patients with ACA typically have high serum concentrations of borrelial IgG antibody, and "seronegative" ACA patients are almost nonexistent (68). There are typical histologic findings with telangiectases, lymphocytic, and plasma cell infiltration of the dermis, with or without atrophy (57,61). Diagnosis of ACA can be further supported by the isolation of borreliae from the involved skin; isolation is successful in about one-third of patients who have not previously received antibiotics (37,43,68,80).

Differential Diagnosis

ACA has a broad differential diagnosis, which partly depends on the stage of the disease. ACA skin lesions on lower extremities can be falsely interpreted as a vascular insufficiency (such as chronic venous insufficiency, superficial thrombophlebitis, arterial obliterative disease, hypostatic eczema, acrocyanosis, and lymphedema) or a consequence of old age ('old skin'). Fibrous nodules are often misinterpreted as rheumatoid nodules or erythema nodosum (37,68,80).

1.6.4. Neuroborreliosis

Lyme neuroborreliosis (LNB) is the central and/or peripheral nervous system involvement in a patient infected with *B. burgdorferi s.l.* (35,37,68,80).

After several weeks to months, about 15% of untreated patients in the United States develop meningitis, encephalitis, cranial neuritis (including bilateral facial palsy), motor, and sensory radiculoneuritis, mononeuritis multiplex, cerebellar ataxia, or myelitis-alone or in various combinations (35,68). The classic full picture of early LNB is defined as meningo-polyradiculoneuritis, also named Bannwarth syndrome or Garin-Bujadoux-Bannwarth syndrome, which consists of neuralgic pain, lymphocytic pleocytosis without headache, and sometimes cranial neuritis, and it is the most prominent LNB manifestation in Europe (35–37,43,68). Bell's palsy is most often cranial neuropathy (mainly in children), which can be the

only neurologic disorder and can be bilateral. In contrast, painful radiculoneuritis, although it can affect all age groups, it rarely has been diagnosed in children (35,36,57,61). Early neurologic symptoms generally begin a few weeks after EM (median 4 weeks), although they can manifest initially alone, especially with *B. garinii* in European Lyme disease (36).

During the first days of illness, headache, and neck stiffness are not associated with spinal fluid pleocytosis or objective neurologic deficit (35). The clinical picture of Bannwarth syndrome begins with pain because of radiculoneuritis. The character of the pain varies, it is usually severe, and patients describe it as burning, tearing, and migrating. Because of its severity, about one-third of patients become depressed, agitated, restless, suffer from sleeplessness, and are full of anxiety (36).

In patients with meningitis, cerebrospinal fluid (CSF) typically has a lymphocytic pleocytosis of more than 100 cells/ mm^3, often accompanied by an elevated protein but a normal glucose level. Specific IgG, IgM, or IgA antibodies are produced intrathecally, and *B. burgdorferi* specific oligoclonal bands may be present (3,37,63). Between one and two-thirds of patients remember arthropod bites preceding the onset of the neurologic involvement. The majority of cases of LNB occur between July and November, with most cases observed in August (37,68,80).

Etiology

LNB in Europe is most frequently caused by *B. garinii*, and less frequently by *B. afzelii* and *B. burgdorferi s.s.* In America, all cases of LNB are caused by *B. burgdorferi s.s*. (37,68).

Histologic Findings

Data on histopathologic findings in the CNS are scarce. In patients with meningoradiculoneuritis, lymphocytic involvement of leptomeninges, ganglia, and afferent and efferent rootlets is present. The CNS may show focal microgliosis. Fibers within the nerve eventually lose myelin. The most striking finding is axonal degeneration (68).

Diagnosis

The diagnosis of early LNB is based on clinical characteristics, the presence of lymphocytic pleocytosis, and demonstration of CNS borrelial infection, as evidenced by seroconversion, intrathecal borrelial antibody production, isolation of borreliae, or demonstration of borrelial DNA in CSF samples (36,37,68). Diagnosis of LNB involving the peripheral nervous system is even more difficult because of the limited possibilities of demonstrating borrelial infection of peripheral nerves (68).

Differential Diagnosis

Differential diagnosis comprises a list of differential diagnoses for each main manifestation of LNB, including meningitis, radiculoneuritis, and cranial nerve involvement (2,11,36,43,85).

Radiculoneuropathy with dermatomal pain may be difficult to diagnose because it is hard to distinguish from mechanical radiculopathy due to spinal disc disease or early shingles before the onset of rash. Lyme disease presenting as psychiatric disorders or rare cases of demyelinating encephalopathy mimicking multiple sclerosis has been reported (36,85).

1.6.5. Chronic neuroborreliosis

The major manifestations of chronic neuroborreliosis are encephalitis, radiculomyelitis, transverse myelitis, stroke-like disorders, and cranial nerve deficits (35,85).

The best-defined late neurological manifestation is progressive chronic encephalomyelitis that develops in up to 10% of European patients with untreated meningopolyneuritis (Garin-Bujadoux- Bannwarth syndrome) caused by *B. garinii*. Moreover, it is characterized by spastic paraparesis, ataxia, cognitive impairment, bladder dysfunction, and cranial neuropathy, particularly of the seventh or eighth cranial nerve, accompanied by intrathecal antibody production of IgG antibody to *B. burgdorferi* (3,35,85). Unlike Banwarth syndrome, progressive chronic encephalomyelitis does not withdraw spontaneously (61). In both the United States and Europe, a chronic axonal polyneuropathy may develop, manifested primarily as spinal radicular pain or distal paresthesias (35).

Diagnosis has to fulfill strict diagnostic criteria with other diagnostic considerations, including neurosyphilis, fungal meningoencephalitis, brain tumor, and multiple sclerosis (68,85).

1.6.6. Carditis

Within several weeks to a few months after the onset of illness, in some patients (about 4-5%) with untreated early disseminated LB, cardiac involvement may develop (35). Carditis linked with borrelial infection is a rarely observed manifestation and usually presents with the acute onset of varying degrees of intermittent atrioventricular (A-V) heart block, from first-degree Wenckebach to a complete heart block (35,37,68). The condition appears to be transient over minutes to hours. Generally, it resolves spontaneously within a few weeks (3 days to 6 weeks), even in untreated patients, and the insertion of a permanent pacemaker is unnecessary (35). Rare cases of clinical evidence of myopericarditis with sudden cardiac deaths have been reported (35,37,68).

Cardiac involvement may be asymptomatic. When symptomatic, the most common complaints include light-headedness, syncope, dyspnea, palpitations, and/or chest pain (85).
The frequency of heart involvement in relation to the other manifestations of Lyme borreliosis appears to be very low (37).

Etiology
There are scarce data on the Borrelia species causing Lyme carditis. In the USA, it appears to be caused by *B. burgdorferi s.s.*, the predominant species causing Lyme borreliosis in humans there (68).
B. burgdorferi s.s. was isolated from the endo-myocardium of a patient suffering from longstanding dilated cardiomyopathy in Europe (37,85).

Histological findings
Based on rare heart tissue examination from autopsy or endomyocardial biopsy, histopathologic findings include an interstitial infiltrate of lymphocytes and plasma cells involving the myocardium, pericardium, and endocardium (68,85).

Diagnosis
Diagnosis of heart involvement with borrelia should be based on demonstration of borrelial infection through a clinical picture in newly developed AV conduction disorder (especially in young patients), or other arrhythmia (86); through the presence of borrelia antibodies in serum or seroconversion; by isolating borrelia or borrelial DNA from an endomyocardial biopsy specimen; through the presence of another typical manifestation(s) of Lyme borreliosis like EM, and/or LNB - together with or manifested previously or proximity to Lyme carditis; and by excluding other causes of cardiac abnormalities (85).

Differential Diagnosis
The differential diagnosis of Lyme carditis includes diseases that may be of infectious etiology, causing conduction disturbances, endomyocarditis, and pericarditis, as well as noninfectious conditions (85).

1.6.7. Arthritis

In the USA, arthritis was the predominant manifestation that drove the investigation to the discovery of the borrelial infection. Lyme arthritis in Europe was recognized subsequently following the reports from the USA (43). Nevertheless, European dermatological literature had repeatedly been describing joint and bone abnormalities in patients with ACA, ever since 1922, referring to it by the term “acrodermatitis atrophicans arthropathica,” and joint symptoms had

been mentioned in case reports on erythema chronicum migrans and lymphocytic meningitis (37,42).

However, in Europe, arthritis is a less common manifestation compared to the USA (37). Lyme arthritis may occur within several months after an untreated EM. Lyme arthritis affects both adults and children, occurring predominantly in the fourth decade, and if present, in childhood, older children are more often affected (37,68,85).

In Lyme arthritis, the evasion of immune mechanisms by changing the expression of several immunostimulatory outer-surface lipoproteins plays an essential role in disseminating synovial tissue and is probably even more critical than in most other manifestations of Lyme borreliosis. *B. burgdorferi s.l.* does not produce proteases, and therefore does not cause rapid joint destruction as observed in classic septic arthritis (68).

Arthralgia may precede, accompany, or follow arthritis but may occasionally be the only rheumatic manifestation of Lyme borreliosis (85).

Lyme arthritis is an intra-articular infection, where a small number of bacteria can induce severe arthritis with mechanisms (including the induction of cytokines and chemokines) that enhance the inflammatory response and is mostly monoarticular or oligoarticular infection (37,85). Large joints are predominantly involved, typically involving the knee in about half of all cases, and the ankle, wrist, and elbow, while smaller joints are rarely involved (68,85). Joint involvement is usually asymmetric; the onset of arthritis is acute and with effusion, and the skin over the affected joint is warm but of normal color (68). Those with arthralgia have had brief episodes of pain in joints, tendons, bones, or muscles without objective signs of inflammation that tend to be migratory and that are a relatively frequent complaint, early in the course of Lyme borreliosis, with onset from 1 day to 8 weeks after the onset of EM. Symptoms can last from 1 month to as long as many years with a relapsing pattern (68,85).

Lyme arthritis can be preceded or accompanied by other manifestations of LB. Several European authors have emphasized that Lyme arthritis often begins in the extremity that was affected by a tick bite or EM (68).

Routine laboratory parameters are often completely normal, and there are no specific radiographic findings (85).

Etiology

Data on the etiology of Lyme arthritis are based predominantly on the detection of borrelial DNA by PCR in joint fluid since the isolation of borrelia is rarely successful. It was convinced that in Europe, Lyme arthritis is caused by *B. burgdorferi s.s.*, the strain that causes LB in North

America, considering a higher prevalence of Lyme arthritis there. *B. garinii* and *B. afzelii* have been identified in cases with Lyme arthritis in Europe as well, concluding that *B. burgdorferi s.l.* strains causing Lyme arthritis in Europe are heterogeneous (68).

Histologic Findings

The histological findings in Lyme arthritis correlate with nonspecific synovitis, with inflammatory infiltrate of lymphocytes and plasma cells (68,85).

Diagnosis

Diagnosis of Lyme arthritis is based on the medical history and clinical features, laboratory findings, exclusion of other causes of arthritis, and demonstration of serum IgG antibodies to Borrelia (36,37,68). The detection of borrelial DNA in synovial tissue or synovial fluid by PCR is much more sensitive (up to 85%). Negative IgG serology essentially rules out Lyme arthritis because borrelial IgG antibodies in serum are almost always strongly positive (36,37,57). The presence of other manifestations of Lyme borreliosis such as EM, Lyme neuroborreliosis, or ACA is of substantial help for accurate diagnosis (37,68).

Differential Diagnosis

The differential diagnosis of Lyme arthritis is broad and generally includes inflammatory rheumatic diseases, bacterial (septic) arthritis, viral arthritis, and crystal-induced arthritis (36,37,68).

1.7. Diagnosis of Lyme Borreliosis

1.7.1. Direct detection of the agent

Lyme disease can be a diagnostic challenge for the clinician and the laboratory alike (36). The proper method for diagnosis of Lyme disease depends on the stage of the disease and clinical manifestations. The gold standard for diagnosis remains isolation of *B. burgdorferi s.l.* from the patient's specimen in Barbour-Stoenner Kelly (BSK) medium. The success of isolation depends on the type of specimen (36,37,68). For example, cultivation of *B. burgdorferi s.l.* from skin biopsies of EM or ACA is usually very successful, at 50–80%, less often from plasma samples (by using high-volume blood cultures of United States patients with EM) and only occasionally (usually 10% to 30% in children) from CSF samples in patients with a very early phase of neurologic disorders such as meningitis (37,57,85). However, this technique is not routinely available, and identification of early disease (EM) remains, primarily, a clinical diagnosis in practice, and cultivation is only rarely needed (36,37,68,72).

Nucleic amplification techniques such as polymerase chain reaction (PCR) have comparable or slightly better sensitivity than the culture for the detection of borrelia in skin biopsy samples (72).

In the later stage of the infection, PCR testing is significantly superior to culture in the detection of *B. burgdorferi* in joint fluid since PCR can detect borrelial DNA in 85% of synovial fluid samples, making it a reliable tool for supporting the diagnosis of Lyme arthritis in Borrelia IgG antibody seropositive patients with joint swelling (3,35–37,68,72).

For the Lyme urine antigen test (LUAT), results are contradictory; hence they should not be used to support the diagnosis of Lyme disease (3).

Because of the problems associated with the direct detection of *B. burgdorferi*, Lyme disease is routinely diagnosed by recognizing a characteristic clinical picture with a serological confirmation (3,35). The limitations of serological tests are that they do not clearly distinguish between active and inactive infection. In addition, 10% of patients are seropositive because of asymptomatic infection (35,72,87).

1.7.2. Indirect Detection of Borrelial Infection

Currently, a countless number of commercial test kits are available for the detection of IgG and IgM antibodies against *B. burgdorferi s.l.* Test systems now comprise numerous techniques, including immunofluorescence, enzyme-linked immunosorbent assay (ELISA), chemiluminescence, luminex, and immunoblot (37,68). Indirect Immunofluorescence Assay (IFA) were the first serodiagnostic tests used to detect antibodies against *B. burgdorferi s.l.* and are still used in numerous countries. Since the two-tier testing principle was introduced, ELISA has become the frequently used serodiagnostic screening method for Lyme borreliosis. Sonicate and recombinant ELISA are in use (3,35,37,68,72).

After more than 20 years of ‘Lyme serology,’ it often appears that for diagnostic purposes, serology has created nearly as many problems as it has solved (37).

Serodiagnosis of LB is currently based on the two-tier testing procedure, meaning that positive and borderline results in a sensitive screening assay are tested for specificity in an immunoblot system because many first-tier assays had relatively poor specificity, with false-positive rates of 5% (37,72,88). The confirmation by immunoblot is required to distinguish between true and false-positive ELISA results (87–89). Immunoblot (Western Blot) is vital in the characterization of immune responses to specific proteins of *B. burgdorferi s.l.* The interpretation criteria for immunoblot results are based on diagnostic antigens, most of which

were identified in the late 1990s (37). Standardization of criteria for interpretation of immunoblot results in Europe was the subject of a multicenter study by The European Union Concerted Action on Lyme Borreliosis/EUCALB (37,68). Although a set of eight bands were identified as significant in each participant laboratory (6 European laboratories using different immunoblot protocols), no single rule could be formulated for use across Europe (37). Since complete standardization of immunoblotting protocols in Europe cannot be achieved, there was hope that new recombinant immunoblots would help in solving this problem (37,68).

A two-tiered approach was recommended by participants in a national conference on serologic diagnosis of Lyme disease held in 1994 under the sponsorship of the CDC (36,88).

