For the last 60 years, only a few cases of tick-borne encephalitis (TBE) have been detected in Bulgaria. Considering the remarkable increase in TBE morbidity in Europe over the past two decades, we conducted a study of TBE among patients with acute viral meningitis who were hospitalised in Bulgaria during 2009 to 2012. A total of 86 patients with viral meningitis of unknown aetiology during this period were tested. Acute TBE was confirmed in three of these patients. The last TBE case was detected in October 2012; the other two were diagnosed in 2009. To the best of our knowledge, these three patients are the first confirmed TBE cases reported in Bulgaria. The risk of TBE is underestimated in Bulgaria due to the low awareness of medical doctors.

Introduction

Tick-borne encephalitis (TBE) occurs in north and central Europe, Russia, Far East, Asia and Japan [1-3]. The aetiological agent, tick-borne encephalitis virus (TBEV), is a member of the genus Flavivirus of the family Flaviviridae, which also includes aetiological agents of yellow fever, dengue, West Nile fever and Japanese encephalitis. Three subtypes of the virus are known: European, Siberian and Far Eastern [4]. The severity of the disease and its outcome depends largely on the causative subtype [4].

TBEV is transmitted to humans through bites of infected Ixodes ricinus ticks or by consumption of unpasteurised milk from infected animals [5], usually goats, but also sheep and cows [6]. The incubation period is between 2 and 28 days, most commonly 7–14 days. A shorter incubation period is connected to milk-borne TBE [5].

Between 70% and 98% of TBEV infections are subclinical [5]. In clinically manifested cases, about two thirds of the patients only develop a non-specific febrile syndrome during the first phase of the infection [5]. Neurological disorders, usually meningitis or meningoencephalitis, appear during a second febrile phase. Biphasic febrile illness is typical for infection with the Western subtype of the virus, while patients infected with the Eastern subtype develop only a monophasic course [5].

In Bulgaria, reporting of TBE has been mandatory since 1953, but is rarely reported. Over the past 60 years, only a few cases of TBE have been detected [7,8] which, according to the current European Union case definition criteria [9] would not be considered as confirmed cases. Before our study presented here, the last TBE cases were reported in 2006 [10]: laboratory diagnosis of these and previous cases was based on detection of TBE-specific antibodies by complement fixation assay. Most of these cases were due to consumption of unpasteurised goats’ milk [10]. Surprisingly, the tick vector, Ixodes ricinus, is widely distributed in Bulgaria and Lyme borreliosis, caused by borreliae transmitted by the same tick species, is endemic in the country, with about 1,000 cases reported annually [11].

Considering the remarkable increase in TBE morbidity in Europe over the past three decades [12], we conducted a study among patients with acute viral meningitis who were hospitalised in Bulgaria during 2009 to 2012, to determine whether some of the acute viral meningitis cases could be due to infection with TBEV.

Methods

Patients and serum samples

Serum samples from hospitalised patients with acute viral meningitis of unknown aetiology (with or without history of a tick bite, according to the anamnesis) were collected between 2009 and 2012. The samples were drawn by physicians at the infectious diseases units of regional hospitals in the largest districts of Bulgaria (Sofia, Pazardzhik, Plovdiv, Burgas). Serum samples from 86 patients were drawn during the acute phase and 49 sera were collected at the convalescence phase, 7–30 days after the first sample.

Case definition

In this study, we used the 2012 European Union case definition for TBE [9]. A case is classified as probable when a patient met the clinical criteria (symptoms of inflammation of the central nervous system (CNS)) and laboratory criteria (detection of TBE-specific IgM antibodies in a unique serum sample). For a confirmed
TBE case, in addition to meeting the clinical criteria, at least one of the following laboratory criteria was met: (i) TBE-specific IgM and IgG antibodies in blood; (ii) seroconversion or fourfold increase of TBE-specific antibodies in paired serum samples; (iii) detection of TBE viral nucleic acid in blood or CSF.

**Enzyme-linked immunosorbent assay**

All 135 serum samples from the 86 patients were tested for TBEV-specific IgM antibodies; those that were positive were also tested for IgG antibodies against TBEV using commercially available enzyme-linked immunosorbent assay (ELISA) (Euroimmun, Germany). The test uses highly purified TBEV proteins. Serum samples were diluted 1:101. Peroxidase-labelled anti-human IgM or IgG antibodies and 3,3',5,5' -tetramethylbenzidine (TMB)/peroxide substrate were used to detect specific interactions. Calculated values below 0.8 were interpreted as negative; those between 0.8 and 1.1 were accepted as borderline and those above 1.1 were considered positive.

**Polymerase chain reaction**

TBEV RNA was detected by reverse transcription (RT) polymerase chain reaction (PCR) based on quantitative real-time technology (TaqMan), as described elsewhere [13]. The system detected a fragment of the 3' non-coding region of the TBEV genome.

**Testing for other pathogens**

Serum samples of patients that were found positive for TBEV-specific antibodies were further tested for IgM antibodies against *Borrelia burgdorferi* by ELISA (anti-Borrelia ELISA IgM, Euroimmun, Germany), West Nile virus by ELISA (West Nile virus IgM capture – DxSelect, Focus Diagnostics, United States) and immunofluorescence assay (IFA) (Euroimmun, Germany) and against yellow fever virus by IFA (Euroimmun, Germany).

**Results**

Samples from 86 patients with viral meningitis of unknown aetiology during 2009 to 2012 were tested to detect acute TBE: three confirmed TBE cases were
found. The last TBE case was detected in October 2012; the other two were diagnosed in 2009. The place of residence of the three confirmed cases, as well as the previous not-confirmed cases reported since 1953, is shown (Figure).

