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Detection of TBEV RNA in *Ixodes ricinus* ticks in north-eastern Poland

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Abstract

TBEV (Flaviviridae, *Flavivirus*) is an etiologic agent of tick-borne encephalitis (TBE), the most important arboviral human infection involving the central nervous system. The disease is endemic in a zone extending from central and eastern Europe to Siberia and Japan, and corresponds to the distribution of the ixodid ticks, which act both as the vectors and reservoir of TBEV. Humans acquire infection mainly by the bite of an infected tick. A continuous increase of TBE cases throughout Europe has been observed over a period of 30 years. The objective of this study was a preliminary determination of the infection level of ticks collected in north-eastern Poland, the endemic area of TBE. Questing *I. ricinus* ticks (adults, nymphs and larvae) were collected by flagging the lower vegetation in 55 locations in Poland in 2006–2009. A total of 2,075 ticks (676 females, 555 males, 799 nymphs and 45 larvae) were collected and examined for the presence of RNA TBEV by nested RT-PCR. The average number of ticks in one pool was 6.98. The minimum infection rate of ticks with TBEV was estimated in the total area as 0.96%. TBEV RNA was detected in all of the investigated developmental stages of ticks. The prevalence of viral infection in ticks is a useful indicator of TBE virus circulation, and may be used for risk assessment of the degree of natural focus activity and of the risk to contact TBE in a particular natural habitat.

Key words

Ixodes ricinus, TBEV, tick borne encephalitis virus, nRT-PCR, Poland

INTRODUCTION

Tick-borne encephalitis (TBE) is a viral zoonotic disease caused by the TBE virus (TBEV) belonging to the tick-borne flavivirus group, family Flaviviridae, genus *Flavivirus*. This is the most important tick-transmitted arbovirus of human pathogenicity in Europe and Asia. TBE is an endemic disease in a zone extending from central and eastern Europe to Siberia and Japan, and corresponds to the distribution of the ixodid ticks, which act both as the vectors and the reservoir of TBEV. TBEV in nature is transmitted by hard ticks (Ixodidae), almost exclusively *Ixodes ricinus* and *I. persulcatus* [1]. Humans acquire the infection by the bite of an infected tick. Infection through the raw milk of goats, sheep and cattle is rare [2]. A continuous increase in the incidence of TBE throughout Europe has been observed over a period of 30 years. TBEV is the most important and widespread tick-borne arbovirus in Europe, causing more than 3,000 human cases of the disease annually [3, 4]. Süss [5] reports a nearly 400% increase of reported TBE morbidity in Europe between 1974 – 2004, and TBE can now be found in new regions; TBEV is present in at least 25 European and 7 Asian countries [6, 7]. The first cases of human TBE in Poland were reported in the Białowieża Primeval Forest over 50 years ago [8]. In the recent years, since the sudden and not entirely explained increase in the incidence of TBE in 1993, Poland recorded annually from 101 (in 1999) to 339 (in 2003) new cases of this disease. Based on the analysis of TBE cases in the years 1999 – 2004, it has been concluded that 93% of them were reported in the Provinces of: Podlaskie, Warmińsko-Mazurskie, Mazowieckie, Dolnośląskie and Opolskie [9], but little is known about the occurrence of TBEV in native *Ixodes ricinus* populations. The objective of this study was a preliminary determination of the infection of ticks collected from vegetation in north-eastern Poland, the endemic area of TBE.

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MATERIALS AND METHOD

Tick collecting. Unfed ticks (adults, nymphs and larvae) were collected by flagging the lower vegetation in north-eastern Poland in the years 2006 – 2009. There were set 55 capture stations, located in the 35 UTM (Universal Transverse Mercator) squares system which splits Europe into 10-km squares, including over 3,000 squares in Poland.

A total of 2,075 ticks were collected in northern and north-eastern Poland in the following Provinces: Pomorskie (UTM squares CE: 18, 25, 35; CF: 06, 16, 17, 24, 25, 30, 31, 32, 33, 34, 43, 44, 72, 92; XA: 71), Warmińsko-Mazurskie (DE: 25, 65; DF: 00, 10, 21; EE: 34, 36, 38, 39, 58, 69) and Podlaskie – mainly Białowieża Primeval Forest (FD: 69, 75, 84, 85, 94) (Fig. 1).