Laboratory testing should be used mainly to confirm clinically suspected active infections in combination with risk factors (exposure time in an endemic region during the ticks feeding period) (36,74). Moreover, the use of serology screening for asymptomatic patients is not reasonable because, in highly endemic areas, there is a high seroprevalence of specific antibodies in the general population, and antibodies can persist for a long time (many years) (36,68,87,88).

Laboratory confirmation of a clinical diagnosis is recommended by the Infectious Diseases Society of America (IDSA) 2006 guidelines in all cases of Lyme disease, with the exception of typical cases of EM in an endemic region. The CDC two-tier system using ELISA and confirmatory Western blot is broadly used as a substitute for bacteriologic isolation. However, it is characterized by poor sensitivity in very early disease, such as in single EM cases. Serology becomes much more sensitive in untreated disseminated infection as in disseminated skin lesions and neurologic, cardiac, and rheumatic manifestations occurring after the first month of infection (36,72,87).

Prompt antibiotic treatment may prevent seroconversion and seroreactivity, especially in the IgG response. Additionally, there may be a relation between seroreactivity and the genotype of the infecting agent (36,72,87).

The serologic response to *B. burgdorferi* is characterized by specific IgM that is detectable a few weeks after the onset of the disease and is directed primarily against flagella of the borrelia. It peaks within 3-6 weeks, with a wideness of the response for other antigens over time. Generally, IgM antibodies fall below the level of detection within 6 months but sometimes remain elevated for longer, and it is hard to distinguish between a persistently positive convalescent serology and a new acute exposure (87). The specific IgG antibody is detectable a few weeks after IgM and is directed against the same antigen. IgG antibodies may not peak

for many months after disease onset. In untreated infection, the two-tier testing strategy becomes more sensitive as IgM antibodies are formed during weeks 3 and 4 of infection 36,87,88).

According to the CDC criteria, an IgM Western blot is considered positive if two of the following three bands are present: 23, 39, and 41 kDa; however, the combination of the 23-and 41 kDa bands may still be a false-positive result. An IgG blot is considered positive if 5 of the following 10 bands are present: 18, 23, 28, 30, 39, 41, 45, 58, 66, and 93 kDa (3,36,72).

The second-generation serologic test IgG ELISA that employs a 26-mer peptide of the sixth invariant region of the VlsE lipoprotein of *B. burgdorferi* called the C6 peptide ELISA is now the most promising test where similar results are gained with this test and the standard two-test approach of sonicate IgM and IgG ELISA and Western blot (36,37,72,87,88). The significant advantage of the C6 peptide ELISA is the early IgG response, and therefore an IgM test is not necessary. Because the C6 ELISA is not quite as specific as sonicate Western blot, two alternative two-tiered assays that employ VlsE have been proposed. Using the conventional sonicate ELISA as the first-tier test and only IgG blot with the addition of VlsE band as the second-tier test (3,36,37,88). C6 antibody testing is more sensitive for the B. burgdorferi species present in Europe, which are often poorly detected by either ELISA or western blot tests in use in the USA (36).

In practice, the interpretation of serologic results by physicians is complicated because of both false-positive and false-negative findings. False-negative results are frequently found in early LB, especially EM (89). The frequency of false-negative findings in late LB has been reported to be extremely low (72,87). False-positive findings can be caused by preceding symptomatic infection and specificity problems of assays, caused by cross-reactivity due to other spirochetal illnesses, particularly *Treponema pallidum* infection, leptospirosis, and tick-borne relapsing fever; acute EBV and CMV infections; rheumatoid factors; multiple sclerosis; and other auto-immune diseases (72,89). Asymptomatic Borrelia infections can also lead to positive antibody responses (72,87,89).

1.8. Treatment of Lyme Borreliosis

Early manifestations of LB, both localized and disseminated, sometimes can heal spontaneously without antibiotic treatment. The main reason to treat such patients is to shorten the duration of the manifestation and to prevent the development of later complications (37,80).

The various manifestations of Lyme disease can usually be treated with oral antibiotic therapy, except for patients with objective neurologic manifestation and occasionally patients with Lyme arthritis who may require intravenous therapy for successful treatment (3).

Treatment with proper antibiotics results in a more rapid resolution of the presenting symptoms of infection. Furthermore, the antibiotic therapy is more effective in the early course of the disease, where EM typically resolves promptly and, in addition, a later-stage disease is prevented with a lower risk of post-treatment Lyme symptoms (3,36,52).

Of antibiotics studied to date, amoxicillin (500 mg q 8 hrs. for 14-21 days), doxycycline (100 mg q 12 hrs. for 10-21 days), and cefuroxime axetil (500 mg q 12 hrs. for 14-21 days) have been the most effective (3,36,80).

Oral amoxicillin or oral doxycycline are first-line options and are adequate to cure patients with erythema migrans. In secondary (early disseminated) borreliosis, oral ß-lactam treatment may fail, suggesting that the use of ceftriaxone or tetracyclines is more effective, though no clinical study has proven this (3,90).

The pediatric dose range of amoxicillin is 50 mg/kg per day, divided into three times a day. If possible, doxycycline should be avoided in children under the age of 8 years and during pregnancy. Moreover, young children who are allergic to penicillin can be treated with macrolides such as azithromycin, clarithromycin, or erythromycin but with less satisfactory results of macrolide compare to those with penicillin, amoxicillin, and tetracyclines (3,36,80).

First-generation cephalosporins such as cephalexin and quinolone antibiotics are not an effective therapy for Lyme disease. Herxheimer-like reactions, with an intensification of fever and arthralgia, may occur shortly after initiation of therapy in some patients (36).

1.8.1. Treatment of Lyme carditis

Patients with carditis Lyme disease and first-degree atrioventricular block with a PR interval of <300 milliseconds can usually be treated with oral doxycycline or amoxicillin. Those with PR intervals >300 milliseconds or with second or third-degree block should receive intravenous ceftriaxone or cefotaxime; patients may be switched from intravenous to oral therapy after resolution of heart block if otherwise stable (3,36).

1.8.2. Treatment of Lyme neuroborreliosis

For patients with neurologic manifestations, a 2 to 4-week course of intravenous ceftriaxone, 2 g q 24 hours, is most recommended, with the exception of isolated facial palsy without meningitis (3,36).

In Europe, oral doxycycline has been studied as an alternative to intravenous therapy in both children and adults with acute neuroborreliosis. The experience with this approach in the USA is limited and oral doxycycline is commonly used in patients who have facial palsy alone (3,36).

1.8.3. Treatment of Lyme arthritis

Either oral or intravenous regimens are usually effective for the treatment of Lyme arthritis (3). The initial therapy is with an oral antibiotic for 28 -30 days. Patients who have simultaneous neurologic involvement or who develop a neurologic disease after the initial oral therapy should receive intravenous treatment with ceftriaxone or cefotaxime for 2-4 weeks (117). Patients with late Lyme arthritis are treated initially with oral antibiotic therapy for 28 days (36). In patients who still have joint inflammation like persistent or recurrent synovitis, a second course of oral therapy or intravenous therapy for 2-4 weeks is recommended (3,36).

If PCR results remain positive after 60 days of treatment, some authors recommend one final 30 days course of oral antibiotics (36). The use of articular injection of corticosteroids after completion of antibiotic therapy can be taken as a possibility. In contrast, if given before antibiotic therapy, it increases the risk for persistent arthritis, as some investigators believe (36). Following appropriately treated Lyme disease, approximately 10% of patients (with a highly variable percentage in different studies) continue to have subjective symptoms, primarily musculoskeletal pain, neurocognitive difficulties, or fatigue, in some instances, persisting for years (3).

1.9. Prognosis of Lyme Borreliosis

Early LB generally has a highly satisfactory prognosis, with a good response to antibiotic therapy and no recurrence after long-term follow-up (57). In late LB, antibiotics are poorly beneficial, except for some situations, e.g., the inflammatory stage of ACA. While there is no apparent benefit for prolonged therapy at this stage, further investigations are needed (3,90).

1.10. Prevention of Lyme Borreliosis

People should avoid tick-infested areas when possible. Insecticides and repellents may be helpful for people living in endemic areas who have daily tick exposures. After exposure to tick-infested areas, tick checks in the body are essential. Because 24 to 72 hours of tick attachment is necessary for borrelia transmission, removal of a tick within 24 hours of attachment is usually sufficient to prevent Lyme disease (3).

1.11. Prophylaxis of Lyme Borreliosis

Chemoprophylaxis of LB is not standard practice for several reasons. Firstly, after a tick bite, the infection rate is low, between 1% and 2%, and, secondly, the rate of preventing eventual LB is not predictable. (3,83) However, in a prospective study with adults bitten by ticks in endemic areas of North America, a single dose of 200 mg doxycycline was effective in the prevention of LB if the drug was given within 72 hours after the tick bite (3,43,91).

Immunoprophylaxis of LB with a commercial Lyme disease vaccine comprising a recombinant OspA was introduced in the USA in 1999, but it was withdrawn. In 2002 the second generation of the OspA vaccine was tested in Europe (3,83). The previous experience proved that prevention of infection can be achieved with vaccination; however, a vaccine is not available anymore (3).

2. Hypothesis

1. Lyme borreliosis is an endemic infection in the Pristina region.
2. The risk of Lyme borreliosis in the Pristina region is low.

3. Aims of the research

GENERAL AIM:

To define the risk of Lyme borreliosis after a tick bite in the adult population of the Pristina region, Republic of Kosovo.

SPECIFIC AIMS:

To determine whether the presence of *Ixodes ricinus* in Kosovo and the considerate number of patients with tick bite affect the risk of Lyme borreliosis.

4. Materials and methods

A prospective epidemiologic and clinical survey on the risk of *Borrelia burgdorferi* (Bb) infection after a tick bite was performed in the region of Pristina, Republic of Kosovo.

The study comprised of consecutive subjects of both genders older than 18 years of age, with an embedded tick (complete tick embedded in the skin or parts of the tick) examined at Outpatient Department of Infectious Diseases Clinic of Kosovo, University Clinical Center in Pristina, and only subjects with signed informed consent approved by local Ethical Committee (University Clinical Center of Kosovo) were included in the study.

In this study, 380 patients were recruited. Patients were included in the study during the four-year period (January 2015-August 2018) followed by a six-month follow-up in order to detect the risk of acquiring LB after a tick bite through the development of clinical LB and/or through seroconversion detected using enzyme-linked immunosorbent assay (ELISA).

At the first visit related to the index tick bite (index visit), subjects were evaluated clinically, ticks (the complete ticks or parts of the ticks) were removed from the skin, blood samples were taken for standard laboratory and serological tests and antibiotic treatment was started when deemed indicated.

At the index visit, the ticks that were embedded in the skin (the complete ticks or parts of the ticks) were carefully removed from the skin with fine-tipped tweezers. Complete ticks that were successfully removed from the skin without being damaged were stored (preserved) for further entomological identification. Tick damaged during the removal (which were cut in pieces during removal or they were cut by the patients trying to remove by themselves) are not preserved ticks. The identification of the ticks was conducted by the entomologist from the School of Veterinary Medicine at the University of Pristina. The blood for initial serology testing was withdrawn during the initial visit. Whereas the second serum sample was taken during the second visit that took place two months after the first one to identify the seroconversion in seronegative patients.

Patients with positive ELISA serology in the first visit were studied to analyze the seroprevalence of Bb infection.

Inclusion criteria:

- Patients older than 18 years of age
- With embedded tick and
- With negative first serology taken during the initial visit

Patients were contacted via telephone to remind them of the second visit, and a contact number was provided for patients to contact the investigator in a case of occurrence of any LB manifestation in between the period from the first-to-second and second-to-third visit (six months apart from the first visit).

A specific questionnaire was filled out during the first and second visits and at the end of the follow-up period.

- The first visit questionnaire contained: Identification data of the patient: name, surname, date of birth, place of residence, occupation, contact phone number. Information about the tick bite: the date of the bite, the geographical location of the patient at the time of the bite (if known), the duration of tick attachment; the number of ticks attached, the history of prior tick bites, antibiotic treatment (for other reasons) at the moment of encountering the tick bite, symptoms and clinical manifestations, data about the onset and the duration of the clinical symptoms, and data on antibiotic prescription.
- The second visit questionnaire contained: Information about the identification of the patient, data on any new tick bites since the previous visit, the appearance of clinical manifestations since the first visit, the date of presentation and duration of clinical manifestation, the administration of antibiotics since the previous visit, and duration of treatment in days.
- Data recorded during the third visit: Identification of the patient, data on any new tick bites, data on any appearance of clinical manifestations.

The photos of patients with embedded tick and skin reactions or later with erythema migrans have been taken.

The list of clinical manifestations taken into account as a subject for further analysis included: chills, fever, muscle and/or joint pain, headache, dizziness; burning sensation and/or itching at the bite site; neck pain, fatigue; meningitis, meningo-radiculoneuritis, cranial neuritis-Bell's palsy, carditis, arthritis, lymphocytoma, erythema migrans, multiple erythema migrans diseminata/ chronicum/erythema recidivans and acrodermatitis chronica atrophicans.

The skin reactions on the site of a tick bite during the first visit were divided into two groups:

1- asymptomatic patients with no clinical manifestation or skin reaction after the tick bite;
2- patients with visible local lesion <3 cm at the site of the tick bite, which were further subdivided into two groups: (a) local reaction to a tick bite (b) local reaction as a result of manipulation during tick removal.

Erythema migrans was diagnosed in cases where the lesion appeared on the site of a tick bite and/or visible lesion in the first visit on the site of a tick bite was enlarged >5 cm.

The serology was carried out using the enzyme-linked immunoassay (ELISA) (NovaLisa Lyme Borrelia, NovaTec, Dietzenbach, Germany) intended for the qualitative determination of IgM/IgG - class antibodies against Bb in human serum or plasma using VlsE (recombinant) and lysatantigens of *B. burgdorferi*, *B. afzelii*, *B. garinii* and flagellin (recombinant) and purified OspC as specific antigens.

The sensitivity of the test is around 85% for specific IgM antibodies and >95% for specific IgG; specificity is 94% and 96.7%, respectively.

4.1. Statistics

In line with the aim and objective of the study (descriptive epidemiology), descriptive statistical methods were almost exclusively used: data are summarized as percentages (proportions) or as means (standard deviations) or, where appropriate, median (quartiles). Where deemed of interest for estimation, proportions are reported with the exact Clopper-Pearson 95% confidence intervals. Occasionally, univariate tests for comparison of proportions (chi2/Fisher exact) were used. In an attempt to identify predictors of the development of clinically manifest diseases, a multivariate modified Poisson regression model with robust sandwich estimation (to model relative risk) was fitted to the probability of the event. For this purpose, we considered calendar year, tick preservation at removal (“yes” or “no”), duration of tick embedment (dichotomized as “>72 hours or unknown” or “≤72 hours”), use of antibiotics already at visit 1 or prescribed at visit 1 (“yes” or “no”) and presence of local reaction (“yes” or “no”) as potential predictors of primary interest. In the process of data analysis, two potential independent variables – duration of tick embedment and presence of local reaction – apparently aliased each-other and precluded model convergence. We therefore excluded “presence of local reaction” based on the rationale that duration of tick embedment should be viewed as factor contributing to the presence of a local reaction (i.e., a more “basic” potential predictor). The effect of “calendar year” (four levels – 2015, 2016, 2017, 2018) was tested for a liner trend, i.e., as a contrast between years 2017&2018 taken together (considering the low number of subjects included during 2017 and 2018 in line with the study duration) and year 2015. Data were analyzed using SAS 9.4 for Windows software (SAS Inc., Cary, NC, USA), licensed to the School of Medicine, University of Zagreb.

5. Results

5.1. Subjects

A total of 380 subjects with tick bites were included in the present study: 179 during the 2015 season, 136 during the 2016 season, and 65 in 2017-18.