Case 1
A teenager residing in Velingrad (Pazardzhik district, south Bulgaria) was admitted to the regional hospital in early 2009 with high fever (40 °C) and malaise. The patient's temperature returned to normal (four days after admission) and then about a week later, the patient's condition again deteriorated, with fever, headache, stiff neck, sore throat, nausea, vomiting and a depressed mood. The patient had a history of possible tick exposure in the forest surrounding the village. Cerebrospinal fluid (CSF) collected four days after admission showed a high number of leucocytes (160/µL; norm: 0–5/µL) with 75% granulocytes (norm: 60–70% lymphocytes, up to 30% monocytes), 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). TBEV was detected by real-time RT-PCR in a serum sample taken the same day. The patient was transferred to a hospital in Sofia and a second CSF sample was obtained on 22 April 2009. CSF pressure was increased, the leucocytes count was increased (400/µL) with 65% lymphocytes, the protein content was decreased slightly but was still high (100 mg/dL) and the glucose level was still normal (0.30 mmol/L). *Mycobacterium tuberculosis* was isolated from this CSF sample. A serum sample drawn the same day showed high titres of TBEV-specific IgM antibodies by ELISA. IgG antibodies against TBEV were not found.

Case 2
In early September 2009, a person aged in their early 20s was admitted to the regional Plovdiv hospital (south Bulgaria) with fever (38.5 °C), headache, fatigue, nausea and vomiting. Physical examination revealed stiff neck, muscle soreness, conjunctivitis, stupor and abnormal reflexes with pain in joints. The symptoms started 5–6 days before hospital admission. The patient lived in a village with a high risk for exposure to tick bites (many village residents had had tick bites). CSF analysis showed an increased count of leucocytes 301/µL, with 82% lymphocytes, slightly elevated protein (56 mg/dL), and normal glucose level (0.38 mmol/L). After initial improvement within a week, the patient's condition worsened again with fever, severe headache and prominent dizziness. CSF analysis of the sample collected at that time reflected the worsened condition of the patient: the leucocytes count reached 442/µL, with 90% lymphocytes and the protein level was remarkably elevated (134 mg/dL); the glucose level was normal (0.28 mmol/L). The patient gradually recovered within a month after symptom onset. Paired serum samples from this patient – one upon admission and a second during the convalescence – were tested by ELISA: the first sample was positive and the second borderline for TBEV-specific IgM antibodies. IgG antibodies were detected by ELISA in the second serum sample, but not in the first.

Case 3
A resident of the Burgas district (east Bulgaria) in their late 20s was admitted to the regional hospital in late September 2012 with fever (37.5–38 °C), considerable numbness in the muscles and weakness. Physical examination revealed mild neck stiffness, mild left hemiplegia and hypesthesia of the limbs. The patient's symptoms started two days before admission. Upon admission, a tick was found on their body and was removed. Four days later, the patient's condition improved, after a further four days, the fever, weakness and numbness in muscles was exacerbated. CSF analysis showed slightly elevated leucocyte count (60/µL) and protein level (74 mg/dL); the glucose level was normal (0.38 mmol/L). A serum sample taken at that time and a later sample taken 9 days later, were tested by ELISA: in both samples, IgM and IgG antibodies specific to TBEV were detected. The patient was discharged in an improved condition three weeks after the admission.

The serum samples of the three patients tested negative by ELISA and IFA for West Nile virus and IFA for yellow fever virus, and were also negative for IgM antibodies to *Borrelia burgdorferi* by ELISA. Bacterial culture from the CSF samples of the three patients was negative.

Discussion
Earlier investigations, carried out between 1974 and 2002, confirmed that TBEV was present in ticks in Bulgaria: 6,849 ticks were investigated and eight TBEV strains were isolated [14]. TBE cases in humans have been occasionally reported in Bulgaria (Figure); however, the fact that cases do occur – even though only diagnosed and reported sporadically – and are associated with tick bites or consumption of unpasteurised milk, shows that TBEV circulates in the country. Given that patients who develop neurological symptoms represent a small proportion of those infected, it can be predicted that the number of TBEV-infected people in Bulgaria is many times higher.

There has been significant increase in the number of registered cases of TBE in Europe, Russia and the Far East since 1990 [12]. Since then, about 10,000 to 12,000 clinical cases are reported per year in 30 TBE-endemic countries in Europe and Russia [12]. The epidemiology of TBE after 1990 is characterised not only by a global increase in the number of cases but also by an expansion of risk areas. For example, a significant increase in TBE was recorded in Sweden in the last decade (2000–2011), especially in 2011, when there was a record annual number of TBE cases in Sweden [15]. New endemic areas in Switzerland were identified by detection of TBEV RNA in field-collected ticks in 2007–2010 [16]. Since September 2012, given the importance and
spread of TBE in the European Union, the European Commission included TBE in the list of communicable diseases covered by epidemiological surveillance in the Member States [9].

In all three patients described here, the typical biphasic course of the disease was noted. These patients are, to the best of our knowledge, the first confirmed cases in Bulgaria, having been laboratory confirmed by PCR and IgG ELISA.

Usually, IgM and IgG antibodies to TBEV are present by the time CNS involvement manifests itself in the second phase of TBE [12]. However, TBEV RNA is very rarely detected by PCR during the vireamic second phase of the disease [13]. Surprisingly, we detected TBEV infection by RT-PCR in the first case. This patient proved to have a mixed infection with M. tuberculosis, which could have promoted the primary progressive course of the meningo-encephalitis, as previously reported [17].

The detection of three cases among the 86 patients tested shows that TBE 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 considered for patients with various manifestations of CNS infection in Bulgaria.

Acknowledgements

This work was supported by the Global Emerging Infections Surveillance Response System (GIES) funds DOD # NAMRU3.2008.0003.

References