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Chapter

Vector-Borne Infections in Bulgaria

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Abstract

Bulgaria is an endemic country for Lyme borreliosis and Crimean-Congo hemorrhagic fever (CCHF). Sporadic cases of tick-borne encephalitis (TBE) and West Nile virus (WNV) encephalitis have been also reported. The reported incidence of Lyme borreliosis in Bulgaria is about 6/100,000 population. Two peaks in the seasonal distribution of cases and more frequent presentation of neuroborreliosis than of Lyme arthritis appear to be characteristics of Lyme borreliosis in Bulgaria. *Borrelia afzelii* is highly prevalent in Bulgarian *Ixodes ricinus* ticks. With sporadic cases or small outbreaks, CCHF appeared every year since the 1950s. For the last 10 years, a total of 60 CCHF cases were officially recorded. There is a tendency for CCHF to spread in previously non-endemic areas. The strains causing CCHF in the country belong to lineage Europe 1. A mean of 3.7% CCHF seroprevalence among the Bulgarian population is established. Both Europe 1 and Europe 2 CCHF virus lineages are present in ticks in Bulgaria. Tick-borne encephalitis (TBE) is very unusual. Only a few cases of TBE have been detected. Overall seroprevalence of 0.6% for TBE virus was found in humans. In 2015, a few WNV human cases were detected caused by lineage 2. Overall WNV seroprevalence in human population in the country is 1.5%.

Keywords: TBE, CCHF, WNV, Lyme borreliosis

1. Lyme borreliosis

1.1 Introduction

Lyme borreliosis is the most prevalent tick-borne infection in the North hemisphere. It is a chronic and multisystem infectious disease caused by several species of *Borrelia burgdorferi* sensu lato complex. Three main species are known to cause the disease in Europe, namely *Borrelia afzelii*, *Borrelia garinii*, and *Borrelia burgdorferi* sensu stricto. The ability to cause persistent infection in humans and a wide variety of mammalian hosts is a common property of Lyme disease.

Early manifestations of Lyme disease include typical skin lesion erythema migrans as a sign of early localized infection and/or unspecific flu-like symptoms, acute meningitis or meningopolyneuritis, and acute arthritis, which are signs of early disseminated infection. Late manifestations may appear as chronic neurological abnormalities, chronic arthritis, or acrodermatitis chronica atrophicans, marking late disseminated infection.

Bulgaria is endemic for Lyme borreliosis and a country with mandatory notification of the disease. The reported incidence of Lyme borreliosis in Bulgaria is about 6/100,000 of the population, but the true incidence is most probably much higher, because the disease often is self-limited and mild cases go unrecognized.

1.2 Clinical features of Lyme borreliosis in humans in Bulgaria

Bulgarian study on clinical manifestations of 1257 patients with Lyme borreliosis, diagnosed by physicians and confirmed in laboratory (except for erythema migrans), showed that the most common clinical presentation is erythema migrans (EM), diagnosed in 868 (69.1%) of the patients, almost uniformly as a sole presentation and rarely as a part of multisystem involvement. Rashes had a median diameter of 11 cm (5–35 cm). The erythema rash was homogenous in 44% and with central clearing and peripheral border in 56%. In 14.7% of the patients, atypical rashes with a vesicular or ulcerated center were found. Flu-like symptoms, such as fever, headache, myalgia, arthralgia, fatigue, neck stiffness, were the most common signs associated with EM. Fever was found on physical examination in 133 (15.3%) of the EM patients. In parallel with EM, lymphadenopathy was detected in 284 (32.7%) of the EM cases. In addition, multiple EM was detected in 59 (6.8%) of the patients.

After EM, the second most frequent presentation of Lyme disease in Bulgaria is neuroborreliosis. It is usually presented as radiculoneuritis as a sole presentation (found in 72% of the patients with neuroborreliosis in Bulgaria) and much rarely manifested as cranial neuritis (mainly in children), myelitis, meningoradiculoneuritis, or encephalopathy.

The third most common presentation of Lyme disease in Bulgaria is Lyme arthritis. In the same study, it was diagnosed in 101 (8%) of the patients with Lyme borreliosis. Lyme arthritis was mainly presented by brief attacks of arthritis and rarely as chronic arthritis.

Rare manifestations of Lyme borreliosis in Bulgaria are those affecting heart and eyes (found in 1.1 and 0.9%, respectively). Very rare syndromes are borrelial lymphocytoma and acrodermatitis chronica atrophicans (0.3%).

Multiple organ involvement was found in 2.1% of the patients. Most commonly it was presented as neurological disorders and skin lesions or arthritis.

In addition, the highest proportion of the patients with Lyme borreliosis is in children aged 5–9. The disease shows two peaks in the seasonal distribution of the cases. Neuroborreliosis is a more frequent presentation than Lyme arthritis in Bulgaria.

1.3 Borrelial C6 peptides as antigens for serological diagnosis of Lyme borreliosis

Diagnosis of Lyme borreliosis depends on clinical signs supported by serological findings—enzyme-linked immunosorbent assay (ELISA) and immunoblotting. Various *B. burgdorferi* sensu lato protein antigens are used for ELISA tests (OspA, OspC, FlaB, VlsE). VlsE is the most promising diagnostic antigen among them due to the conserved immunogenic epitopes.

VlsE gene consists of expression site and 15 silent cassettes with a high degree of homology and high rate of reassortments. Each cassette consists of six variable and six invariable regions. The 26-amino-acid-long sixth invariable region (IR6) is immunodominant and much conserved among *B. burgdorferi* sensu lato species. It was shown that IgG antibodies to IR6 (C6) are often detected in early and late Lyme borreliosis [1, 2]. These two statements, i.e. that C6: (1) possess high immunogenicity and (2) is highly conserved among species of the complex B. burgdorferi sensu lato, we decided to test in practice with serum samples from Bulgarian patients with Lyme disease.

Four 26-amino-acid-long peptides were synthesized (ProteoGenix SAS, France), which corresponded to IR6 regions of VlsE proteins from *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*. Because of the previously described difference in reactivity [1], two peptides were synthesized from two strains of *B. burgdorferi*

sensu stricto—B31, isolated from a tick and 297, isolated from cerebrospinal fluid of a patient with neuroborreliosis.