5.2. Geographical distribution of the included subjects

By far, most subjects with tick bites (274 or 72.1%) were identified in the Pristina municipality: geographical distribution of included subjects is depicted in Figure 1. Overall, 74/380 were positively diagnosed with *erythema migrans* – 19.5% (95%CI 15.6-23.8) – during the study.

5.3. Demographic characteristics

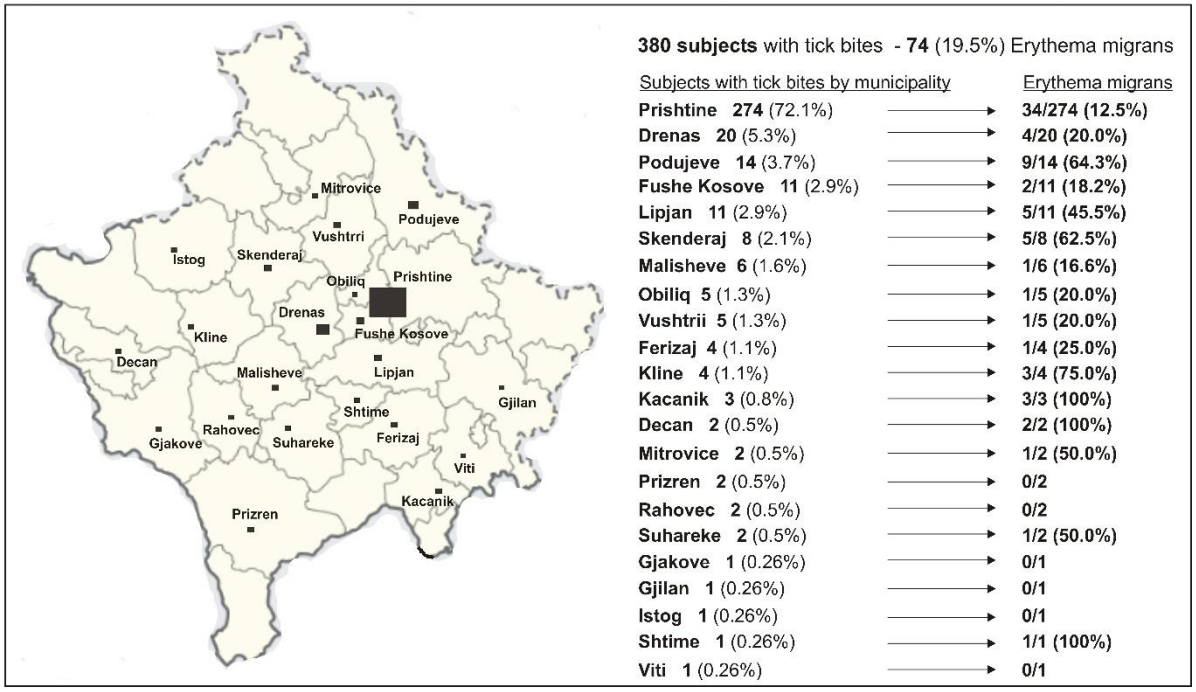


Figure 1. Geographical distribution of patients included in the present study.

The age of subjects included in the study ranged between 18-91 years old, but there was a predominance of younger and middle-aged adults (median consistently around 48 years) with a slight prevalence of male sex (Table 1).

The majority of subjects (365, 96.0 %) included in the study belonged to the general population in whom tick bites were related to recreational activities, with only 15 (3.9%) study participants who were bitten during a professional activity (Figure 2). Eight subjects were farmers, three were soldiers, two gardeners, one forestry worker, and one forestry engineer.

Table 1. Subject characteristics at the 1st visit for the index tick bite.

	All	2015	2016	2017-18
N	380	179	136	65
Age (years)	48 (18-91)	48 (18-80)	49 (18-91)	43 (19-79)
Male sex	226 (59.5)	111 (62.0)	78 (57.4)	37 (56.9)
Any concomitant chronic disease	121 (31.8)	54 (30.2)	50 (36.8)	17 (26.2)
Tick bites history (prior to index bite)	179 (47.1)	85 (47.5)	64 (47.1)	30 (46.1)
No clinical signs	173	80	64	29
Local clinical signs	6	5	0	1
Using antibiotics for other reasons at the time	7 (1.8)	1 (0.6)	5 (3.7)	1 (1.5)
Number of ticks 1 / ≥2	360 (94.7) / 20	166 (92.7) / 13	130 (95.6) / 6	64 (98.5) / 1
Visible local lesion	176 (53.7)	83 (46.4)	68 (50.0)	25 (38.5)
Local reaction to tick bite	124 (32.6)	69 (38.6)	36 (26.5)	19. (29.2)
Completely removed (preserved) tick	117 (30.9)	63 (35.2)	41 (30.2)	13 (20.6)
IgG positive / negative / intermediate	28 (7.4) / 345 / 7	13 (7.3) /164 / 2	11 (8.1) / 122 /3	4 (6.2) / 59 / 2
IgM positive / negative / intermediate	15 (3.9) / 347 / 18	7 (3.9) / 167 / 5	5 (3.7) / 123 / 8	3 (4.6) / 57 / 5
Antibiotics prescribed	69 (18.3)	15 (8.4)	39 (28.7)	15 (23.8)
Amoxicillin+clavulanic acid	42	11	25	6
Amoxicillin	8	1	5	2
Doxycycline	12	0	6	6
Erythromycin	3	1	1	1
Ceftriaxone	1	0	0	0
Not specified	4	2	2	0

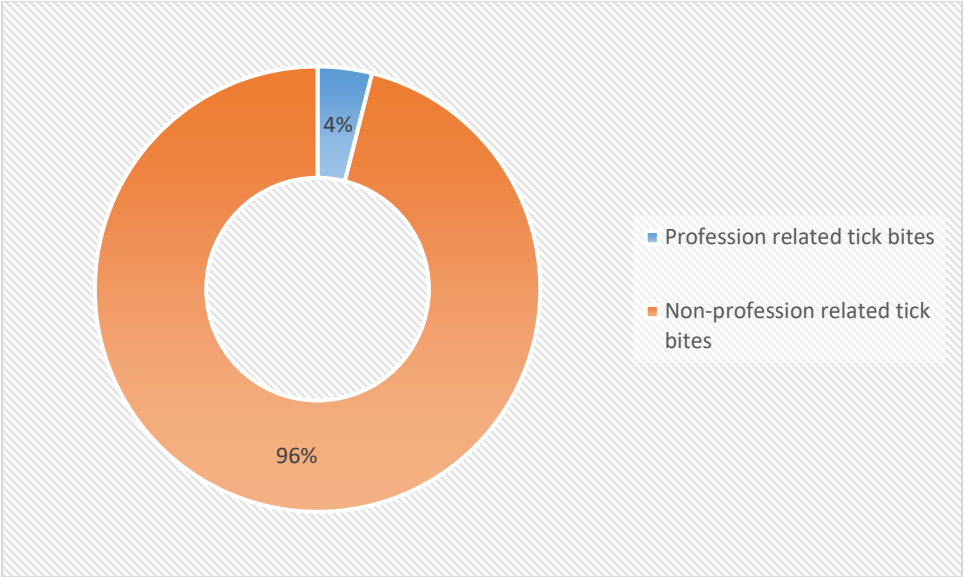


Figure 2. Profession and tick bites.

5.4. Tick bites seasonality

The highest number of bites occurred in May, followed by June. Only in 2015, the peak tick activity month was June. The earliest tick activity was registered in late February, and the latest in November (Table 2, Figure 3).

Table 2. Tick bite seasonality through years 2015-2018.

Year	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total
2015	0	0	0	18	45	79	28	5	2	2	0	0	179
2016	0	0	2	21	54	38	13	2	1	3	2	0	136
2017	0	1	1	8	17	4	7	0	1	1	0	0	40
2018	0	0	0	1	15	3	5	1	0	0	0	0	25
Total	**0**	**1**	**3**	**48**	**131**	**124**	**53**	**8**	**4**	**6**	**2**	**0**	**380**

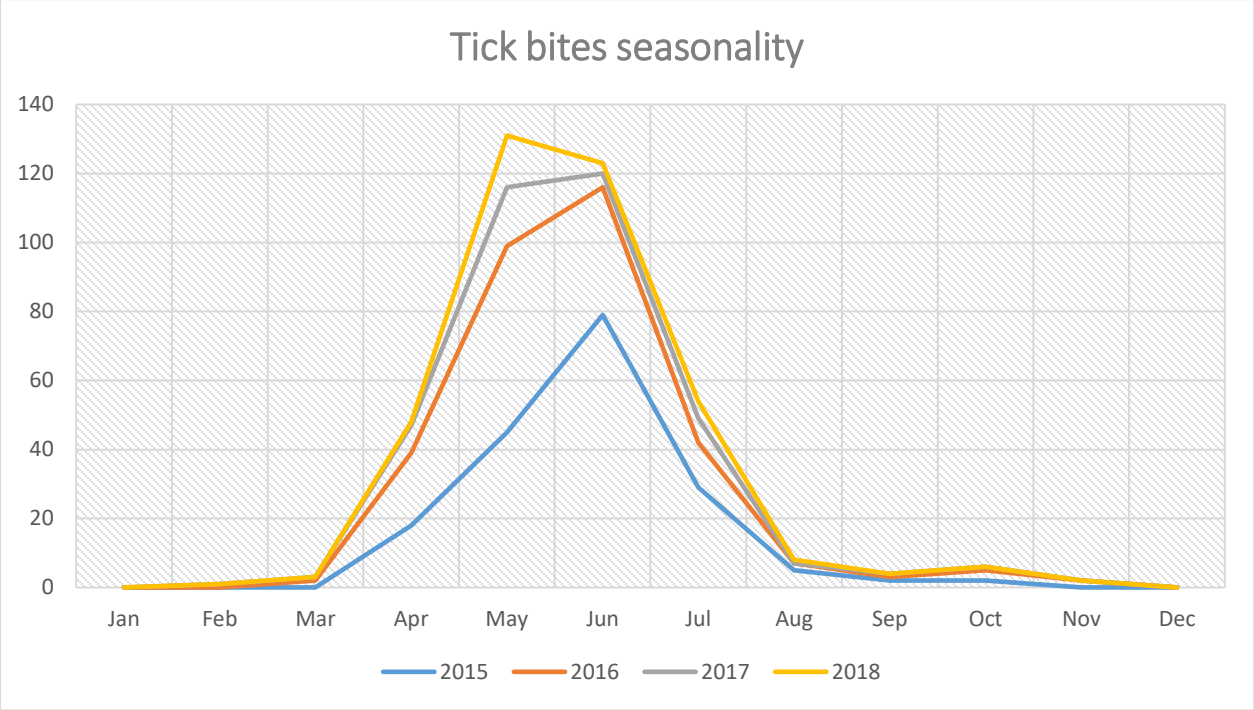


Figure 3. Tick bites seasonality. Axis represents the number of tick-bites.

5.5. Previous tick bites

Nearly half of the subjects reported having experienced previous tick bites, which, however, were only sporadically (6/179 cases) accompanied by only local clinical signs (Table 1).

5.6. Local manifestation from the tick bite

20 (5.2%) subjects had more than one tick embedded in the skin simultaneously, as observed during the first visit (Table 1).

When considering the index bite, in half of the cases, it was accompanied by a visible local lesion, and in 1/3 of the cases, there was a local reaction (Table 1).

5.7. Tick identification

Similarly, in around 1/3 of the cases, the tick was completely removed and preserved during removal (Table 1). Moreover, 98 ticks have been possible to identify (preserved during 2015, 2016, and 2017), and all were identified as *Ixodes ricinus;* 73 of these ticks were adult females and 25 nymphs.

During 2015, from 54 identified ticks, 39 were adult females, and 15 were nymphs.

During 2016-2017, from 44 identified ticks, 34 were adult females, and 10 were nymphs.

From the ticks identified in three patients who developed EM, two were *Ixodes ricinus* adult females and one nymph.

5.8. Current active and chronic diseases of the patient (at the moment of tick bite)

Around 1/3 of the subjects suffered from some systemic chronic disease. The majority of the subjects suffered from arterial hypertension (medicated), then diabetes mellitus, dyslipidemia, rheumatismal problems, thyroid gland disease, depressive syndrome, thrombosis, and chronic viral hepatitis B. One patient was with prostate carcinoma, and one patient with pulmonary carcinoma. There were three pregnant women.

5.9. Duration of tick attachment

Over half of study participants (198/380) were able to estimate (obviously not with accuracy) the duration of tick attachment. Only one subject declared less than 24 hours, 95 of them declared for about 24 hours of the tick attachment, 48 subjects for 48 hours, 27 for 72 hours, 19 for 96 hours, 8 for 7 days. In 16 (16/34) patients with EM that were able to estimate the time of tick attachment, only three patients reported for 24 hours; the others reported for more than 48 hours (one for 7 days) of the duration of the tick attachment. The identification of ticks, swallowed or engorged ticks, approximately corresponded with the time of embedded tick estimated from subjects.

5.10. Tick bite and body part

The body parts affected with tick bite were: head 10/380 (2.6%), neck 10/380 (2.6%), trunk 121/380 (31.8%), upper extremity 69/380 (18.3%), and lower extremity 170/380 (44.7%) (Table 3, Figure 4, Figure 5).

For both men and women, the lower extremities were the primary anatomical location of tick attachment (46.5% of 105 ticks on men and 42.2% of 65 ticks on women) (Table 3); followed by the trunk (29.2% on men and 35.8% in women) and upper extremities (21.2% in men and 13.6% in women). Women were more likely to be bitten by ticks in the head (5.2%) compared to males (0.9%). Only in a small number of males (1.3%), tick bites in the genital area were recorded.

Table 3. Tick bites by region of body part and gender.

Body Region	M	F	Total		% Male	%Female
Head	2	8	10	2.6%	0.9%	5.2%
Neck	5	5	10	2.6%	2.2%	3.2%
Trunk	66	55	121	31.8%	29.2%	35.8%
Upper extremity	48	21	69	18.3%	21.2%	13.6%
Lower extremity	105	65	170	44.7%	46.5%	42.2%
Total	**226**	**154**	**380**	**100.0%**	**100.0%**	**100.0%**

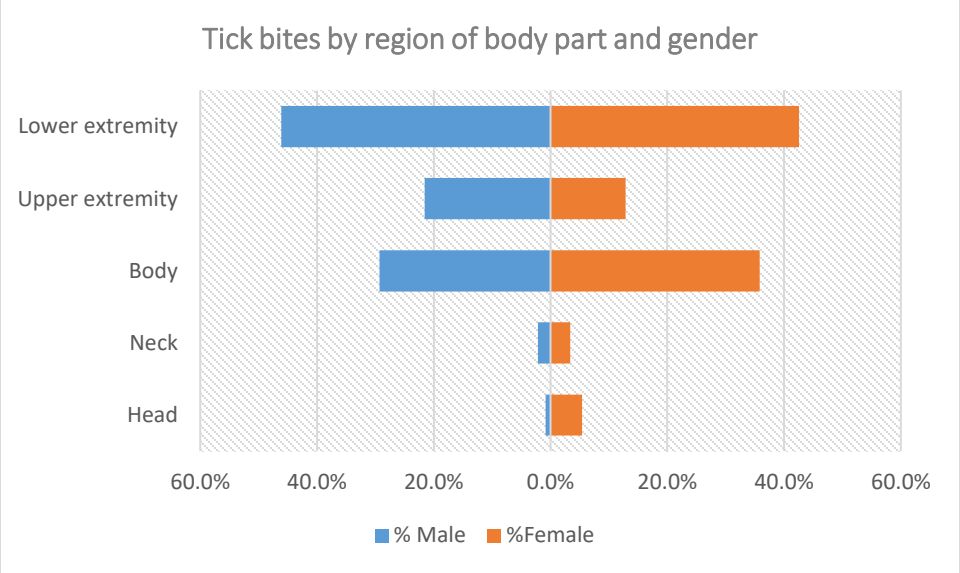


Figure 4. Tick bites by region of body part and gender.