Ticks were sorted according to the collecting site, stage of development and sex, placed in pools of not more than 20 ticks in a mixture of phenol and chaotropic salts (Fenozol, A&A Biotechnology) and frozen at -20 °C.

RNA extraction. After homogenisation in Fenozol with glass pearls using IKA Ultra Turrax Tube Disperser, total RNA was extracted by the phenol-chloroform method according to the protocol of A&A Biotechnology, and frozen at -80 °C for further investigation.

Nested RT-PCR (reverse transcription-polymerase chain reaction): Reverse transcription reaction. Reverse transcription reactions were performed as described previously [10]. To make cDNA, 5 µl RNA, 5 µl 0.5 mM dNTP's mixture

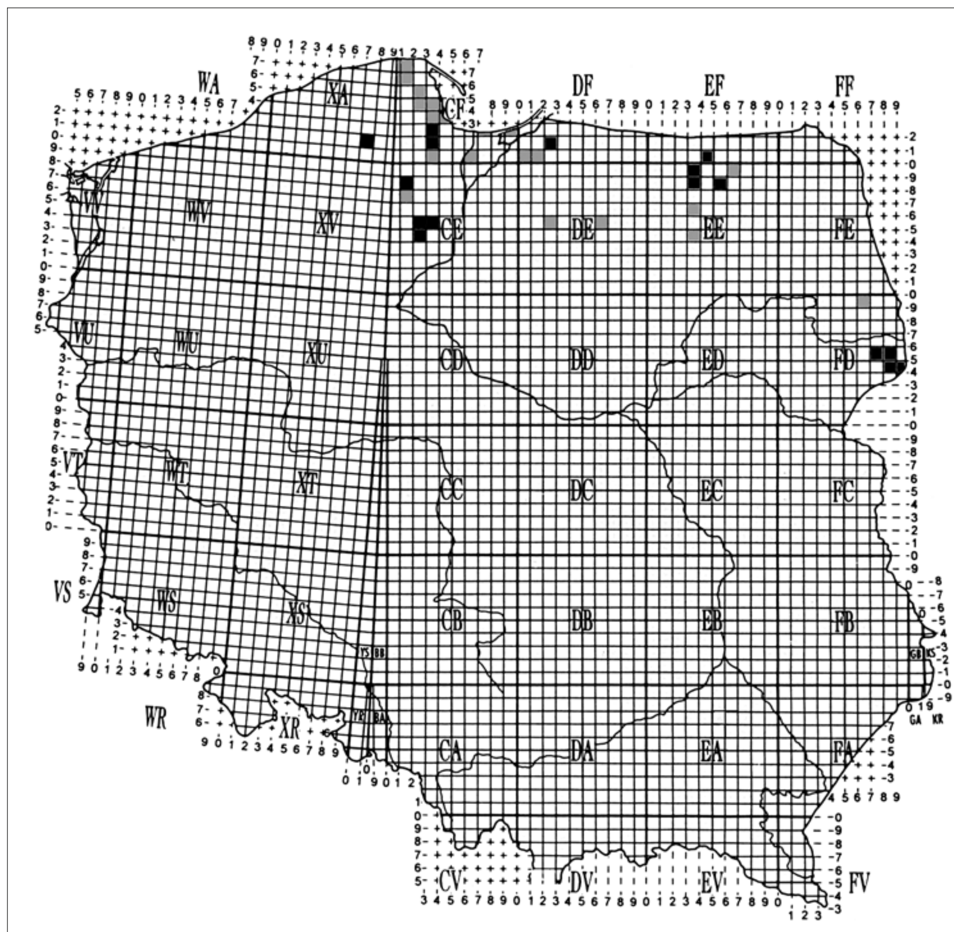

Figure 1. Distribution of collected *Ixodes ricinus* ticks.
Black squares – TBEV infected ticks; grey squares – non-TBEV infected ticks

(MBI Fermentas), 2.5 µl 0.8 µg random primers (Invitrogen) and 8 µl RNase-free water (A&A Biotechnology) were heated at 99 °C for 5 min, cooled to 4 °C for 5 min, and then added to the RT reaction mixture containing: 6 µl RT buffer (Invitrogen), 2 µl 0.1M DTT (Invitrogen), 0.1 µl ribonuclease inhibitor (Invitrogen) and 1 µl M-MLV reverse transcriptase (Invitrogen). Reaction was conducted at 37 °C for 1 hour.