Four different peptide ELISA tests were developed based on IR6 regions of two *B. burgdorferi* sensu stricto strains (B31 and 297), of one *B. afzelii* (PT7) and one *B. garinii* (IP90) strain. Two different serum panels were tested. The first one consisted of serum samples from Bulgarian patients with Lyme borreliosis—50 sera from patients with erythema migrans (clinical hallmark of early Lyme borreliosis), 20 sera from patients with neuroborreliosis, and 10 sera from patients with Lyme arthritis. This serum panel was used to analyze sensitivity of the tests. It contained 40 serum samples from patients with known cross-reactive serological results—patients with syphilis (n = 10), leptospirosis (n = 10), rheumatoid arthritis (n = 10), and sclerodermia (n = 10). The second serum panel was applied to test specificity of the developed peptide ELISA tests.

Test results showed that the two C6 peptides from *B. burgdorferi* sensu stricto had higher reactivity than the corresponding C6 peptides from *B. afzelii* and *B. garinii* with sera from patients with erythema migrans and those with Lyme arthritis. On the contrary, the C6 peptide from *B. garinii* was more reactive with sera from patients with neuroborreliosis [3]. The two peptides from *B. burgdorferi* sensu stricto showed different reactivity with sera from patients with erythema migrans (**Table 1**).

Concerning non-specific reactivity of the peptide antigens with sera from patients with syphilis, leptospirosis, rheumatoid arthritis, and sclerodermia, the lowest level of specificity (87.5%) was found for the C6 peptide from *B. afzelii*; specificity was higher (90% and 92.5%) with the C6 peptides from *B. burgdorferi* s.s. and highest (100%) with the C6 from *B. garinii*. Overall, specificity of the four peptides was high [3].

In order to test applicability of the C6 peptide for serological diagnosis, we used peptide ELISA tests for detection of antibodies in Lyme borreliosis. Four peptide antigens from the C6 regions of VlsE proteins from the three *Borrelia* species that mainly cause Lyme disease in Europe were tested. The findings were very promising since up to 80% of the patients with early Lyme borreliosis and neuroborreliosis and all patients with Lyme arthritis can be diagnosed by the peptide ELISA tests. In addition, overall specificity of the C6 tests was high (87.5–100%). Notably, the tests are easy to perform and cheap as the peptide synthesis is much more easy to implement than the production of recombinant protein antigens.

The C6 peptides from *B. burgdorferi* sensu stricto showed the highest sensitivity in detection of specific anti-borrelia antibodies in patients with early Lyme disease

Serum panel	C6 B31 (*B. burgdorferi* s.s.)	C6 297 (*B. burgdorferi* s.s.)	C6 IP90 (*B. garinii*)	C6 PT7 (*B. afzelii*)
Erythema migrans (n = 50)	36 (72%)	39 (78%)	26 (52%)	29 (58%)
Neuroborreliosis (n = 20)	11 (55%)	11 (55%)	16 (80%)	9 (45%)
Lyme arthritis (n = 10)	10 (100%)	10 (100%)	8 (80%)	8 (80%)
Total number of reacted samples (% sensitivity)	57 (71.3%)	60 (75%)	50 (62.5%)	46 (57.5%)
Number of reactive sera from patients with other diseases (% specificity)	3/40 (92.5%)	4/40 (90%)	0 (100%)	5/40 (87.5%)

Table 1.
Reactivity of peptide C6 ELISA with serum panels of patients with Lyme disease in Bulgaria.

from Bulgaria. Our previous studies on borrelia infections of Bulgarian ticks have shown that the ticks are mostly infected with *B. afzelii*, followed *by B. burgdorferi* sensu stricto and *B. garinii* [4]. The discrepancy between the abundance of *B. afzelii* in our ticks and predominant *B. burgdorferi* sensu stricto reactivity of Lyme borreliosis patients could be explained by different pathogenic potential of the *Borrelia* species.

It is well known that different *Borrelia* species cause predominantly certain clinical manifestations: neuroborreliosis is associated with *B. garinii* and Lyme arthritis with *B. burgdorferi* sensu stricto [5]. This finding may explain the higher reactivity of sera from patients with neuroborreliosis with the C6 peptide from *B. garinii* as well as the predominant reactivity of the sera from Lyme arthritis with the C6 from *B. burgdorferi* sensu stricto.

1.4 PCR detection of *Borrelia*, *Ehrlichia*, and *Rickettsia* DNAs in *I. ricinus* ticks from Bulgaria

A total of 298 *I. ricinus* ticks, collected by flag from the vegetation in 2000 and 2001, were examined by the reverse line blotting technique for *Borrelia, Ehrlichia,* and *Rickettsia* DNAs [6].

Prevalence of *Borrelia*, *Ehrlichia*, and *Rickettsia* in 202 ticks, collected in 2000, was as follows. Overall *Borrelia* prevalence in adult ticks was 41% (44% in males and 39% in females) and 10% in nymphs. *B. afzelii* was the predominant species. Its prevalence was 23% (26 of 112) in adult ticks and 6% (5 of 90) in nymphs, representing 56% (31/55) of all *Borrelia*-positive results. *B. burgdorferi* sensu stricto was detected in 15 (13%) of 112 adult ticks and in 1 (1%) of 90 nymphs. Prevalence of *B. garinii* was 3% in adult ticks and 7% in nymphs. *B. valaisiana* was detected in 3 and *B. lusitaniae* in other 3 of 202 examined ticks.

Overall *Borrelia* prevalence in adult *I. ricinus* ticks, collected in 2001, was 29% (21 of 72) in adult ticks (8% in males and 40% in females). No *Borrelia* infection was found in nymphs from 2001. *B. afzelii* was again the prevalent species—prevalence rate 15% (11 of 72) in adult ticks, representing 52% (11/21) of all *Borrelia*-positive results. Prevalences of *B. garinii* and *B. valaisiana* were 7% and 8%, respectively.

Anaplasma phagocytophilum was detected in 36 (32%) of 112 adult ticks and in 5 (6%) of 90 nymphs, collected in 2000. Of the ticks, collected in 2001, *A. phagocytophilum* was detected in 28% of adult ticks (male 17% and female 33%) and 21% of nymphs.