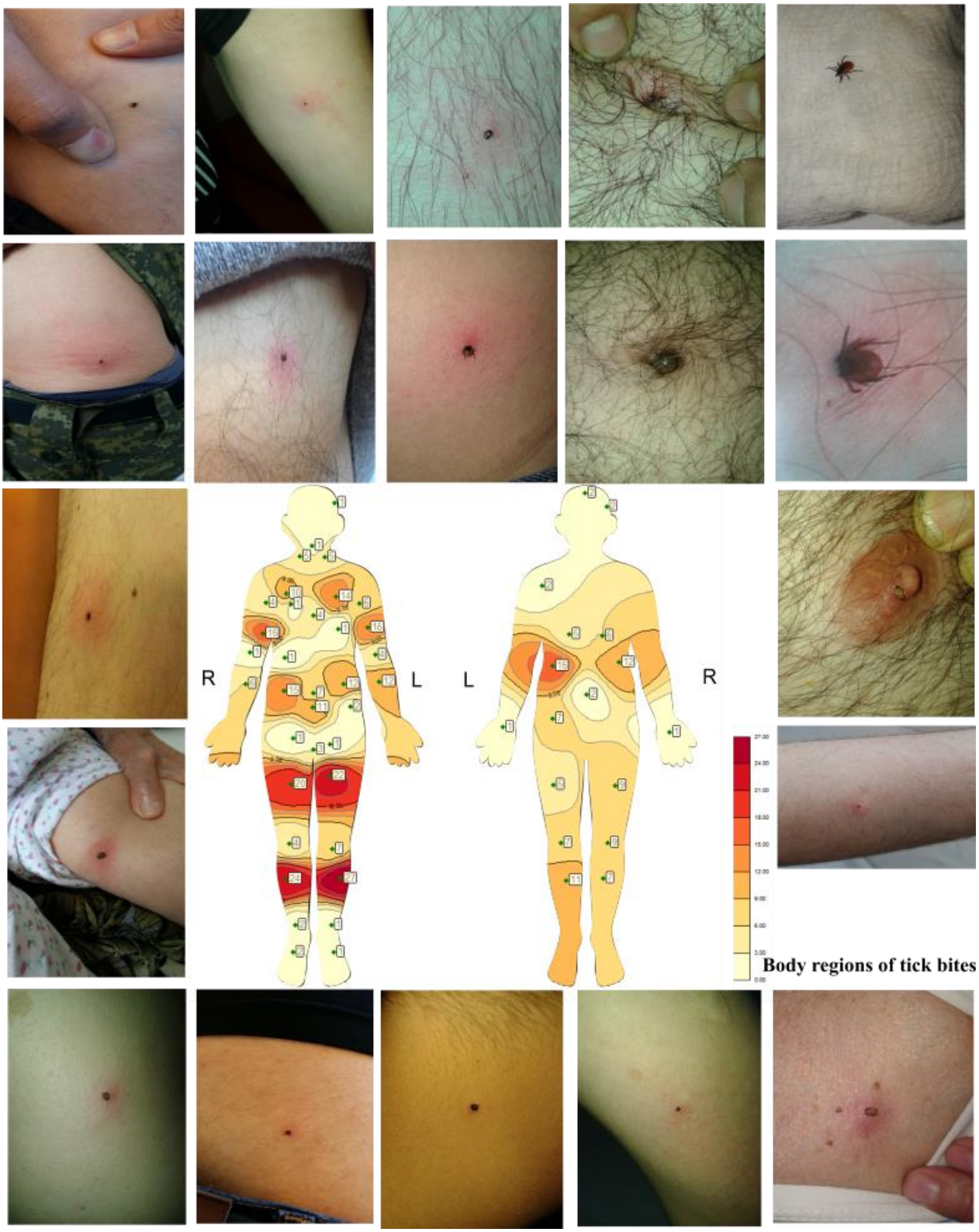


Figure 5. Body regions of tick bites.

5.11. Serological results in the first visit

At the first visit, 43 (11.3%) of 380 subjects had positive serology for IgG or IgM. The majority of these patients were from the urban area (33/43; 76.7%).

IgG seroprevalence among subjects during the first visit in the study was 28/380 (7.4%), with 17 male and 11 female. Among subjects with detectable specific IgG antibodies, 16 were >59 years (7 of them were > 70 years of age) of age, while 12 were < 59 years of age.

In 31/43 (72%) subjects with positive serology in the first visit, the second blood sample during the second visit was taken. From 15 IgM positive subjects in the first visit, the second blood sample during the second visit was taken in 11 of them (4 of them refused to give the second sample), and 8/11 subjects were positive for IgM again in the second visit. Further on, from 28 patients who were positive for IgG in the first visit, the second blood sample was taken in 20 of them, and 16/20 subjects were positive for IgG in the second visit too. More than half of subjects (16/28; 57.1%) with IgG positive in the first visit declared a previous tick bite. Only one patient with detectable IgG on the first and the second visit reported possible early LB in the past.

5.12. Antibiotics prescribed in the first visit

Overall, for 69 (18.3%) patients, antibiotics were prescribed, typically amoxicillin+clavulanic acid. However, 7 patients were being treated with antibiotics – for other reasons - at the time of the bite (Table 1).

In the first visit, doxycycline was prescribed as prophylaxis in 12 patients; and amoxicillin in 8 patients (Table 1). In all of them, embedded ticks were swollen, suggesting a longer duration of attachment.

5.13. Clinical characteristics of the subjects in the second visit

At the 2nd visit, occurring 55-65 days after the first one, 377 subjects were observed: Three were lost for further follow-up; 2 were lost to follow-up, and 1 died of prostatic cancer (Table 4); 38 (10.1%) of these subjects experienced another tick bite in the meantime. Overall, 13 (3.5%) experienced at least one systemic symptom since the 1st visit – most commonly fatigue, joint pain, headache, fever, and chills (Table 4). Only 7 subjects experience two or more systemic symptoms (Table 4). Local symptoms were more common: burning and itching were each reported by 15 (4.0%) subjects, and EM was observed in 74/377 – 19.6% of those seen at the 2nd visit (Table 4) and 74/380 -19.5% of all initially included in this study.

Table 4. Subject characteristics at the 2nd visit for the index tick bite (3 drop-outs; one died of prostate carcinoma, 2 lost to follow-up) and at the 3rd visit (38 additional lost to follow-up).

	All	2015	2016	2017-18
2nd visit (55-65 days since the 1st visit) N	377	179	135	63
Another tick bite since the last visit	38 (10.1)	24 (13.4)	11 (8.1)	3 (4.8)
Any systemic symptom since 1st visit	13 (3.5)	8 (4.5)	4 (3.0)	1 (1.6)
Increased body temperature	4 (1.1)	---	---	---
Chills	4 (1.1)	---	---	---
Muscle pain	2 (0.5)	---	---	---
Joint pain	5 (1.3)	---	---	---
Headache	4 (1.1)	---	---	---
Dizziness	2 (0.5)	---	---	---
Cognitive impairment	1 (0.3)	---	---	---
Neck pain	1 (0.3)	---	---	---
Appetite loss	0	---	---	---
Weight loss	0	---	---	---
Fatigue	6 (1.6)	---	---	---
Two or more systemic symptoms	7 (1.9)	5 (2.8)	2 (1.5)	0
Burning at bite site	15 (4.0)	9 (5.0)	6 (4.4)	0
Itching at bite site	15 (4.0)	8 (4.5)	7 (5.2)	0
Erythema migrans	74 (19.6)	30 (16.8)	23 (17.0)	21 (33.3)
≥2 systemic OR local OR *erythema migrans*	76 (20.2)	30 (16.8)	25 (18.5)	21 (33.3)
IgG positive/negative/intermediate/not done	25 (6.6)/335/5/12	15 (8.4)/160/2/2	6 (4.4)/121/2/6	4 (6.3)/54/1/4
IgM positive/negative/intermediate/not done	34 (9.0)/320/11/12	17 (9.5)/156/4/2	10 (7.4)/117/2/6	7 (11.1)/47/5/4
3rd visit (6 months after the 1st visit) N	339	161	121	57
Any late disease manifestation	3	2	0	1
Palpitation and dyspnea+arthralgia+fatigue	1	---	---	---
Erythema migrans recidivans and multiple erythema disseminata	2	---	---	---

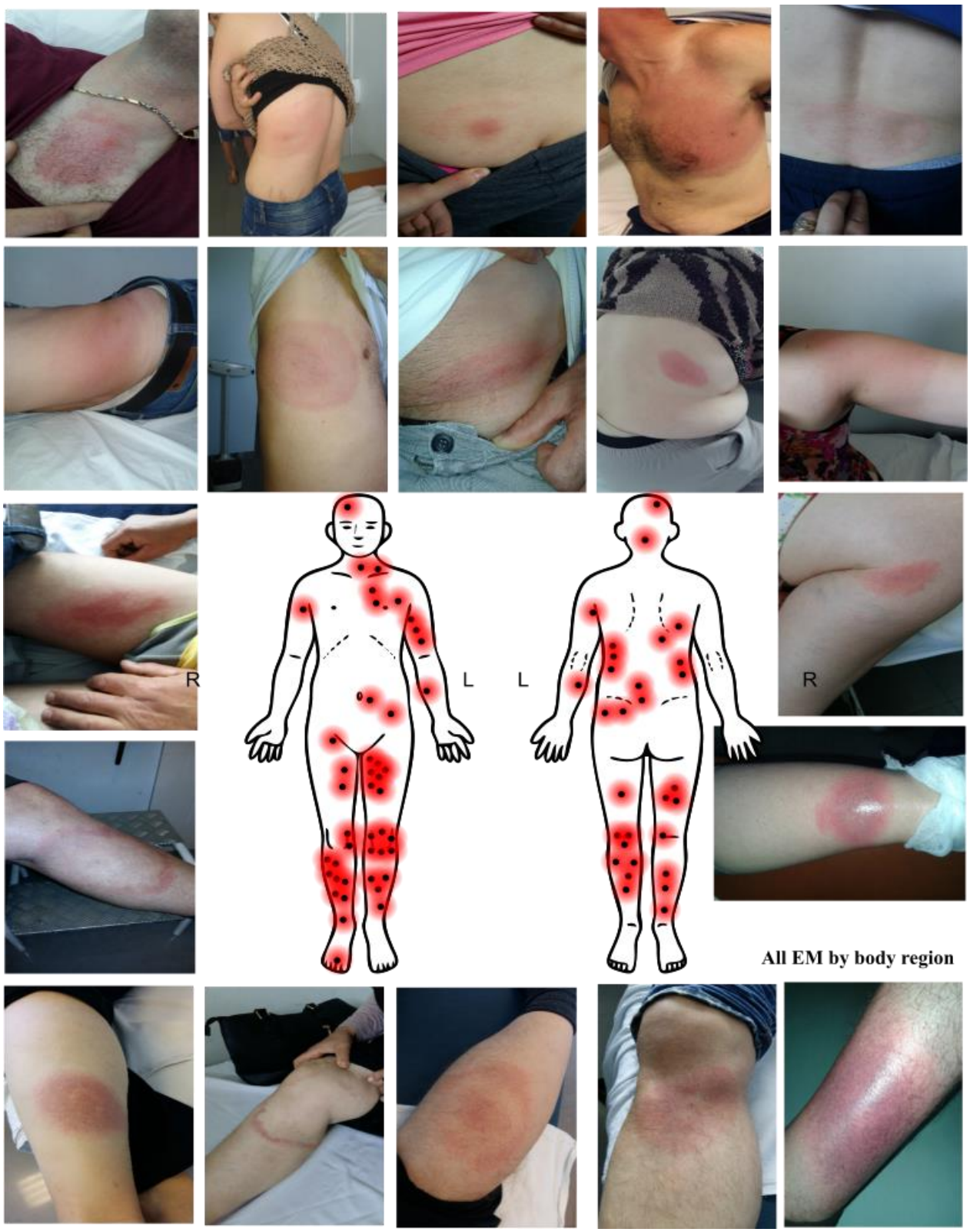


Figure 6. All 74 EM by body region and some photos taken from the patients included in the study.

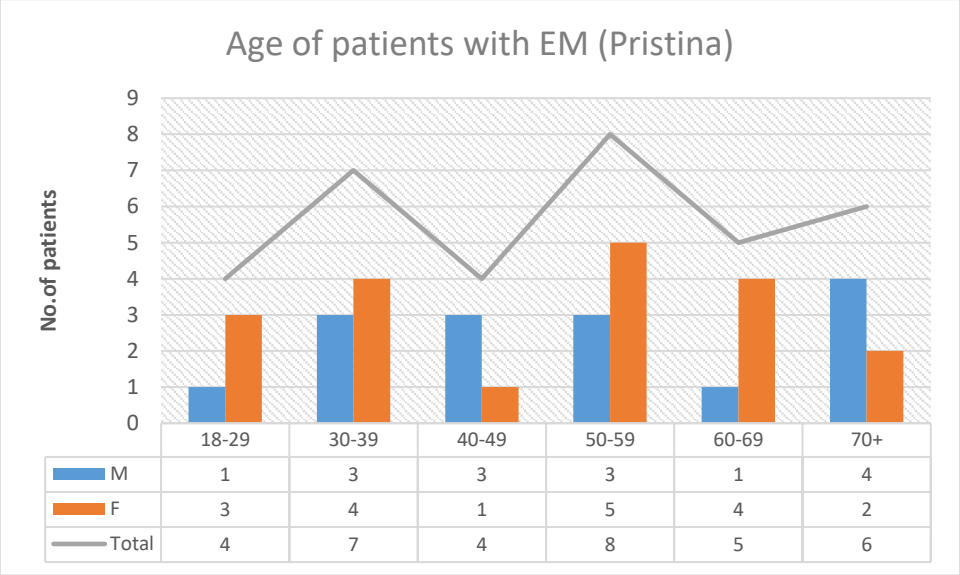
EM was accompanied by itching and burning in 12 (16.2%) patients.

Accompanied joint pain was present in three (4.0%) patients with EM. One patient treated as EM manifested chills, lymphadenopathy, headache, and neck pain.

It can be considered that subjects who between the two visits had developed two or more systemic symptoms, or had local symptoms (itching, burning), or had developed *erythema migrans* – (Figure 6), actually developed a clinically manifest disease: there were 76/377 such patients, i.e., 20.2% (95%CI 16.4-24.5) of those examined at the 2nd visit (Table 4) or 76/380 - 20.0% (95%CI 16.1-24.4) of all included in the study.

5.14. Demographic and geographic characteristics of patients in the Municipality of Pristina

34/274 (12.5%) patients from the Pristina municipality were clinically diagnosed with EM, 19/34 (56%) female, and 15/34 (44%) male. The patients were aged 26-76 years, with an average age of 50 (SD±15.5), with the predominance female sex (Figure 7).



	18-29	30-39	40-49	50-59	60-69	70+
M	1	3	3	3	1	4
F	3	4	1	5	4	2
Total	4	7	4	8	5	6

Figure 7. Age-group and gender of patients with EM in the Pristina region.

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Among patients with EM from the Pristina municipality, 26 of 34 (76.4%) were from Pristina city, and 8 (23.5%) patients were from villages around Pristina: 2 patients from Siqeva village, 2 from Matican and 1 from Mramor, Hajvali, Llukar and Koliq villages (Figure 8).