Nested PCR. For nested PCR, two pairs of primers were used: 1) 5'-CTCTTTCGACACTCGTCGAGG-3', 2) 5'–GCGTTTGCT(C,T)CGGA-3'. 3) 5'-CCTTTCAG(A,G)ATGGCCTT-3', 4) 5'-CGGA(C,T)AGCATTAGCAGCG-3') for the 5'-NCR and the 5'-terminus of the C protein coding region, which are highly conserved among the TBEV isolates [11]. PCR reactions were carried out in a reaction mixture that contained: 2.0 µl of cDNA template, 0.5 U (0.5 µl) *Taq* polymerase RUN (A&A Biotechnology), 2.0 µl of 10 × PCR reaction buffer with Mg^{++}, 2.0 µl of 2.5 mM dNTPs mixture (MBI Fermentas), 0.5 µl of 10 µM primer (1,2 in the first round and 3 and 4 in the second) and nuclease free water to give a total volume 20 µl. In this assay, the size of the first round amplification product was 175 nucleotides and that of the second round amplification – 128 nucleotides. For the second round, 1 µl of product of the first round was used. Both amplification rounds were performed under this same conditions: 1 min at 96 °C for initial denaturation, followed by 38 cycles: 1 min at 92 °C denaturation, 1 min at 37 °C annealing, 2 min at 72 °C extension and 7 min final extension at 72 °C [12].

Amplification products were analysed by electrophoresis in 1.5% agarose gel stained with ethidium bromide. One positive (TBEV Langat strain, courtesy of dr Ch. Klaus, Friedrich Loeffler Institute, Jena, Germany) and 2 negative (sterile RNase-free water instead of tick RNA after the RT reaction and DDW instead of cDNA in nested PCR reactions) controls were run with each PCR reaction.

DNA sequencing. Amplification products of the second round of the nested PCR (128 bp) were purified using the Clean-up Purification Kit (A&A Biotechnology), and sequenced using Big Dye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems) and the ABI PRISM 310 Genetic Analyser (Applied Biosystems), according to the manufacturer's protocol. Sequences were edited and compared with representative gene sequences deposited in the GenBank database using the NCBI BLAST programme (United States National Institute of Health, Bethesda, Maryland). Since the obtained amplification products did not exceed the length of 200 bp, their sequences could not be submitted to the GenBank database according to the latest guidelines.

RESULTS

Altogether 2075 *Ixodes ricinus* ticks were tested: 676 females, 555 males, 799 nymphs and 45 larvae in 301 pools, of which 20 were RNA TBEV-positive in the nRT-PCR assay (6.73%)

Table 1. Presence of tick-borne encephalitis virus in *Ixodes ricinus* ticks collected in north- eastern Poland.