Of 202 *I. ricinus* ticks, collected in 2000, 94 (47%) were found to carry *Rickettsia* DNA: 78% of males, 61% of females, and 19% of nymphs. Prevalence of *R. helvetica* was 28% (56/202) and prevalence of IRS4 rickettsia was 30% (60/202). *R. conorii* was found in only two of the ticks. Ticks, collected in 2001, showed also high *Rickettsia* infectivity rate, 40% (38 of 96): 83% in males, 29% in females, and 17% in nymphs. *R. helvetica* was again the prevalent species, detected in 18% and 23% of the ticks, respectively.

A high proportion of Bulgarian *I. ricinus* ticks contains *Borrelia* DNA. Analysis of *Borrelia* prevalence revealed that ticks, collected from the same location and even in the same month (May) but in 2 adjacent years, 2000 and 2001, had different *Borrelia* prevalence (41% and 29%, respectively). Apart differences in overall *Borrelia* prevalence, there were also differences in *Borrelia* prevalence in males, females, and nymphs, collected in the 2 years in the same place. In ticks from 2000, *Borrelia* was most prevalent in adult males, less so in adult females and least so in nymphs, while in ticks from 2001, the prevalence was higher in females and lower in males and nymphs.

B. afzelii is highly prevalent among Bulgarian *I. ricinus* ticks, giving more than half of the *Borrelia*-positive results, followed by *B. burgdorferi* sensu stricto, *B. garinii, B. valaisiana*, and *B. lusitaniae* in that order. Prevalence of coinfection is high (17–45%) in *Ixodes* ticks, representing double or even triple infection with *Borrelia, Ehrlichia*, and/or *Rickettsia*. Even coinfection with two different *Borrelia* or *Ehrlichia* species was often detected, showing that the tick hosts are infected with multiple tick-borne pathogens. Since these ticks were collected from vegetation, a risk for simultaneous transmission of these pathogens during the same tick bite exists.

2. Crimean-Congo hemorrhagic fever

2.1 Introduction

Crimean-Congo hemorrhagic fever (CCHF) is a tick-borne human viral disease with a fatality rate up to 30%. It is characterized by a sudden onset of fever and muscular pain, often progressing to hemorrhagic manifestations [7]. Various wild and domestic mammals are natural reservoir hosts of CCHF virus (CCHFV). Ticks of the genus *Hyalomma* are primary vector and serve also as reservoir hosts. *Rhipicephalus* and *Dermacentor* ticks may play an additional role in maintaining the circulation of CCHFV in an enzootic cycle between vertebrates and ticks. The vector-competent ticks stay infected through the molt ("transovarial transmission") [7]. Humans are infected by infected ticks and also by contact with tissues or body fluids of infected animals or patients. Nosocomial outbreaks are described [8]. A case of probable airborne transmission is reported [9].

CCHF is spread in over 50 countries in Africa, Southern Asia, the Middle East, and Southeastern Europe, including the Balkan Peninsula. Cases have been reported in Bulgaria, Turkey, Kosovo, Albania, and Greece. CCHFV (genus *Orthonairovirus*, family *Nairoviridae*) is a negative-stranded RNA virus with a three-segmented genome: large (L), medium (M), and small (S) segments. CCHFV strains belong to seven genetic lineages. Lineage V, also named Europe 1, contains pathogenic CCHFV strains. Lineage VI, called Europe 2, contains genetically different Greek AP92 strains and recently reported similar strains from Turkey, Greece, Kosovo, and Algeria. Besides the detection in ticks, CCHFV lineage Europe 2 has been detected in a mild CCHF case in Turkey [10]. A fatal case due to an AP92-like strain has been recently reported in Iran [11].

With sporadic cases or small outbreaks, CCHF appeared in Bulgaria every year since 1950s. CCHFV was first detected in 1952 in Stara Zagora region [12]. Over 1500 cases have been reported in the country since then. For the last 10 years, 2009–2018, a total of 60 CCHF cases are officially recorded in the country. Case fatality rate of CCHF was an average 15.0%. There is a tendency for CCHF to spread in previously non-endemic areas. The strains causing CCHF in the country are closely related to others in the Balkan peninsula, belonging to lineage Europe 1 [13].

2.2 Countrywide seroprevalence study on Crimean-Congo hemorrhagic fever in general population of Bulgaria

To test current circulation of CCHFV in the country, we conducted a seroepidemiological study. The main objective of the study was to estimate the prevalence of IgG antibodies to CCHFV, as stable and long-persisting antibodies, in general human population of Bulgaria.

Serum samples were collected prospectively from 1500 residents of all 28 districts in Bulgaria. Participants were selected randomly among persons referred

to public biochemistry laboratories in the regional primary healthcare centers to follow noninfectious diseases or for routine prophylactic checkup. Persons previously vaccinated against CCHFV were excluded from the study.

All serum samples were tested for anti-CCHFV IgG antibodies using commercially available ELISA kits according to the manufacturer's instructions (Vector-Best, Novosibirsk, Russia). Positive serum samples were tested also for specific IgM antibodies against CCHFV by ELISA kits from the same manufacturer. Positive samples for CCHFV IgG antibodies were additionally tested by commercial immunofluorescent kits (Euroimmun, Lübeck, Germany).

Specific IgG antibodies to CCHFV were found in 55 (3.7%) of the 1500 people tested by both ELISA and IFA tests. No CCHFV IgM antibodies were detected in these 55 samples. Analysis of the risk factors revealed that age over 40 years, tick bites, contact with livestock and residency in Haskovo district, are associated (95% CI) with an increased risk of CCHF [14].

Positive samples were found in residents of 20 out of the 28 districts in Bulgaria (**Figure 1**). The highest seroprevalence rate was observed in Southeastern Bulgaria: in districts of Haskovo (28%) and Yambol (12%), both well-known endemic regions. Notably, considerable seroprevalence rates were detected in districts where no CCHF cases have been reported, like in some northern and western districts [14].