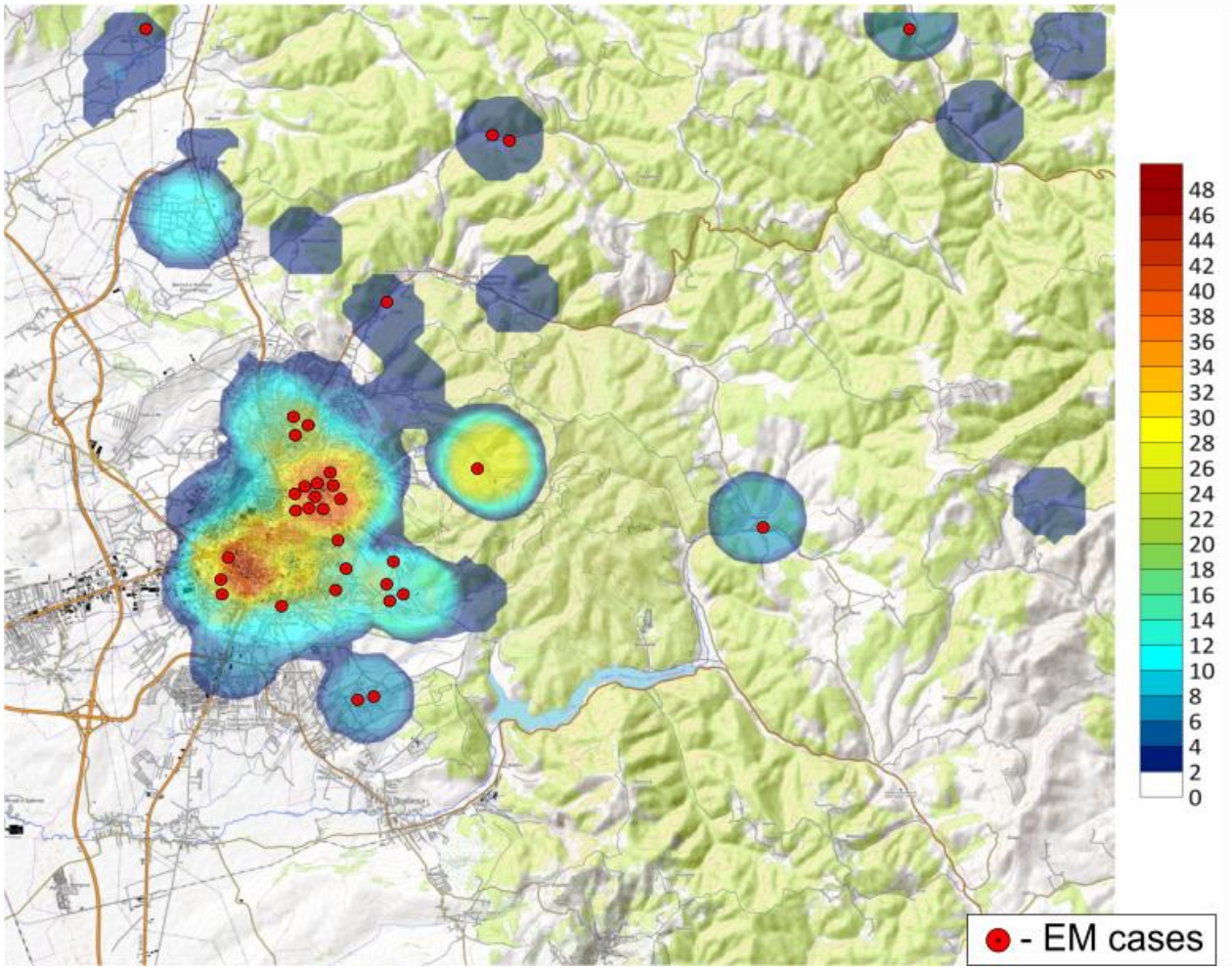

Figure 8. Map of the Pristina Municipality: distribution of tick bites (represented in color-bar) and EM.

5.15. Demographic characteristics of patients with EM from other municipalities of Kosovo

Among subjects from other parts of Kosovo, EM was diagnosed in 40 out of 106 patients; 18 (45%) were female and 22 (55%) male. The patients were 18-80 years old, with an average age of 44 years (SD±16.5), with a slight predominance of males (Figure 9).

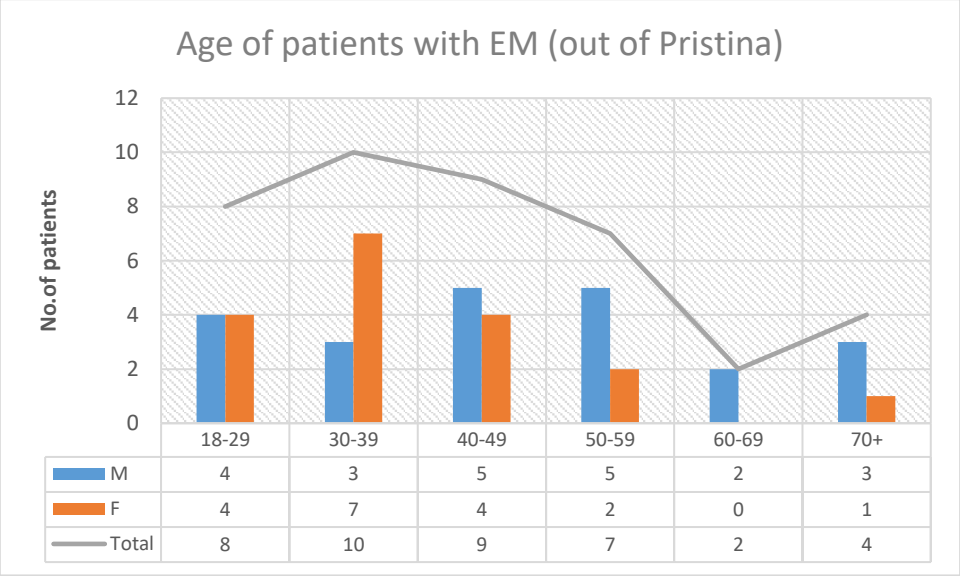

Figure 9. Age-group and gender of patients with EM outside of Pristina Municipality.

5.16. Seasonality of clinical manifestations

EM was mainly diagnosed in May and June. One patient with EM was diagnosed in March 2017, and one in November 2016. No EM cases were recorded in the winter months (December to February) (Table 5, Figure 10).

Table 5. Seasonality of EM 2015-2018. The number of cases per month.

Seasonality of EM

Year	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total
2015	0	0	0	3	4	19	4	1	0	0	0	0	31
2016	0	0	0	4	7	5	2	1	0	2	1	0	22
2017	0	0	1	2	3	1	5	0	0	0	0	0	12
2018	0	0	0	0	3	2	4	0	0	0	0	0	9
Total	**0**	**0**	**1**	**9**	**17**	**27**	**15**	**2**	**0**	**2**	**1**	**0**	**74**

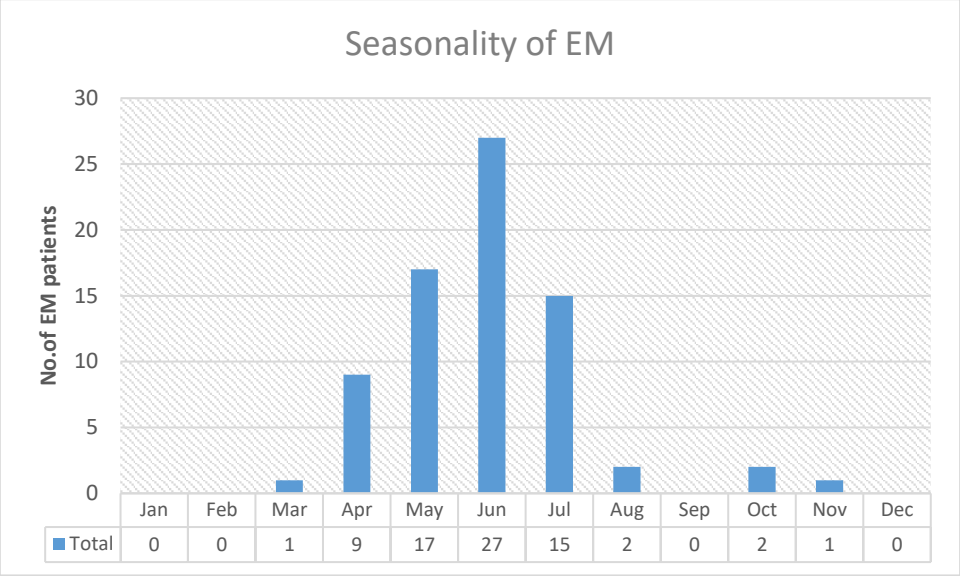

Figure 10. Seasonality of EM patients 2015-2018. The number of patients per month.

5.17. Clinical manifestation and serology

From the overall of 74 patients with EM, 57 of them were seronegative in the first visit. Whereas the second serology was provided in 56 of them (one of them refused to give the second sample), and 15/56 (27%) patients experienced seroconversion, 3.9% of all subjects included in the study. Seven patients from IgG and IgM negative in the first visit had become positive for only IgM in the second visit, two patients seroconverted only for IgG, and six patients became positive for both IgG and IgM in the second visit. On the other hand, 8 patients with positive IgG in the first visit manifested EM.

Fifty-six of 74 patients with EM had short incubation <14 days and prompt access to treatment. Positive serology (seroconversion) from that of negative in the first visit in this group was only in 5 patients (8.9%). While 18 of 74 patients with EM who had long incubation equivalent or >14 days, or delayed treatment (EM without treatment for more than 1 week –range 1-6 weeks) which correlated with the large size of EM, resulted with positive serology in 10 of them (55.5%), resulting in statistically significant for *p*-value<0.05 (Chi-square statistic is 18.3241, the *p*-value is 0.000019).

Ten (2.6%) asymptomatic patients had positive serology for IgM in the second visit, while in the first visit, six of them had intermediate IgM.

5.18. Treatment of the patients with EM

Doxycycline (100 mg PO bid) was prescribed in 63 (85.1%) patients for treatment of EM, amoxicillin (500 mg PO tid) in eight (10.8%) patients while in three patients (4.0%) a 3rd

generation cephalosporin (ceftriaxone - 2g per day i.v.) was applied, with a duration of treatment from 10 to 21 days. EM was treated successfully, without complications in all patients, and the skin change withdrew in all patients within 3 to 14 days. Only two patients manifested with EM *recidivans* and multiple EM *diseminata* two months later. The patient with EM *recidivans* was initially treated with amoxicillin, while the patient with multiple EM was treated with doxycycline. After the second course of treatment with doxycycline (100 mg bid) for 14 days, both patients experienced prompt recovery. Three patients with EM were treated with topical corticosteroids before the diagnosis of EM was established, which was followed with exacerbation of the skin change. Nevertheless, prompt recovery of the skin change in those patients was achieved after proper antibiotic therapy.

5.19. Clinical characteristics of the follow-up (third visit)

At the final visit, 6 months after the 1st one, 339 subjects were observed (the rest were lost to follow-up). During this period, only 3 had developed some late disease manifestation, two patients developed *erythema migrans recidivans and multiple erythema migrans*, respectively, three months after tick bite; and one patient experienced: fatigue, arthralgia, palpitation, and dyspnea after more than two months from the tick bite (77 days) (Table 4), with ECG (electrocardiography) showing of right bundle branch block-RBBB (without any treatment from the cardiologist), and with seroconversion (IgM positive) in the second visit. The patient was admitted to the Clinic of Infectious diseases for monitoring. Chest x-ray revealed signs for bilateral mild bronchopneumonia and was treated with a 3rd generation cephalosporin – Ceftriaxone. The same patient was treated five days with Amoxicillin + Clavulanic acid in the first visit for local skin reaction from tick removal manipulation.

5.20. Factors related to the probability of developing a clinically manifest disease

Since only 3 subjects developed a late disease manifestation, we considered findings of the 2nd visit as primary to evaluate factors related to the probability of developing any clinical form of the disease (which most commonly was *erythema migrans*, Table 4). We considered data depicted in Table 1 (calendar year, geographical location, tick preservation at removal, use of antibiotics at the 1st visit/prescription of antibiotics at the 1st visit, age and sex) and in the textual part of results (duration of tick embedment; subheading 5.9) as independent, i.e., potentially explanatory variables. Table 6 summarizes the multivariate model fitted to the probability of clinically manifest disease. Data suggest a higher risk in years 2017 and 2018 (combined) vs.

2015 (RR=1.67, 1.00-3.04), and association of complete tick removal (RR=0.10 (0.02-0.47) and of use/prescription of antibiotics at visit 1 (RR=0.29, 0.13-0.64) with a reduced risk of a clinically manifest disease. Patients from the Prishtina region also less commonly developed a manifest disease compared to patients from other Kosovo regions (Table 6). The length of tick embedment apparently was not associated with a risk of developing a manifest disease, but, as already pointed-out (subheading 5.9), there was uncertainty about accuracy of the anamnestic data in this respect.

Figure 11 shows the estimated probabilities of developing a clinically manifest disease (as assessed during the 2nd visit), derived from the fitted model (Figure 11 is for illustrative purposes).

Table 6. Predictors of developing clinically manifest disease after a tick bite (as assessed at visit 2).

		Model-generated effects		Multiplicity-adjusted[2]	
	Effect levels	RR (95% CI)	P	95% CI	P
Of primary interest					
Calendar year (linear trend)[3]	2015 (n=179)	1 (reference)			
	2017&2018 (n=63)	1.67 (1.07-2.63)	0.025	1.00-3.04	0.046
Tick preservation at removal	No (n=260)	1 (reference)			
	Yes (n=117)	0.10 (0.03-0.33)	<0.001	0.02-0.47	<0.001
Tick embedment (hours)	≤72 (n=170)	1 (reference)			
	>72/unknown (n=207)	1.01 (0.66-1.55)	0.971	0.57-1.78	0.971
Antibiotic use at visit 1/after	No (n=304)	1 (reference)			
	Yes (n=73)	0.29 (0.13-0.64)	0.002	0.10-0.83	0.007
Geographical location	Not Prishtina (n=105)	1 (reference)			
	Prishtina (n=272)	0.40 (0.28-0.57)	<0.001	0.25-0.64	<0.001
Further adjustments					
Men (vs. women)	---	0.73 (0.51-1.04)	0.077	---	---
Age (by 1 year)	---	1.01 (0.99-1.02)	0.229	---	---

[1]A modified Poisson regression model with robust error variance was fitted to probability of a manifest disease in which calendar year, tick preservation, use/prescription of antibiotics, duration of tick embedment (>72 hours or unknown vs. ≤72 hours) and geographical location (Prishtina region vs. all others) were independents of primary interest, while age and sex were further adjustments.

[2]Estimates and covariance from the fitted model were retained and used to adjust all estimates of the effects of primary interest and P-values for multiplicity (logical stepdown with a simulation method).

[3]2016 (n=135) was not included because with a three-level polytomous predictor, as calendar year in this case, linear trend is tested as a contrast between the highest ordered, e.g., years 2017&2018, vs. the lowest ordered level, i.e., 2015. The probability of a manifest disease in year 2016 was slightly higher than in 2015 and lower than in years 2017&2018. To avoid uninformative tests/contrasts and to reduce multiplicity – we tested the linear trend and not "all possible comparisons".

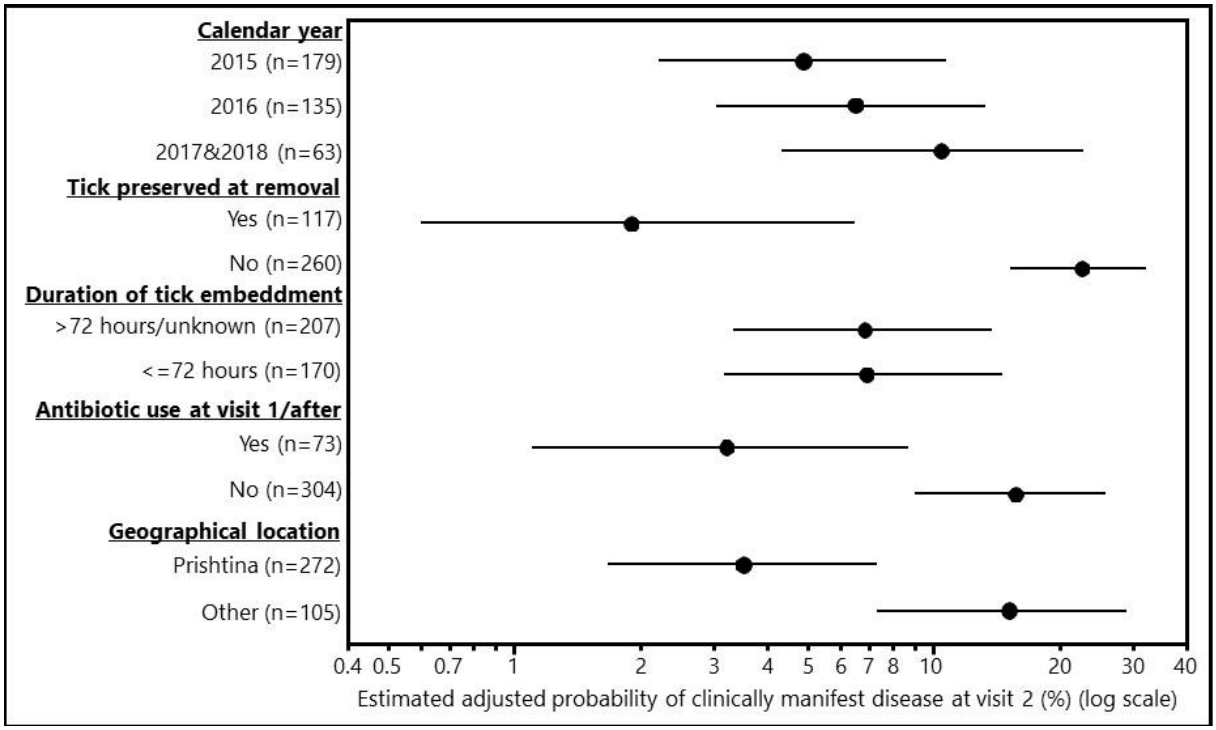

Figure 11. Predicted probabilities of developing a clinically manifest disease –derived from the model in Table 6. Circles are point-estimates, bars are 95% confidence intervals.

6. Discussion

At the time of its discovery, Lyme borreliosis was considered as a recently emerged, rare disease with regional occurrence. Although published evidence now indicates that infection with similar bacteria existed in Europe from the ice age, several aspects of the disease still remain controversial due to scarce evidence-based data (44,75).

According to the World Health Organization estimates, the annual number of reported LB cases in Europe exceeds 85,000, which is probably an underestimated number because of the inconsistent case report mechanism and under recognition of disease manifestations (46,52,58,59).

In the United States, around 30,000 new LB cases are reported annually to the Centers for Disease Control and Prevention, even though the current estimation of the total number of cases suggests being tenfold higher, considering unreported cases (59,75,88,92,93).

Investigation over the past decades revealed that the effect of climate change on the spread of vector and reservoir hosts, and also increased awareness for the disease among healthcare workers and the general population might have reflected on the reported LB incidence increase in Europe and the United States (46,59,93).

Several factors, such as the geographical distribution of the tick population, the rate of *Borrelia burgdorferi* infection in the tick population, and the tick species presence in certain regions could influence the risk for developing LB after a tick bite. Other factors such as climate, the intensity of human recreational and/or professional activities, and the presence of the vertebrates that are reservoir hosts for the tick-borne pathogens play a significant role, as well (94). On an individual level, the risk for developing LB depends on the duration of tick attachment, too (2,13,18,19).

Investigation performed by Kurteshi et al. on Kosovo's tick population showed the presence of *I. ricinus* in the country (40). They sampled and tested 795 ticks (only adult ticks could be collected) in the years 2014/2015. The most abundant questing tick species in Kosovo collected by flagging (n = 340) was I. ricinus (64.2%), followed by *D. marginatus* (28.8%) and Haemaphysalis spp. (7%). *Borrelia burgdorferi s. l.* was detected in seven of the 340 flagged ticks (2.0%) from Kosovo, two of 134 I. ricinus (1.5%), and five of 98 *D. marginatus* (5.1%), and was found only in the Mitrovica region (northern Kosovo) (40).

In the present study, 117 ticks were preserved, 98 of these ticks were removed entirely, and they were preserved for further identification - all of which were identified as *Ixodes ricinus* adult females or nymphs. Our finding is consistent with previously published results founding

Ixodes ricinus nymphs and adult females as tick's life stage forms mainly responsible for the majority of tick bites in humans (18,94–96).

In endemic areas, 30% or more of nymphs may be infected with *B. burgdorferi*, the rate of infection in adult ticks may be even higher, while infection rates in unfed larvae are less than 1% (36,52,93).

In the study of Jouda et al., in Switzerland, adult ticks were also significantly more infected (129/366, 35%) than nymphs (109/552, 20%), and there was no correlation between nymphal density and infection prevalence as well as between adult density and infection prevalence. However, there was a correlation between the density of ticks and the density of infected ticks (97).

Our study reflects the risk of human LB after a tick bite. There are only a few publications that study the development of LB from tick bite perspective, which is considered one of six crucial key indicators for surveillance of LB proposed by Wijngaard et al. (63). Online citizen-based reporting of tick bite has been implemented in the Netherland, Switzerland, and Belgium, reflecting the distribution of tick bites and, consequently, the human exposure to the risk of LB. In the future, this approach should also be considered in our country (63).

A total of 380 subjects with tick bites were included in the present study. Subjects in the study were older than 18 years of age (18-91) with a median age of around 48 years and a male predominance. A Dutch research project based on ticks collected from human patients in participating hospitals found children below 10–15 years and adults between 50 and 69 years were bitten most commonly, suggesting differences in tick exposure and leisure behavior as possible explanations (98–100).

Tick bites, during this study, were recorded in the period from March to October. Only one case occurred in February and two cases in November.

The majority of bites occurred during May; only in 2015, the peak tick activity month was June. Previous studies have also shown that the questing activity of the *I. ricinus* ticks lasts from March to October, although local climatic conditions can modulate the beginning and the end of tick activity (53).

The climate of Kosovo is mainly continental with the Mediterranean and Alpine influences, characterized by warm summers and cold winters (average daily temperature within the country ranges from +30 °C (summer) to –10 °C (winter)). However, due to unequal elevations in certain parts of the country, there are significant differences in temperature and rainfall distribution (101,102).

Depending on regions, ticks start to search for blood meals in early or late spring: in the Czech Republic, it is reported that this usually occurs in March or April, whereas in Latvia, where the climate is colder, the tick's searching for blood meal starts later (52). A Swiss study reported that the majority of tick bites from May to July during 3 consecutive years (103), which is similar to findings in our study. Another study from southern Sweden showed that tick bites occur most frequently in July. These seasonality patterns are explained by the fact that July is the main summer holiday month in Sweden, also related to the hunting season and mushroom/berry picking season (52).

Overall, two patterns (unimodal and bimodal) of seasonal tick questing activity have been reported in Europe and may vary between years and regions. The months of spring and early summer seasons are important ones for the questing activity of *I. ricinus*, while tick activity decreases during warm summer months and increases again in autumn (53). With early spring and autumn peaks, a bimodal questing activity has been described for all life stages of *I. ricinus* in highly seasonal climates such as that of central Europe (96). In milder climates, with less climatic variation between seasons, only one peak of activity was observed for all life stages: either spring or early summer (96).

The peak months of tick bites in our study were May and June, which indicates a unimodal pattern of seasonal tick questing activity.

We found that for patients of both genders, the lower extremities were the most common site for tick attachment (46.5% in males and 42.2% in females); followed by the trunk (29.2% in men and 35.8% in women), and the upper extremities (21.2% in men and 13.6% in women).

Other studies have also shown the lower extremities as the primary site for tick bites in adults, as in the study performed in the highly endemic Swiss area. In this study, lower limbs were the most common site of tick attachment in both men and women (44.4% and 40.7%, respectively). Only children younger than 10 years old were bitten more frequently on the head (41.2%) and the neck (38.5%) (103). In the study of Wilhelmsson et al. for Northern Europe, the legs were the major site for tick attachment (49% of 1051 ticks on women; 51% of 597 ticks on men), followed by the trunk (20% and 24%, respectively) and the upper extremities (19% and 17%, respectively). With a significantly greater proportion of men (9%) than women (5%) recorded ticks attached to the groin/genital area, and with a greater proportion of woman (5%) than men (1%) in the head/neck area (104), which is similar to findings observed in our patients.

In Bennet et al. study on solitary EM distribution, the legs (63.6%) were the most common site of the lesion, followed by the trunk (24.6%), arms (10.2%), and genitalia (1.7%) corresponding

with a distribution of tick bites which has been observed in the patients in the present study, as well (35,104).

According to the patients' declaration of the time when they last visited the location of a possible tick bite, more than half of the subjects (198/380) in our study were able to estimate the duration of tick attachment. Additionally, they reported that the majority of ticks were removed within 24–48 hours (obviously not with accuracy) from the attachment. In Cook's review on transmission time after tick attachment, only 50% of subjects were able to estimate tick attachment times and thought to be more than 24 hours (105). During the identification of preserved ticks in our study, swallowed or feed ticks corresponded approximately with the duration time of embedded tick estimated from the subjects. In the study conducted in Sweden, the majority of ticks were removed 24–48 hours after the attachment. However, the researchers found that the tick-bitten persons usually underestimated the duration of tick attachment, compared with the calculation based on scutal and coxal indices (104).

In the municipality of Pristina, 16 patients from 34 with EM were able to estimate the time of tick attachment. Only three patients reported 24 hours; the others reported the tick attachment for more than 48 hours (one case for 7 days). In patients who manifested EM, the tick could be identified in three of them: two adult females and one nymph.

Hugli et al., in their study in a highly endemic area in Switzerland, have found that most nymphs were removed after 24h of a blood meal. In contrast, most adult females were removed in less than 24h, which was expected that the larger, adult female ticks would be detected sooner than the smaller and less visible nymphs (103). In contrast, in the study from Northern Europe (Sweden and Åland Islands), it was not found any significant difference between adult female ticks and nymphs regarding the time from attachment until the tick was detected and removed, suggesting that this could be due to differences in the participants' awareness as well as people's knowledge about tick-infested habitats (104). Moreover, it was found that the location of the attachment site might influence how soon a tick will be detected when Wilhelmsson et al. in their study found that ticks attached to the head/neck area or the groin/genital area were seemingly more challenging to detect than ticks attached to other sites (104).

Several studies have shown that increased risk for infection is related to occupations and hobbies that increase tick exposure (forestry workers, hunters, and hikers) (94). Our study comprises the general population, and only 15 (3.9%) of tick bites were related to professional activities. A study on tick bites and the risk of human infection by *B. burgdorferi* showed that this can also happen during visits to a vector habitat, when host mammals and their associated

ticks migrate into the urban environment, or when companion animals bring ticks into areas of human habitation (94). 26 of 380 (6.8%) subjects in our study reported that the garden was the place where the tick bite occurred. Enkelman et al., in their study, also reported that in the majority of cases, tick exposure occurred in a residence district. A citizen science research program on tick-bites in the Netherlands suggests that tick-bites might often happen close to home. After bites in forests, gardens were the second most commonly mentioned places (31%) where tick-bites occurred (98–100).

Similarly, in another study in Europe, EM was the most common clinical manifestation observed during our study (98,100). In the study of Enkelman et al., the most commonly reported clinical manifestation was EM only, with similar proportions as the 94% recorded in French sentinel data (98,100).

In our study, 74/380 patients were diagnosed with EM only – 19.5% (95%CI 15.6-23.8). In the municipality of Pristina, 34/274 (12.5%) of subjects had EM as a clinical manifestation of LB after the tick bite. EM is the hallmark of the early disease; it is a localized skin infection, which occurs at the site of a tick bite (94). Accurate clinical identification of this lesion is essential for a correct diagnosis, as serology is negative in 60% of patients early in infection (106).

EM developed in 34 patients in the municipality of Pristina with an average age of 50 years, with female predominance - 19/34 (55.8%). In patients from other Kosovo regions, EM was diagnosed in 40 patients, with a predominance of males - 22/40 (55%). However, the number of females and males was equal in the overall number of EM during the study. The predominance of women and girls among EM patients has been reported in various epidemiologic studies in Europe (93). The incidence of LB is higher among adult women (55%) than among men (45%) in Slovenia and Germany (94). In most European countries, studies on epidemiological data have revealed that there is a bimodal age for disease occurrence, with peaks incidence occurring in the age groups of 5 to 15 and 40 to 60(46). In a Finnish study by Sajanti et al., 59.7% of clinically diagnosed LB cases occurred in women and girls. In the 50–79-year age group, EM incidence was notably higher among women. With a higher incidence of clinically diagnosed LB only among boys 5–15 years of age and men >80 years of age (93). In our study, the predominance of male gender among patients with EM > 70 years of age was observed throughout Kosovo.

Enkelman et al., in their study, likewise observed that in Germany, females more frequently experienced EM after a tick bite, whereas extracutaneous forms were more frequent among males. The same findings were reported in a Slovenian study with gender differences between

cutaneous and non-cutaneous manifestations of LB (22,98). The biologic or immunologic mechanisms that might explain why older men would be more likely to have disseminated LB than women are unknown. Enkelman et al., in their study, try to give the biological hypotheses that lower infection rate with genospecies that have an affinity to affect the neural tissue or joints and different duration of spirochetemia leading to disseminated forms according to gender, albeit there is not sufficient evidence (98). In another study, attempts were made to give another explanation highlighting that women might tend to notice EM more often, or they might seek the healthcare services more actively while still in the EM stage of the infection, thus probably preventing the development of late clinical manifestations (93).

In the United States, the age distribution of Lyme borreliosis is typically bimodal, with peaks in children 5-15 years of age and adults 45-55 years of age. In those <60 years of age, the incidence of LB is higher among males than females, whereas in older age groups is slightly higher among females (94).

We found that June was the peak month of EM in the study. In most of Europe and the northeastern United States, the peak months of disease onset are June and July, following the peak months of tick bites (80,94). In Finland, the EM manifestations are registered during the warm summer months, respectively, during June–September, and few EM cases are registered in Avohilmo during November–April (93).

Interestingly, close to 50% of patients had a history of previous tick bites in the present study. Only 6/179 (3.3%) reported that they noticed “clinical signs”- and exclusively local, and it is unlikely that they would have forgotten EM in the case that they had it with the previous bite! - EM may be missed, as they are often asymptomatic, sometimes unseen by the patient, when located in areas such as the back, neck, trunk, and groin and will eventually resolve without antibiotic therapy (94,106).

In a Dutch study of Den Boon et al., the observed increase in the number of tick bites and erythema migrans since 1994 was associated by the authors with increases in tourism in highly dense tick areas, newly planted forests in urban regions, and an increased number of horses, which also may be attributed partly to changes in ecological risk factors and human behavior (107).