A few CCHF cases are reported every year in Bulgaria. Nevertheless, results of the seroprevalence study revealed that actual significance of the disease is much higher with a high rate of subclinical infections. The mean established CCHF seroprevalence is comparable to those in other Balkan countries: 4.2% in Greece [15] and 4.0% in Kosovo [16]. The seroprevalence in the endemic Bulgarian districts is close to that in the endemic Turkish regions [17, 18], experiencing a large outbreak.

As found in previous studies, risk factors for CCHFV seropositivity are contact with livestock and tick bites. At higher risk are also residents of Haskovo district, where the highest seroprevalence is detected. There is no significant difference between the age groups. Nevertheless, there is significant difference in the
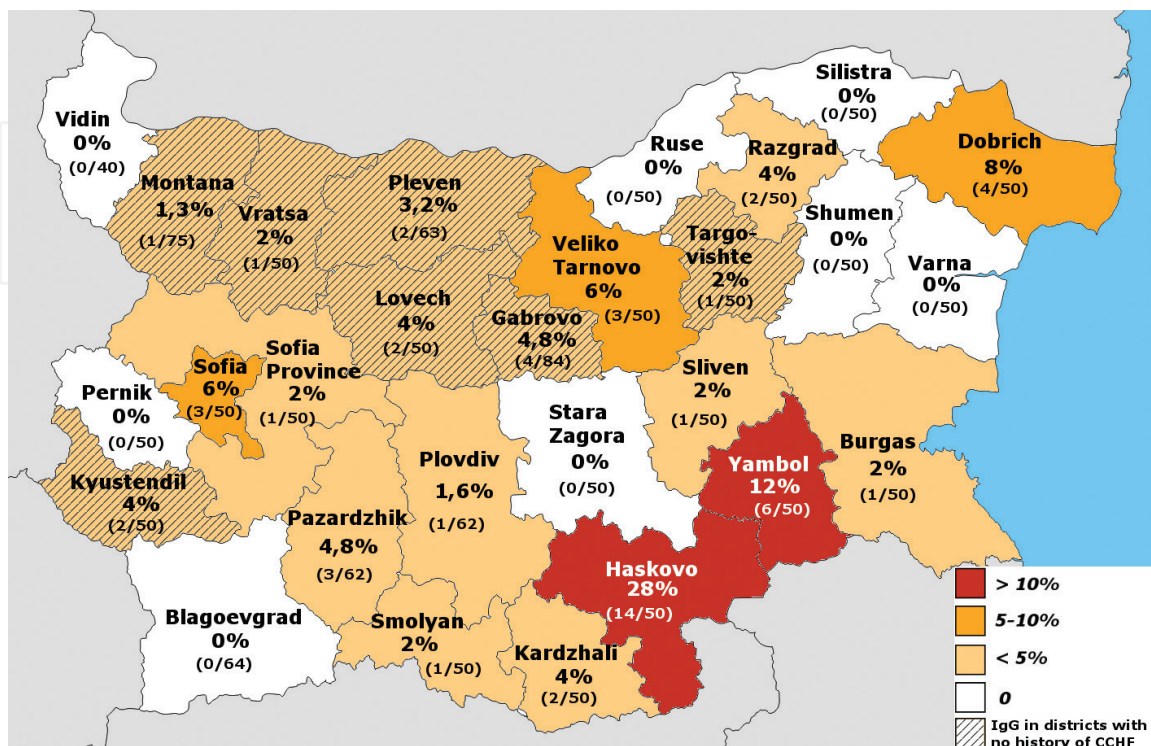

Figure 1.
CCHF seroprevalence by districts in Bulgaria.

seroprevalence rates in groups over and bellow 40 years. However, probability of contacting the virus also increases with age.

In the last years, almost all CCHF cases in Bulgaria are reported in southeastern districts, close to the national borders with Greece and Turkey (districts of Kardzhali, Yambol, Haskovo, and Burgas). On the other side, specific IgG antibodies against CCHFV were found in almost all Bulgarian districts (in 20 of 28 districts), high seroprevalence in the endemic districts, located in Southeastern Bulgaria, Haskovo (28%) and Yambol (12%), and significant seroprevalence in non-endemic areas in Northern and Western Bulgaria. The data presented indicate two important findings: (1) CCHFV increased substantially its circulation in the endemic regions and (2) CCHFV was introduced in many new areas.

In addition, the seroprevalence data revealed that CCHF may go asymptomatic or present with very mild manifestation in many cases. Increased awareness among physicians about subclinical forms of CCHF is needed.

2.3 Crimean-Congo hemorrhagic fever virus lineages Europe 1 and Europe 2 in Bulgarian ticks

In order to determine prevalence of CCHFV in ticks, a total of 2315 ixodid ticks were collected from 216 animals (cattle and sheep) in five Bulgarian districts, where CCHF cases were reported in the last 5 years, namely, Blagoevgrad, Kardzhali, Haskovo, Yambol, and Burgas. The majority of the ticks (2231/2315) were adults and the rest (84/2315) were nymphs.

By real-time reverse transcription polymerase chain reaction (RT-PCR), 623 *H. marginatum* and 107 *R. sanguineus* s.l. ticks were examined [19]. CCHFV RNA was detected in none of *R. sanguineus* s.l. ticks and in 39 of *H. marginatum* ticks (6.3%). The CCHFV RNA-positive ticks were collected from 13 animals: 9 cattle and 4 sheep (6% of all 216 domestic animals tested) in the districts of Burgas and Kardzhali, where the mean percentage of infected animals was 10.0% and 11.4%, respectively. (range 3.7%–17.7%). All positive samples were further tested by the RT-nested PCR. A total of 28 (71.8%) of them were again positive. Sequencing of these samples showed that they clustered into CCHFV lineage Europe 1. Average 8.8% of investigated *H. marginatum* ticks in Burgas and 8.6% in Kardzhali districts were infected with CCHFV. Infestation rate ranged per village from 2.7 to 15.2% [20].

Specific RT-nested PCR for CCHFV lineage Europe 2 [10] was applied to test negative real-time RT-PCR *R. sanguineus* s.l. ticks. A total of 49 (11.8%) of 415 investigated *R. sanguineus* s.l. ticks were positive. Very high rate of AP92-like CCHFV was found in *R. sanguineus* s.l. ticks from Kardzhali district (40.4%). In the district of Haskovo, infestation rate was 2.6%. Europe 2 lineage CCHFV was not detected in districts of Blagoevgrad, Burgas, and Yambol (**Figure 2**). Sequences were submitted to the GenBank DataBase (accession numbers KR092373, KR092374, and KX227372-KX227377) [20].