During our study, subjects were all advised to report if they noticed any change in the skin at the site of the tick bite or any other complaints during the follow-up period (for six months). A bizarre behavior of the patients with EM was observed - some of the patients came for a visit to the clinic after two or more weeks after the EM had appeared (EM was enlarged). They did

not seek medical help because they did not have any other complaints. The other patient with EM in the back did not take the therapy because she was a breastfeeding mother. When she came for the second visit, in the examination, EM was two or three times larger. Only upon coming for the appointed second visit, the other patient reported that the skin change at the site bite had been present for around five weeks. She did not have any other complaints and did not seek medical help immediately.

EM was accompanied by local itching and burning in 12/74 (16.2%) patients. Accompanied joint pain was present in 3 (4.0%) patients with EM. One patient treated as EM (localization on the head) experienced chills, lymphadenopathy, headache, and neck pain.

Previous studies have shown that EM in Europe is caused by any of the *B. burgdorferi s.l.* genospecies; it expands slower than that evident in cases in the United States and is not usually accompanied by other symptoms. In the United States, where the infection is caused by *B. burgdorferi s. s.*, EM is often accompanied by malaise, fatigue, headache, arthralgias, myalgias, fever, and regional lymphadenopathy (94).

EM is the clinical manifestation of LB in 80% of patients in the United States; 18% of patients have nonspecific symptoms during summer without recognition of EM, and the remaining 2–3% present with a manifestation of early or late disseminated infection, such as facial palsy, trigeminal neuropathy or Lyme arthritis (94).

The clinical characteristics of *B. burgdorferi* infection in Europe more closely resemble the clinical characteristics of *B. afzelii* or *B. garinii* infection rather than the *B. burgdorferi* infection in the United States (94). Although *B. garinii* infection also starts in most cases as a solitary skin lesion, itching and burning symptoms within the lesion are more common, and local spreading is faster than with *B. afzelii* or *B. burgdorferi* infection (94).

EM is well-recognized among healthcare workers in Pristina and specialists in the Clinic of infectious diseases, especially in cases that recall a tick bite. Accuracy of EM diagnosis is highly dependent on the healthcare professional's skill in recognizing the clinical presentation (93).

Although typical EM is considered as a pathognomonic sign of LB, in general, the ability of clinicians to accurately diagnose EM is ambiguous because both over- and under-diagnosis have been reported (106). Moreover, if the EM is initially missed, misdiagnosed, or inadequately treated, the diagnosis of LB may be significantly delayed or completely missed (106).

Typically, EM can be circular or oval, but it can also be irregularly shaped as rectangular or triangular, especially when appearing on the neck or head (80,106). The average diameter of

EM is 15 cm but varies widely from lesions greater than 80 cm to very small ones, although extremely sized lesions are very rarely seen (37). A minimum diameter of 5 cm is required by definition for the diagnosis. Atypical EM lesions with a persistent small size called mini EM (miniature size) occur in 1% to 3% of European patients and microbiologic confirmation of the presence of *B. burgdorferi s.l.* or its DNA is required in such cases (80). Nevertheless, the size of EM lesion depends on disease duration. In our study, the patients with EM were more likely to have small EM and short duration of the disease before treatment since they have been advised to immediately report if noticing changes in the site of the tick bite. Previous studies showed that the average daily growth rate (largest diameter divided by disease duration) is 1.0 cm (80,108).

The common misconception that a bull's eye EM is the only diagnostic manifestation of Lyme disease continues to misguide both patients and practitioners in the United States. While 80% have a uniform red appearance of EM, only 19% have the stereotypical bull's eye appearance (106). In the United States, the most common misdiagnosis for EM is spider bites, which may be commonly over-diagnosed (106).

Atypical morphology of EM may make the clinical diagnosis more difficult (80). Linear lesions are often found in the center of the popliteal fossa at the skin, similar to the findings in the present study (80). Taking into consideration, atypical features of EM described in the literature with central induration, urticarial like lesions, bullae, confluent red-blue lesions mimicking ecchymosis, vesicles mimicking shingles, and central vesiculation, ulceration, and necrosis mimicking spider bites, make the differential diagnosis quite challenging (80,106). Convalescent serology may be falsely negative in patients exposed to antibiotic treatment early in the course of the disease (106). This is common with patients in our study, where the majority of them started treatment very early in the course of the disease.

Three patients with EM in the present study were treated with local corticosteroids prior to coming to the clinic and had an exacerbation of the skin change. After appropriate antibiotic therapy was prescribed, the prompt disappearance of the skin change was achieved. Additionally, other studies showed that diagnosing of early LB continues to depend on experience with accurate identification of typical and atypical EM (36). In some cases, atypical or alternative presentations of EM, or unfamiliarity of physicians with EM, result in an inaccurate diagnosis and the use of ineffective antibiotics or steroids (106).

Only three patients during our study developed any late disease manifestation like EM recidivans and multiple erythema migrans disseminata; and one patient manifested symptoms

of dyspnea, palpitations, fatigue, and dizziness with positive IgM for *B. burgdorferi* in the second visit (ELISA seroconversion). No other clinical manifestations had developed during the six months follow-up.

In general, there are no data about early or late disseminated LB in Kosovo. There is only one case report of a 13 years old girl diagnosed with meningitis and facial palsy two years ago in the Clinic of infectious diseases in Pristina with positive serology of *B. burgdorferi* (109). While classical forms of LB are usually easy to recognize and manage, clinicians can be confused in cases with pleomorphic nonspecific symptoms (85). Investigation on LB showed that the disease might mimic chronic inflammatory or degenerative diseases, including a wide range of auto-immune diseases, and if the organism had not been identified, it would be considered as a classic autoimmune disease (110). Regardless of how skilled clinicians from every medical specialty are, they sometimes can fail to recognize LB with clinical manifestations in joints, heart, and nervous system (75). On the other side, it is shown that only 70–80% of patients present with the initial pathognomonic lesion of EM, and only 30% declare a history of a tick bite (75). The lesion of EM sometimes may go unrecognized, especially in cases that do not recall a tick bite, where may be taken by mistake for an "insect bite" or an "allergic rash"; while mini-erythema migrans are less likely to be diagnosed (75). Considering these difficulties and numerous complexities of LB on diagnosing of the late LB, could be one reason to explain the seldom or total absence in the diagnosis of the late stage of LB in Kosovo.

Another reason that could explain the clinical manifestation of EM only in our study, with only three patients with late disease manifestation, could be that successful treatment of all patients with EM possibly had prevented the other stages of the disease during the study. Alternatively, the heterogeneity among *B. burgdorferi* species that are the main factor for the regional differences in the clinical manifestations of human LB could be another explanation. In Europe, the LB is caused primarily by *B. afzelii* and *B. garinii*, whereas in America, it is caused exclusively by *B. burgdorferi*. With each of the *Borrelia spp.*, the infection usually begins with EM. However, *B. burgdorferi* often disseminates widely; it is particularly arthritogenic and may cause antibiotic refractory arthritis. *B. garinii* typically disseminates less widely, but it is especially neurotropic and may cause borrelial encephalomyelitis. *B. afzelii* often infects only the skin but may persist in that site, where it may cause several different dermatoborreliosis, including acrodermatitis chronica atrophicans (8,94).

B. afzelii, which often infects only the skin, is more widespread in the Northern, Central, and Eastern parts of Europe and *B. garinii* in Western parts (40). In the Greater Hanover region in

Northern Germany comparison of genospecies distribution in ticks removed from humans with those from questing ticks flagged in the same geographical area revealed that ticks removed from humans were significantly more frequently infected with *B. afzelii* (p=0.0004), while 11.5% of ticks carried more than one genospecies (111). In *I. ricinus* adults collected in Bulgaria, the prevalence of *B. burgdorferi s. l.* was almost 40%, while *B. afzelii* was the main species with >50% (40). In Kosovo, as a South-Eastern part of Europe, *B. afzelii* might be the main etiologic agent too, and this remains a target for further investigations to reveal *Borrelia* species in our country.

However, it is also *B. spielmanii,* a borrelia that has been associated only with EM (80). This is a novel borrelia which is transmitted by *I. ricinus* (has been found in *I. ricinus* ticks in Germany and France), and it is classified as a pathogenic species because it has been isolated from human skin biopsies, initially in patients in the Netherlands, and later reported from different European counties: Czech Republic, France, and Poland, among others (80).

We found that 43 (11.3%) of 380 of our participants in the study had positive serology at the first visit for IgG or IgM. IgG Seroprevalence among subjects during the first visit in the study was 28/380 (7.4%) with a predominance of male and with age distribution in subjects >59 years old 16 subjects, while 7 of them were > 70 years old (43.7%).

For a valid estimate of the seroprevalence in a region or country, a representative sample of the general population in specifically designed seroprevalence studies are needed, with additional epidemiological data collected on risk factors (63).

Previous studies have shown higher seroprevalence among subjects belonging to high-risk occupational groups in many parts of Europe, as well as increasing in seroprevalence rate with age and years of exposure risk. However, at the same time, the incidence of the occupationally acquired clinical disease seems to be low (46).

In a Wilking et al. study about the incidence of the LB in Germany, where 9.4% of the general population were found to have IgG antibodies to *B. burgdorferi,* IgG seroprevalence showed male predominance similarly to our findings, and similar age distribution as well (that increases by age) (74,87,112). Seroprevalence rates reported previously from different European regions/countries varied widely – from lower seroprevalence (4-5%) in Italy and Romania to higher seroprevalence (16-20%) shown in serosurveys in endemic areas in southwestern Germany and Finland (112). While immunity above 20% has been described in selected populations with high exposure to ticks (forestry and agricultural), for example, 26-35% in a

high-risk population in Switzerland, while seroprevalence of the general population is 9% (18,74,87).

The majority of subjects from this present study, including those who were seropositive at the first visit, were urban area residents. Wilking et al. showed that persons living in urban areas had a lower probability for *B. burgdorferi s.l.* seropositivity, suggesting that exposure to infected ticks is higher in rural areas. However, urban populations are also at substantial risk for infection (112). Seropositivity is not equivalent to clinical disease and may not necessarily reflect cases of clinical manifestation of infection (63,112).

From overall 74 patients with EM, 11 patients were seropositive in the first visit for IgM or IgG, and from 63 patients with negative serology in the first visit, only 15 (23.8%) patients with EM had a specific immune response in ELISA in the convalescent phase.

One of the limitations of this study is the absence of the confirmation of positive ELISA results with Western Blot. However, considering that all the subjects in the study had a tick bite, they certainly had a potential risk for infection. The specificity of tests used for serologic diagnostics of LB is extremely important, especially in scenarios of low possibility for the disease transmission - a situation where a positive result is more likely to be a false positive (92). Physicians need to be aware that these types of tests should only be requested when there is reasonable clinical suspicion (74).

While the discovery of VlsE and its C6 peptide as markers of antibody response in Lyme disease studies have shown that it has been a significant advance (59,88,92). The WCS ELISA and C6 ELISA are not completely independent tests, and the use of two assays increases the specificity compared to that of either test alone (69). An alternative two-tiered testing strategy in which a whole-cell sonicate (WCS) ELISA is followed by a C6 ELISA - Modified two-tiered test (MTTT) for subjects with a positive or equivocal first-tier result or the reverse strategy in which a C6 ELISA is supplemented by a WCS ELISA, has been shown to provide sensitivity comparable to that obtained with either of these first-tier ELISAs followed by immunoblots (WB) -Standard two-tiered testing (STTT), while preserving the specificity, for LB patients with noncutaneous manifestations or with erythema migrans (88,92). MTTT approach is much simpler than the STTT algorithm in all aspects and is less expensive. Nevertheless, WB has the advantage of giving information with respect to the expansion of the immune response, and the WB will still have a place in evaluating difficult cases (88,92).

Serological tests performed for the diagnosis of Lyme disease are based on the detection of the antibody responses against *B. burgdorferi* in serum. The majority of patients with clinical

manifestation of EM have a negative result, while the sensitivity of antibody-based tests increases with the duration of the infection (92,94). On the other hand, studies showed that approximately 50% of patients with EM remained permanently seronegative (87).

In the United States, 20–50% of patients have detectable specific antibodies, usually of the IgM isotype, during acute, early infection (113,114). During the convalescent period at the end of antibiotic treatment, 70–80% of patients became seroreactive, still usually of the IgM isotype (94). However, after 4–8 weeks of untreated infection, virtually 100% of patients showed specific IgG antibody response (94).

Our study found a low seroconversion rate (8.9%) in patients with short incubation and early introduction of antimicrobial treatment for EM, compared to 55.5% seroconversion rate among patients with long incubation or delayed treatment of the large size EM.

Similar studies showed that a positive antibody immune response and a persistent positive IgG titer after treatment were found mostly in patients with long duration or large size of EM before therapy (115).

In a Quebec study following 278 patients after a tick bite, 44 of them presented with EM. Laboratory diagnosis was based on 2-tiered testing, and from 44 patients with EM, 15 had positive serology, 14 were IgM-positive, 1 was positive only for IgG, while 29 remained seronegative for IgG and IgM (116).

Glatz et al. found that IgG and IgM antibody responses to borrelial infection developed through 3 different profiles, with valid results for European and American patients because the ELISA used is based on the flagellum antigen that is phenotypically stable between *B. burgdorferi* strains of different geographic origin (115). The most common profile of antibody response was a persistent negative serology (negative seroconversion), and because of the absence of a specific immune response, they thought that it could be questioned whether the diagnosis of EM was correct in those patients (115). Another study also postulated that most of the rashes or rash-like lesions in the seronegative patients were probably not due to Borrelia but rather to some other pathogenic agent or allergens transmitted by *Ixodes ricinus* considering that the serological investigation has an expected sensitivity of around 80% 2 months after a tick bite (18). In the study from New Jersey, the authors proposed that toxins from tick salivary glands may cause skin lesions and laboratory abnormalities in tick-borne diseases since no organisms have been isolated from many EM changes (68,117). In the present study, although cases with an atypical form of EM that remained seronegative in the convalescent phase and in which correct diagnosis of EM remained doubtful were observed, all fulfilled the criteria including

tick bite, redness or skin change at the bite site, that did not withdraw for 24 - 48 hours after the tick bite, expanded for more than 5 cm, or reappeared some days later after the tick bite at site bite was taken for EM, as the guidelines recommend. However, it is generally agreed that the clinical diagnosis of EM is reliable, and international guidelines do not require laboratory confirmation (115).

Since in European patients with EM, infection with *B. burgdorferi* is often limited to the skin, Glatz et al. suggest that this could be the reason for the absence of a systemic antibody response (115). Consequently, since only skin manifestations were observed in the present study, this rationale could be an acceptable explanation for the absence of a serologic response in some of the patients. Low convalescent seropositivity in patients with EM in our study could also be the consequence of early antimicrobial therapy, as was shown in previous studies (74). Other studies showed that a certain number of early treated, initially nonreactive patients remained seronegative (106,113). Alternatively, in the case of positive detection of IgM antibodies, there does not have to be a regular continuation of the immune response in the sense of conversion from IgM to IgG (74).

On the other hand, other studies show that even patients receiving early and effective treatment for culture-confirmed erythema migrans, still the majority of them seroconverted (113,115). All this has shown that serology has some limitations and is very complicated in *B. burgdorferi* infection.

The antibody response to LB often regresses very slowly, both after an infection that is latent or after successful treatment. However, reinfection cannot be excluded in these cases, as well (72, 74). No current specific serologic test can be used to follow the response to antibiotic therapy (92). Posttreatment antibody testing during follow- up of patients with EM is improper for assessing treatment efficacy, and the serologic profile is not dependent on the type or duration of antibiotic therapy (113,115). Patients may continue to be seropositive for years, including long term IgM persistence (92). IgM or IgG antibody responses to *B. burgdorferi* may persist for 10-20 years, even after adequate antibiotic treatment (69,94).

Ten (2.6%) patients in this study seroconverted (specific IgM detectable during the second visit), although no symptom had been observed. More than half of subjects (16 of 28, 57.1%) with positive IgG in the first visit reported a previous tick bite; however, only one of those patients reported possible LB seven years before entering the study.

Asymptomatic or minimally symptomatic infections are common in many endemic areas (46).

In the United States, *B. burgdorferi* can cause asymptomatic infections in 10% of patients (114). In European LB, the asymptomatic seroconversion seems to be frequent, considering the relatively high level of seropositivity in populations in endemic areas in Europe and the population at risk (18). However, there is always a question: what happens to these patients in the long-term follow-up? (18). In a study of seroprevalence in Sweden, >50% of participants who were seropositive by ELISA did not recall symptoms of LB (94). Thus, it has been hypothesized that if patients with past or asymptomatic Borrelia infection develop another illness with neurological or joint symptoms, these symptoms might sometimes be incorrectly connected with Lyme borreliosis (94). In our study, eight patients with detectable IgG antibodies in the first visit manifested EM. Other studies suggest that seropositivity indicates the persistence of antibodies in a patient but does not prevent reinfection upon the development of an EM (18). This seropositivity does not indicate a higher risk of developing clinical manifestations or disseminated LB than untreated patients with EM. However, there is little information on the long-term follow-up of these patients (18). A US study showed that persons can be consecutively infected by different *B. burgdorferi* strains and experience clinical manifestations with each infection (112).

However, the clinical significance of asymptomatic infection with *B. burgdorferi* remains still unclear, and its management is controversial. In the Vaccine study where most of the volunteer participants that were identified with asymptomatic IgG seroconversion (by Western blotting) were treated with antibiotics and were followed for 4 to 14 months after the treatment, only one patient with asymptomatic seroconversion (who was not treated with antibiotics), developed Lyme arthritis confirmed by PCR from synovial fluid (3,80).

In our study, 85.1 % of patients with EM were treated with doxycycline and 10.8% with amoxicillin. Both antibiotics successfully treated all patients.

Due to the scarcity of studies that directly compare treatment outcomes of LB in Europe and North America, the overall results of antibiotic treatment seem to be very similar on both continents, despite differences in the *B. burgdorferi s.l.* genospecies (94). The most comprehensive treatment guidelines are the 2006 Infectious Diseases Society of America (IDSA) guidelines that were authored by both US and European experts. The IDSA Lyme borreliosis guidelines were reviewed by an independent panel and found to be current and valid. A recent review includes slight modifications of the IDSA guidelines (94).

A range of antibiotics such as tetracycline, penicillin, and cephalosporin are available for LB treatment, but their selection and use vary depending on clinical manifestation and local clinical

practice (36,37,52). Doxycycline is the first-line drug. Amoxicillin is as effective as doxycycline and is preferred for children younger than 8 years of age, as well as pregnant or lactating women (3,36,37,94). Only one out of eight cases in the present study, who was treated with amoxicillin, manifested EM recidivans two months later. Among 63 patients treated with doxycycline, one patient developed a disseminated form of multiple EM more than two months later. Both of them were treated with the second course of antibiotics, with doxycycline (100 mg bid) for 14 days, resulting in the prompt recovery of EM (within 5 days).

There are only a few published studies that validate a 14-day treatment course with amoxicillin, which is otherwise a wide clinical usage for EM or early LB treatment (118). The findings of Wormser et al. provided additional evidence that a 14-day course of 500 mg amoxicillin given 3 times per day is highly efficacious for patients with early LB. Amoxicillin is well tolerated and uniformly successful in resolving the EM skin lesion and preventing the development of late manifestation (118).

In Europe, more than 80% of the recovery rate can be achieved if the treatment of LB is initiated in a local or disseminated early stage (52).

Overall, in 69 (18.3%) of the cases, the patients were prescribed antibiotics in the first visit, mostly amoxicillin+clavulanic acid (42 cases).

In patients with skin lesions where the differential diagnosis of cellulitis is suspected, empiric antibiotics should be chosen that will have activity against both Lyme disease and common agents of cellulitis (106,119).

Doxycycline was prescribed as prophylaxis in 12 patients and amoxicillin in eight patients in our study. These patients had an embedded tick that was engorged or swollen or was attached for a couple of days (up to seven days as was estimated from patients), and followed by skin reaction at the site bite. Indication for antibiotic prophylaxis after a tick bite is controversial; the strongest indication is in the highly endemic area when an engorged tick is attached for ≥36 hours, but it would also be reasonable when the duration of tick attachment is uncertain (36,94). In a study by Nadelman et al., in 482 patients who had removed an attached *I. scapularis* tick, a single 200 mg dose of doxycycline within 72 hours of tick removal was 87% effective in preventing the development of EM at the site bite (91,94,119).

In Europe, only observation is recommended for individuals with tick bites since the efficacy of prophylactic antibiotic therapy has not been determined (94).

The studies revealed that the risk of infection is very low if the tick is removed within 24–48 hours, while a literature review has determined that in animal models, transmission can occur

in <16 hours, and the minimum attachment time for transmission of infection has never been well-known. Compared with the USA, the relatively early transmission in Europe is supported by findings in laboratory experiments on transmission (18,37). Early transmission of spirochetes has been suggested based on the presence of spirochetes in different organs of the tick. Systemic infection and the presence of spirochetes in the tick salivary glands prior to feeding indicate that Borrelia transmission can occur in humans within a short time after tick attachment (105).

Transmission within the first 12 hours has rarely been observed in laboratory animals (104). Thus, if the tick is removed quickly, other treatments are generally not required (94).

In Europe, the frequency of clinically symptomatic infection after I. ricinus bites is 1–5% (18,94). In the United States, the frequency of the infection after an I. scapularis bite has been shown to be 1–4%, similar to Europe (94). Based on the study by Hofhuis et al., an individual's risk of Lyme borreliosis after a tick bite can be predicted with tick engorgement, patient-estimated duration of tick attachment, and detection of *Borrelia burgdorferi s.l.* DNA in the tick (120).

In the present study, the duration of tick attachment was unknown in 50% of the patients and was >72 hours in additional 6.8%, while in around 25% of the patients, it happened within 24 hours, but this was not the main criterion for antibiotic prophylaxis- doxycycline was administered in patients with engorged or swollen ticks. In the multivariate analysis – which should be taken with caution considering the study design likely resulting in residual confounding – early antibiotic use was associated with a reduced risk of clinically manifested diseases. This finding is suggestive of a possible effect of prophylaxis. In line with the expectations (74), proper removal of the entire tick was associated with a considerably reduced risk of disease. Our findings are consistent with previous studies showing that immediate and appropriate tick removal remains an essential step in preventing Borrelia transmission (74). The ticks should be removed immediately, with a tick tweezer. Removing ticks before the engorgement is very important. If the ticks are engorged with blood, their bodies should not be squeezed in order to prevent the transfer of the borrelia. Whereas, if parts of the ticks (suction organ) remain in the skin, they can be removed with a needle or a curettage (74). As mentioned earlier, the observed apparent differences between the location of Pristina and other parts of Kosovo are likely due to confounding-by-indication. Finally, although there appeared a trend of a higher risk of LB in the years 2017 & 2018 vs. 2015, this is highly uncertain considering the small number of subjects seen during the last two years of the study. The present study is

limited by its design: it did not record all tick bites (most certainly the unknown number of clinically unremarkable bites and those seeking medical advice elsewhere were missed), but just those cases where patients felt the need to specifically contact the single research site. Hence, the true risk of LB could not be estimated; however, it likely provides a relatively accurate data about clinical manifestations of the LB following a tick bite in this specific subset of patients (seeking medical help at a specialized institution).

7. Conclusion

1. These are the first data about tick bites and the related human LB in Kosovo. Investigations of ticks collected in the environment had revealed the presence of *I. ricinus* in Kosovo. However, this study supplements the picture with the identification of ticks removed from humans. All the ticks preserved from participants in the study were identified as *I. ricinus*. Furthermore, LB is present in Kosovo and could be related to the distribution of *I. ricinus* in the country.

2. The study comprised a general population older than 18 years of age. The majority of the tick bites occurred in the urban area and were related primarily to recreational and leisure behavior; only 3.9% of tick bites were professionally related.

3. Overall, 74/380 were positively diagnosed with erythema migrans – 19.5% (95%CI 15.6-23.8) – during the study. Only three patients developed a late manifestation (two had early disseminated disease and one EM recidivans), but none of the patients developed late LB. It could be suggested that successful treatment of all patients with EM probably had prevented the development of other stages of the disease during the study. The heterogenicity of species determining the clinical manifestation of the disease could be the other reason for missing other stages of the disease during the six months follow-up of participants. *B. afzelii,* which often infects only the skin, was found in a high percentage of ticks in East Europe and regions nearby, and may probably be present as the main species also in Kosovo. This finding urges us for further investigation to reveal the species of Borrelia in Kosovo.

4. The peak month for tick activity during the study was May, and for EM development, it was June that suggests the unimodal pattern of seasonal tick questing activity, with the largest number of tick bites occurring during spring and early summer in Kosovo.

5. Few study patients with EM did not seek medical help immediately after EM occurrence or did not take medicine when it was prescribed, which delayed the treatment of EM, fortunately without any other consequences. Education of patients about recognition of EM and increasing awareness about the LB, explaining the potential complications and possible development of advanced stages of the disease in untreated cases, could greatly help in the compliance of the therapy. Counseling about the seasonality of ticks' activity and appropriate ways of removing attached ticks may help in the reduction of potential risk for borrelia transmission during a tick bite.

6. For both men and women, the primary site for ticks' attachment was the legs, followed by the trunk and arms. Knowledge regarding the body areas that are most affected by tick bites

could shorten and improve the process of body examination after coming back home from places where contact with ticks is probable. Nevertheless, it has to be emphasized, and there should be awareness that almost all parts of the body can be affected by tick bite irrespective of the subject's age or gender.

7. The ELISA has some limitations, and the number of patients with clinical manifestation with reactive tests was very low. Asymptomatic patients who seroconverted without symptoms did not receive any antibiotic treatment. Further investigations are required in the long-term follow-up of subjects, which could contribute to gaining information about rather they must treat without symptoms or not, as well as about the potential risk for the development of late LB in the future.

8. Accurate identification of typical and atypical EM is the key to appropriate antibiotic treatment. In general, early LB has an excellent prognosis and effective response to antimicrobial therapy. Doxycycline and amoxicillin as first-line therapy options were successful in treating patients with EM in the study, with prompt disappearance of skin lesion. Only two patients had a recurrence of EM and disseminated multiple EM with no further complications and effective clinical response after the second course of antibiotics.

9. This assessment of the disease following tick bite can be helpful in indicating the need for disease awareness and for reinforcing the importance of primary prevention measures particularly tick awareness, early removal of attached ticks, and also in the provision of appropriate information about the offset of ticks' activity season and proper ways for tick removal in order to prevent Borrelia transmission due to tick bites. Also, the importance of early diagnosis and appropriate treatment to prevent late severe stages of the disease.

8. Abstract in Croatian

Rizik pojave Lajmske borelioze nakon ujeda krpelja u regiji Prištine, Kosovo

Cilj istraživanja: Ne postoji nijedna objavljena studija o kliničkim osobitostima Lajmske bolesti na Kosovu, kao ni ona o seroprevalenciji. Ove činjenice potakle su nas na prospektivno istraživanje s glavnim ciljem utvrđivanja rizika nastanka lajmske borelioze nakon uboda krpelja na području Kosova.

Materijali i metode: U studiju je ukupno uključeno 380 ispitanika starijih od 18 godina koji su se, s pričvršćenim krpeljom, javili na pregled u Odjel hitnog prijema Klinike za infektivne bolesti Sveučilišnog kliničkog centra Kosova u Prištini, tijekom četverogodišnjeg razdoblja (siječanj 2015. – kolovoz 2018). Ispitanici su praćeni šest mjeseci nakon uključivanja u studiju, s ciljem utvrđivanja rizika aktiviranja Lajmske borelioze na osnovu pojave klinički manifestne bolesti, odnosno pojave serokonverzije protiv *Borrelia burgdorferi*.

Rezultati: Najveća aktivnost krpelja utvrđena je tijekom proljetnih mjeseci, svibnja i lipnja. U 74 ispitanika – 19,5% (95%CI 15,6-23,8) dijagnosticiran je solitarni erythema migrans (EM). Samo 15 klinički dijagnosticiranih EM (u seronegativnih bolesnika) serološki je potvrđeno serokonverzijom (2 mjeseca kasnije). U samo tri bolesnika razvile su se druge manifestacije infekcije borelijom – jedan je bolesnik imao recidivirajući formu EM, dok su druga dvojica imala ranu diseminiranu bolest. Bolesnici sa kožnom klinički manifestnom bolešću liječeni su sa doksiciklinom ili amoksicilinom.

Zaključak: Ova analiza lokalne epidemiološke situacije ukazuje na potrebu podizanja svijesti o ovoj bolesti i važnosti mjera primarne prevencije, rane dijagnoze i odgovarajućeg liječenja.

9. Abstract in English

The risk of Lyme Borreliosis infection following a tick bite in Pristina region, Kosovo

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PhD Thesis 2021

Aim: No clinical and seroprevalence studies for Lyme borreliosis (LB) in Kosovo have been publicly available thus far. Therefore, we performed a prospective observational study with the primary objective of defining the risk of developing Lyme borreliosis after a tick bite in Kosovo.

Materials and methods: A total of 380 adult participants (≥18 years of age) with an embedded tick in the skin examined at the Clinic of Infectious Diseases, Pristina, were included in the study during the four years (January 2015-August 2018). Participants were followed by a six-month follow-up in order to detect the development of clinical manifestations of LB and/or seroconversion against *Borrelia burgdorferi*.

Results: Most cases were seen in May and June in all study years. Erythema migrans (EM) was clinically diagnosed in 74/380 patients (19.5%, 95%CI 15.6-23.8). Only 15 clinically diagnosed EM (in seronegative patients) were serologically confirmed with seroconversion (2 months later). Three cases developed clinical manifestation with erythema migrans recidivans, multiple erythema, or several non-specific systemic symptoms. Doxycycline and amoxicillin were mainly used for the treatment of borrelial skin lesions.

Conclusion: This assessment indicates the need for disease awareness and the importance of primary prevention measures, early diagnosis, and appropriate treatment.

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11. Brief curriculum vitae

Albina Ponosheci Biçaku was born on 24.07.1978 in Gjakova, Republic of Kosovo. She graduated from the Faculty of Medicine, the University of Pristina in Kosovo in 2004. She worked as a Medical doctor from 2005 to 2007 in Primary Health Care. In November 2007, she started residency in the Clinic of Infectious Diseases at the University Clinical Center of Kosovo in Pristina to continue working from 2014 as a specialist of Infectious Diseases in Clinic of Infectious Diseases. For five years, she has been an assistant (part-time) for the subject of Infectious Diseases at the Faculty of Medicine, University of Pristina.

She participated as an investigator in the Multi-center European study of Major Infectious Disease Syndromes (MERMAIDS) project in (site) Kosovo as part of the Platform (Platform for European Preparedness Against (Re-)emerging Epidemics) in MERMAIDS-ARBO-Arboviral compatible febrile illness - observational study, from 2016 to 2019.

She has actively participated as an author and co-author in numerous national and international congresses and conferences and as a co-author in several scientific papers. She is a member of the Infectologists Society of Kosovo, a member of the Kosovo Medical Chamber, and a member of ESCMID. She is married and has two children.