Detection of CCHFV lineage Europe 1 only in *H. marginatum* ticks supports its leading role as competent vector for CCHFV in Bulgaria. It is of interest that *H. marginatum* tick species is the predominant species in the two CCHF endemic districts: Kardzhali and Burgas. In some villages in these districts, *H. marginatum* ticks were infected with CCHFV up to 13.9 and 15.2%, respectively. In other Balkan countries, rates of CCHFV infection in *H. marginatum* ticks are as follows: 9.1–10.9% in Turkey [21, 22] and 11–15% in Kosovo [23, 24], although in another study in Kosovo, CCHFV was not detected in ticks collected from livestock in otherwise highly endemic regions [16].

CCHFV lineage Europe 2 was detected for the first time in Bulgaria: in 40% (46/114) of ticks in Kardzhali district. The previous study showed that the seroprevalence in human population in Kardzhali and Burgas districts is also high [25].

Figure 2.
Detection of CCHF virus lineages Europe 1 and Europe 2 in tick from Bulgaria.

Since CCHFV strain of lineage Europe 2 has been related with mild human disease in Turkey, the high infection rate of ticks in Kardzhali and Burgas districts may be connected with possible undetected mild or asymptomatic CCHF cases in these regions.

Summarizing the data, CCHFV lineages Europe 1 and Europe 2 were found in the district of Kardzhali. Both lineages were never detected simultaneously in ticks from an individual animal. One possible explanation could be superinfection exclusion of closely related viruses. All sequences from *R. sanguineus* s.l. ticks belong to CCHFV Europe 2 lineage. This lineage has been originally detected in *R. bursa* ticks from Greece, and much later similar sequences were detected in *R. bursa* and *H. marginatum* ticks in Turkey, in *R. bursa* ticks in Kosovo, in *H. aegyptium* ticks in Algeria, as well in a fatal CCHF case in Iran.

The tick study showed that Bulgarian ticks are infected at higher rate with the low pathogenic CCHFV lineage Europe 2 than with the high pathogenic lineage Europe 1 [20]. Possible widespread circulation of the low pathogenic CCHFV lineage Europe 2 strains might explain the discrepancy between high seroprevalence rates in humans and only few CCHF cases detected per year.

Further studies are needed especially where Europe 2 CCHFV strains were detected in order to investigate any association with human disease. In the area where Europe 1 lineage was detected, increased awareness about its pathogenicity to humans is needed.

3. Tick-borne encephalitis

3.1 Introduction

Tick-borne encephalitis is the most common tick-borne viral infection in humans. The disease occurs in North and Central Europe, Russia, Far East Asia, and Japan. In the last few decades, the number of reported cases increased within the endemic regions along with expanding of these areas.

Tick-borne encephalitis virus (TBEV) belongs to genus *Flavivirus*, family *Flaviviridae* like etiological agents of dengue, yellow fever, Zika infection, West Nile fever (WNF), and Japanese encephalitis. Three subtypes of the virus, European, Siberian, and Far Eastern, cause TBE with different severity and outcome of the disease.

Transmission routes of TBEV include bites of infected *Ixodes ricinus* ticks and consumption of row milk from infected goats, sheep, and cows. The incubation period is usually 7–14 days (between 2 and 28 days).

Like infections with other flaviviruses, most of the human infections with TBEV are asymptomatic (75–98%). Among the symptomatic patients infected with European subtype of TBEV, most develop unspecific febrile disease. In some cases only, infection of the central nervous system appears—meningitis (about 50% of the patients), meningoencephalitis (about 40%), and meningoencephalomyelitis (about 10%).

Tick-borne encephalitis is very unusual in Bulgaria. Over the past 40 years, only a few cases of TBE have been detected. Most of the TBE cases in the country are due to consumption of row goat milk. However, the tick vector, *Ixodes ricinus*, is widely distributed in Bulgaria, and Lyme borreliosis, transmitted by the same tick species, is endemic in the country.

Since 2009, reliable laboratory diagnosis of TBE, based on PCR and ELISA, was introduced, and the first three confirmed TBE cases in Bulgaria were identified: two cases in 2009 and one case in 2012 [26]. Two more TBE cases are identified in 2015.

3.2 A nationwide seroprevalence screening for tick-borne encephalitis virus in the population of Bulgaria

To assess local circulation and risk for human infections with TBEV, nationwide seroprevalence study was conducted in 2015 for the first time in Bulgaria.

Serum samples were prospectively collected from persons visiting laboratories for routine checkup in primary healthcare centers in all districts of Bulgaria: Blagoevgrad (n = 64), Gabrovo (n = 63), Vidin (n = 40), Montana (n = 78), Dobrich (n = 52), Plovdiv (n = 62), Targovishte (n = 42), and 50 samples from each of the rest 21 districts. Information about age, sex, and area of residence for each sampled person was collected in the laboratories.

Using ELISA kits (Euroimmun, Lübeck, Germany), all serum samples were tested for TBEV IgG antibodies. Then, all IgG-positive samples were tested for specific IgM antibodies by ELISA. In addition, avidity tests (from the same manufacturer) were applied to distinguish between acute and non-acute TBEV infections.

A total of 1451 residents of all districts in Bulgaria (population 7.2 million), 622 male and 829 female, the mean age ± standard deviation was 53.2 ± 18.8 years, were tested for TBEV-specific IgG antibodies.

Nine persons were found reactive by IgG ELISA; mean seroprevalence was 0.6%. IgM antibodies were not detected. IgG avidity index ranged from *33–85%*, main 60%. The nine people were residents of six districts. The highest seroprevalence rate was found in districts of Gabrovo (4.8%) and Ruse (4%) (**Figure 3**). No significant association between age and TBEV infection was detected [27].

The first and nationwide seroprevalence survey on TBEV circulation in Bulgaria found overall seroprevalence of 0.6% for TBEV. However, district analysis showed TBEV seroprevalence up to 4–4.8%. The great variability of IgG avidity indices is suggestive of recent and past infections.

TBE is endemic in Central and Northern European countries. By occasion, TBE has been detected in Southern Europe and the Balkan Peninsula in particular. Nevertheless, data obtained from the seroprevalence study indicates noticeable appearance of TBEV infections in Bulgaria.
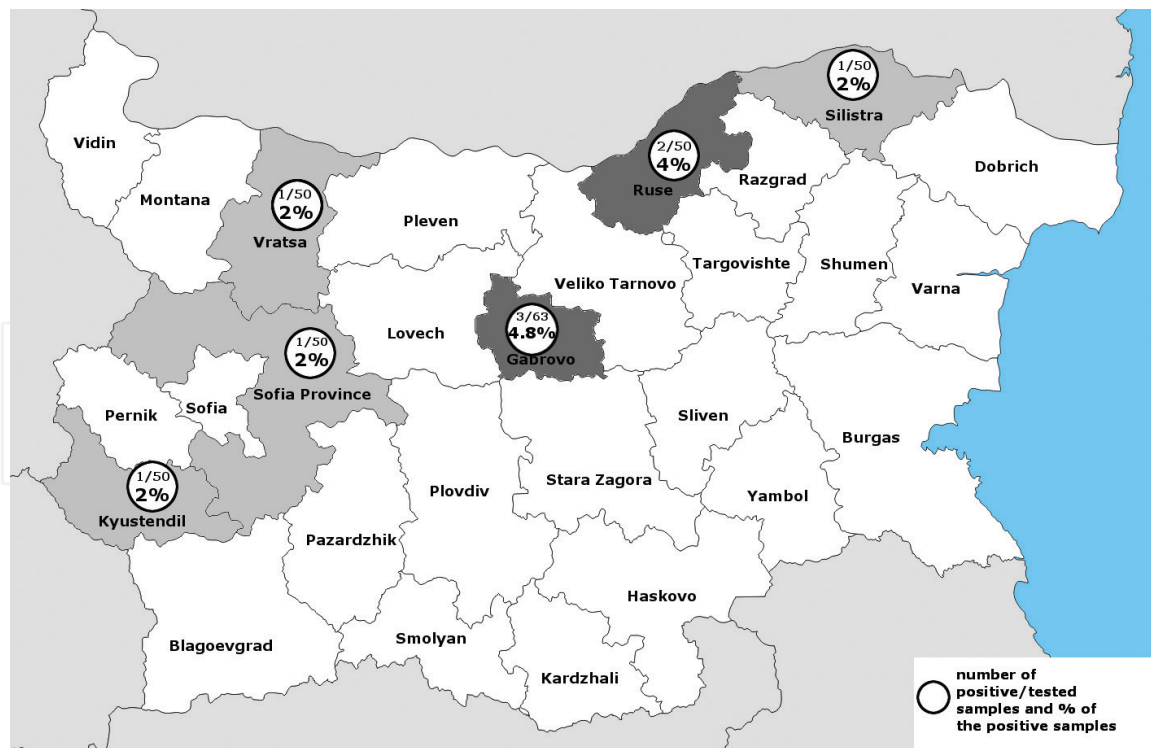
Figure 3.
TBEV seroprevalence in Bulgaria, 2015.

The level of TBEV seroprevalence in Bulgaria showed that the infection seems to be more widespread in the country as has been described so far. It is evidence that some viral encephalitis or meningoencephalitis cases in the country are underdiagnosed and underreported.

3.3 Tick-borne encephalitis among patients with viral meningitis in Bulgaria

Considering the remarkable increase in TBE morbidity in Europe over the past two decades [28], we organized and conducted a study of TBE among patients with acute viral meningitis, who were hospitalized in Bulgaria during 2009–2012.

A total of 86 patients with acute viral meningitis were investigated between 2009 and 2012 by physicians at the infectious diseases units at regional hospitals in districts of Sofia, Pazardzhik, Plovdiv, and Burgas. A total of 86 serum samples were collected during the acute phase and 49 sera at the convalescence phase up to 30 days after the first sample.

All 135 serum samples from patients were tested for IgM antibodies, and positive were also tested for IgG antibodies against TBE virus using commercially available ELISA tests (Euroimmun, Germany), according to the manufacturer's instructions.

TBE virus RNA was detected by reverse transcription polymerase chain reaction based on quantitative real-time technology (TaqMan) as described [29]. The system detected a fragment of the 3′ noncoding region of the TBE virus genome.

A total of 86 patients with viral meningitis of unknown etiology during this period were tested to detect acute TBE. Three TBE cases in Bulgaria were found. The last TBE case was detected in October 2012 and the other two were diagnosed in 2009.

3.3.1 Case no. 1

A girl aged 16 years residing in Velingrad (South Bulgaria) was admitted to the regional hospital on April 10, 2009. The patient had a high fever (40°C) and malaise. The temperature went to normal 3–4 days after admission, and then again

her condition deteriorated with fever, headache, stiff neck, sore throat, nausea, vomiting, and depressed mood. The patient had a history spending some time in the forest. The cerebrospinal fluid (CSF) collected on April 14 showed a high number of leucocytes (160/μL; norm, 0–5/μL) with 75% granulocytes, high protein content (125 mg/dL, norm 15–45 mg/dL), and normal glucose level (0.31 mmol/L; norm, 0.22–0.44 mmol/L). The patient was transferred to a hospital in Sofia and a second CSF sample was obtained on April 22, 2009. The CSF flow was at increased pressure, leucocytes count was 400/μL (norm: 0–5/μL) with 65% lymphocytes, the protein content was 100 mg/dL (norm 15–45 mg/dL), and glucose level was normal. *Mycobacterium tuberculosis* was isolated from this CSF sample. TBE virus was detected by real-time RT-PCR [29] in the serum sample drawn on April 14. The serum sample drawn on April 22 showed high titers of specific IgM antibodies against TBE virus by enzyme-linked immunosorbent assay (ELISA) (Euroimmun, Germany). IgG antibodies against TBE virus were not found.

3.3.2 Case no. 2

On September 11, 2009, a 21-year-old man was admitted to the regional hospital in Plovdiv (South Bulgaria) with fever (38.5°C), fatigue, headache, nausea, and vomiting. Stiff neck, stupor, muscle soreness, conjunctivitis, and abnormal reflexes with pain in joints were found during physical examination. The onset of the disease was 5–6 days earlier. Exposure to tick bites could be excluded. The CSF analysis showed increased count of leucocytes 301/μL (norm: 0–5/μL) with 82% lymphocytes, slightly elevated protein (56 mg/dL; norm, 15–45 mg/dL), and normal glucose level (0.38 mmol/L; norm, 0.22–0.44 mmol/L). The patient initially improved and after a week, the patient's condition worsened again. He manifested fever, significant dizziness, and severe headache. The CSF analysis also supported worsening of the patient. Leucocyte count reached 442/μL (norm: 0–5/μL), with 90% lymphocytes, and protein was remarkably elevated (134 mg/dL; norm, 15–45 mg/dL); glucose level (0.28 mmol/L, norm, 0.22–0.44 mmol/L) was normal. Within a month, the patient gradually recovered. Examination by ELISA of paired serum samples from the patient, one upon admission and a second during the reconvalescence, revealed high level of IgM antibodies and no IgG antibodies in the first serum sample and borderline level of IgM antibodies in the first sample and significant levels of IgG antibodies against TBEV in the second serum sample [26].

3.3.3 Case no. 3

A 28-year-old woman, resident of Burgas area (East Bulgaria), was admitted to the regional hospital on September 23, 2012, with fever (37.5–38^0 C), significant numbness in muscles, and weakness. Physical examination revealed mild neck stiffness, mild left hemiparesis, and distal-type hypoesthesia. Her medical history started 2 days before. Upon admission, a tick was found on her body and removed. On September 27 the patient's condition improved, but starting from October 1, the fever, weakness, and numbness in muscles exacerbated. CSF analysis showed slightly elevated leukocytes (60/μL; norm, 0–5/μL) and protein (74 mg/dL; norm, 15–45 mg/dL), normal glucose level (0.38 mmol/L; norm, 0.22–0.44 mmol/L). Two serum samples, taken on October 1 and October 10 were tested by ELISA, and both antibodies, IgM and IgG, specific to TBE virus were detected. The patient was discharged in improved condition.

The serum samples of the three patients tested negative by ELISA and IFA for West Nile and yellow fever viruses, also negative for IgM antibodies to *Borrelia burgdorferi* by ELISA. Their CSF samples tested negative for bacterial culture. Though TBE cases

are reported sporadically, TBE virus circulates in the country, causing human cases associated either with tick bites or consumption of unpasteurized milk.

In all three patients described, typical biphasic course of TBE infection was revealed. About two-thirds of the patients develop only febrile syndrome in the first phase of the disease [30]. Neurological disorders appear during the second febrile phase. Biphasic febrile illness is typical for infection with Western subtype of the virus. Patients infected with Eastern subtype of TBEV develop only monophasic course [30].

TBE cases in humans are occasionally reported in Bulgaria. However, the fact that TBE cases occur in Bulgaria, even sporadically, and are associated with tick bites or consumption of unpasteurized milk shows that TBE virus circulates in the country. Taking into account that patients who develop neurological symptoms are only "the tip of the iceberg"; one can predict that the real amount of infected people is many times more.

There is significant increase in the number of registered cases of TBE in Europe, Russia, and Far East, starting with 1990 [28]. Since then, about 10,000–12,000 TBE cases are reported annually in Europe and Russia. There is a tendency to global increase in the number of cases and to expansion of areas at risk. In Sweden, a significant increase in TBE cases reported was recorded in the last decade [31]. New endemic areas in Switzerland were confirmed by detection of TBE virus RNA in field-collected ticks [32]. Since September 2012, considering the importance and spread of TBE in the European Union, European Commission included TBE in the list of communicable diseases covered by epidemiological surveillance in the member states [33].

The three cases reported considered the first clinically and laboratory confirmed cases in Bulgaria since. The first case proved to have mixed infection with *M. tuberculosis* that could promote the primary progressive course of the meningoencephalitis, as previously reported [34]. The second case showed clinical manifestation of subacute viral meningitis, while the third case presented as subacute encephalomyelitis.

Usually, IgM and IgG antibodies to TBEV are present by the time that central nervous system involvement manifests in the second stage of TBE. Nucleic acids of the TBEV are very rarely detected by PCR during the viremic stage of the disease [29]. Surprisingly, we detected TBE virus infection by RT-PCR in the first patient. Thus, we confirmed not only the case but also the real circulation of the virus in Southeast Europe, where no information is available so far. The first case described above was also remarkable by the two coinfections ongoing—TBE and tuberculosis, responsible for aggravation of the course of the illness.

The TBE cases described showed that the disease is probably not uncommon in Bulgaria. The risk of TBE is underestimated in Bulgaria because of the low awareness of medical doctors. TBE should be taken into consideration in patients with various manifestations of central nervous system infections in Bulgaria.

4. West Nile fever

4.1 Introduction

West Nile virus (WNV) is a member of the genus *Flavivirus* within the *Flaviviridae* family. Widespread *Culex* mosquitoes transmit WNV.

About 80% of human infections with WNV are asymptomatic [32]. Around 20% of infections with WNV present as febrile syndrome and less than 1% manifest as neuroinvasive disease such as encephalitis, meningitis, or polio-like paralysis [35].

First in 2015, a few probable WNV human cases appeared in Bulgaria. Then, one confirmed WNV neuroinvasive infection was described [36]. The causative strain belonged to WNV lineage 2, closely related to Greek strains that caused

the largest outbreak of WNV in Europe 2010–2013 [37] and also close to the WNV that caused outbreak in Hungary in 2008, when the WNV lineage 2 emerged for the first time outside Africa [38].

4.2 A nationwide seroprevalence screening for West Nile virus in the population of Bulgaria

To assess local circulation and risk for human infections with WNV, a nationwide seroprevalence study was conducted.

Serum samples were collected prospectively from persons visiting laboratories for routine prophylactic checkup in all districts of Bulgaria: Blagoevgrad (n = 64), Gabrovo (n = 63), Vidin (n = 40), Dobrich (n = 52), Plovdiv (n = 62), Targovishte (n = 42), Montana (n = 78), and 50 samples from each of the rest 21 districts. Information on age, sex, and area of residence for each sampled person was recorded by the staff in the laboratories.

Using ELISA kits (Euroimmun, Lübeck, Germany), serum samples were tested for WNV IgG antibodies. IgG-positive samples were further tested for specific IgM antibodies and for IgG avidity using the tests from the same manufacturer. Microneutralization assay (MNTA) was used to test all IgG-positive samples to exclude infection with closely related Usutu virus (USUV).

Serum samples from 1451 residents of all districts in Bulgaria, 622 male and 829 female, mean age ± standard deviation 53.2 ± 18.8 years, were tested for WNV-specific IgG antibodies.

Specific WNV IgG antibodies were detected in 22 participants tested by ELISA giving mean seroprevalence rate of 1.5%. Neutralizing antibodies were found in 6 (27.3%) of the IgG-positive samples; titer of these antibodies ranged between 1:10 and 1:100. The MNTA-positive samples originated from four districts (**Figure 4**). IgM antibodies were detected in two of the IgG-positive samples, and one of them was also MNTA-positive (titer 1:100) with IgG avidity index 48%. IgG avidity index for the rest of the MNTA-positive samples was between 70 and 97%. IgG avidity index of all samples ranged between 14 and 97%, mean 59%. USUV was not found in any serum samples.

The highest seroprevalence rates of WNV IgG antibodies were detected in districts of Sofia Province and Vidin—10 and 7.5%, respectively, followed by districts of Ruse and Silistra—6% each (**Figure 4**). There was no significant association of WNV seroprevalence neither with gender or age [27].

The first and nationwide seroprevalence survey on WNV circulation in Bulgaria found overall seroprevalence of 1.5% for WNV. However, district analysis showed WNV seroprevalence up to 7.5–10%. Recent and past infections could be suspected in accordance with variability of the IgG avidity indices.

Analysis of the WNV seroprevalence rates in Bulgaria showed that they are lower than the rates in the endemic European countries (Greece, Northern Italy, and Southern France) [37, 39, 40]. Nevertheless, they showed that WNV is widespread in the country. The highest WNV seroprevalence rate was detected in Sofia Province, where the first confirmed neuroinvasive case was described in 2015 [36] and an additional case was confirmed in 2016. WNV IgM antibodies were detected in people only from this district, giving a certainty that it is a "hot spot," and more cases from this area could be expected in the future. WNV antibodies were detected in almost all districts near the river Danube, the border of Bulgaria with Romania. WNV outbreaks in Romania in 1996–1997 and 2010 appeared in areas close to the Bulgarian border [41]. This area represents excellent conditions for mosquito reproduction. At high risk for attracting WNV infection, according to the seroprevalence data, are also people in some central districts along the big rivers Maritsa and Tundzha as well as in a southern district, close to the border with Greece. The
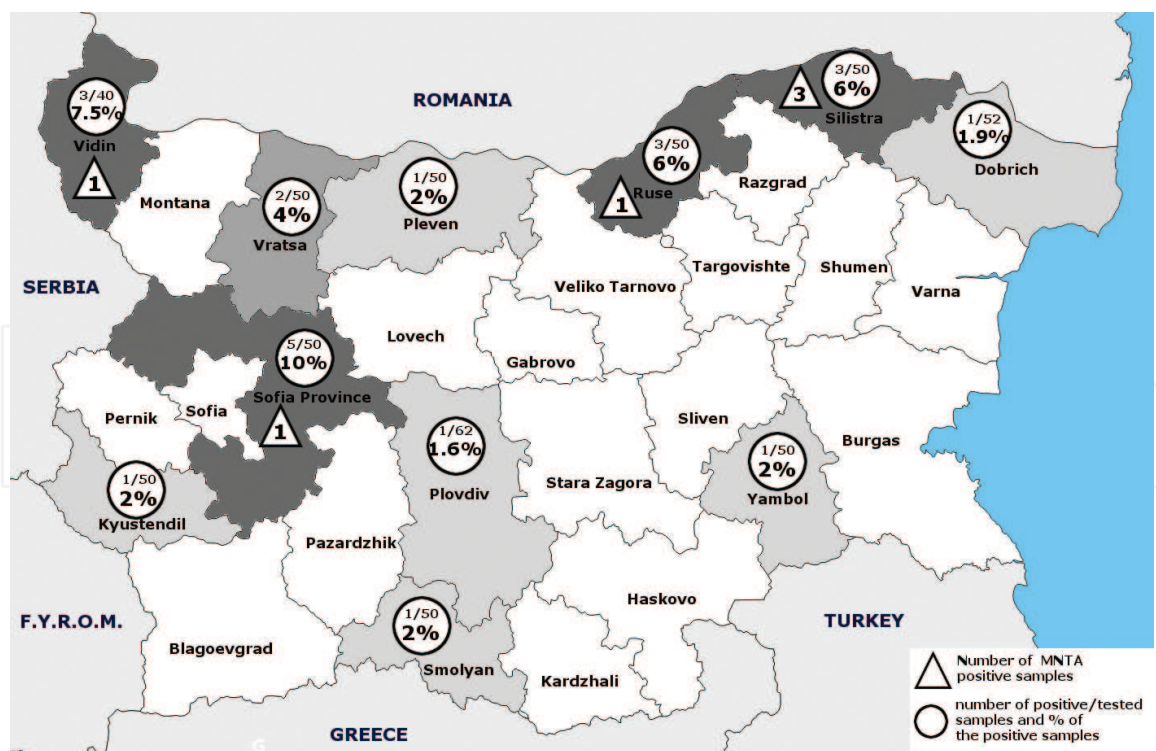
Figure 4.
WNV seroprevalence in Bulgaria, 2015.

big WNV outbreak in Greece, 2010–2012, affected northern parts of the country, not far from Bulgarian territory. The causative WNV was a recent introduction of WNV lineage 2 strain [42]. In the last years, WNV expanded and was reported also in other Balkan states.

WNF infection seems to be more widespread in the country as has been described so far. The level of WNV seroprevalence found in Bulgaria is evidence that some viral encephalitis or meningoencephalitis cases in the country are under-diagnosed and underreported.

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