	No. infected/No. examined (%)				
District/UTM squares	***Females***	***Males***	***Nymphs***	***Larvae***	***Total***
		Podlaskie Province			
Białystok FD: 69, 75	0/43 (0.0)	1/35 (2.85)	-	-	1/78 (1.28)
Hajnówka FD: 84, 85, 94	3/145 (2.06)	1/139 (0.71)	1/100 (1.0)	-	5/384 (1.30)
subtotal	**3/188 (1.59)**	**2/174 (1.14)**	**1/100 (1.0)**		**6/462 (1.29)**
		Pomorskie Province			
Gdańsk CF: 30, 31, 32	3/132 (2.27)	1/90 (1.11)	0/29 (0.0)	1/13 (7.69)	5/264 (1.89)
Gdynia CF: 34, 44	0/56 (0.0)	0/68 (0.0)	0/376 (0.0)	-	0/500 (0.0)
Kartuzy CF32, XA71	1/68 (1.47)	0/27 (0.0)	0/8 (0.0)	-	1/104 (0.96)
Kościerzyna CE18	-	-	1/6 (16.66)	0/12 (0.0)	1/18 (5.55)
Nowy Dwór Gd. CF: 72, 92	0/22 (0.0)	0/33 (0.0)	-	-	0/55 (0.0)
Puck CF: 16, 17	0/8 (0.0)	0/7 (0.0)	-	-	0/15 (0.0)
Sopot CF43	0/13 (0.0)	0/11 (0.0)	-	-	0/24 (0.0)
Starogard Gd. CE: 17, 25, 35	2/62 (3.22)	0/37 (0.0)	1/148 (0.67)	0/20 (0.0)	3/267 (1.12)
Wejherowo CF: 06, 24, 25	0/19 (0.0)	0/30 (0.0)	0/60 (0.0)	-	0/109 (0.0)
subtotal	**6/381 (1.57)**	**1/303 (0.33)**	**2/627 (0.31)**	**1/45 (2.22)**	**10/1.356 (0.73)**
		Warmińsko-Mazurskie Province			
Braniewo DF21	1/51 (1.96)	0/29 (0.0)	-	-	1/80 1.25)
Elbląg DF: 00, 10	0/9 (0.0)	0/3 (0.0)	-	-	0/12 (0.0)
Giżycko EE: 38, 58	0/2 (0.0)	0/2 (0.0)	1/4 (25.0)	-	1/8 (12.5)
Kętrzyn EE: 38, 39	2/6 (33.33)	0/2 (0.0)	0/2 (0.0)	-	2/10 (20.0)
Mrągowo EE36	0/10 (0.0)	0/11 (0.0)	0/3 (0.0)	-	0/24 (0.0)
Olsztyn DE65	0/5 (0.0)	0/5 (0.0)	0/14 (0.0)	-	0/24 (0.0)
Ostróda DE25		0/1 (0.0)	0/8 (0.0)	-	0/9 (0.0)
Pisz EE34	0/23 (0.0)	0/17 (0.0)	0/41 (0.0)	-	0/81 (0.0)
Węgorzewo EE69, EF40	0/1 (0.0)	0/8 (0.0)	-	-	0/9 (0.0)
subtotal	**3/107 (2.8)**	**0/78 (0.0)**	**1/72 (1.38)**		**4/257 (1.55)**
TOTAL	**12/676 (1.77)**	**3/555 (0.54)**	**4/799 (0.50)**	**1/45 (2.22)**	**20/2075 (0.96)**

(Tab. 1). Among the 35 UTM squares tested, ticks were founds in 15 squares (Fig. 1) which constitutes 42.85% of the tested area.

Positive results were noted in all investigated Provinces. TBEV RNA was detected in all of the investigated developmental stages of ticks. The average number of ticks in one pool was 6.98 and average number of ticks in one positive pool: 8.2. The total number of ticks in infected pools was 164 (102 females, 19 males, 30 nymphs and 13 larvae). Provided that each positive pool always contained only one infected specimen, the minimum infection rate (MIR) of ticks with TBEV was calculated as 0.96%,

In adult ticks, the MIR was 1.21% and was higher in females than in males (1.77% and 0.54%, respectively), whereas in nymphs the MIR was 0.50%. For the first time in Poland, larvae of *I. ricinus* were tested for the presence of TBEV RNA. The larvae were collected in the Pomorskie Province (UTM square: CF32) (Fig. 1), and one pool of 13 larvae was positive.

The specificity of the obtained amplifiaction products was confirmed by the sequence analysis of 20% of randomly chosen samples. All the obtained sequences were 100% identical to each other, and 100% homologous to the sequences deposited in the GenBank: strain Ljubljana JQ654701.1, Kumlinge GU183380.1 and Neudoerfl U27495.1.

DISCUSSION

The obtained results confirm the presence of TBEV endemic foci in all 3 Provinces: Podlaskie, Warmińsko-Mazurskie and Pomorskie [13]. Until the publication of the present study, in Poland, the research for the presence of TBEV in ticks had been conducted mostly in the Lublin Province [14] and resulted in the isolation of one strain of TBEV. The minimum infection rate with TBEV in ticks from this region was estimated at 1.8%. A subsequent study on *I. ricinus* from the Lublin Province, which involved the use of nRT-PCR as the detection method, resulted in a similar minimum infection rate value of 1.6% [15]. Also, parallel tests of raw milk conducted in the same region showed the presence of TBEV RNA in about 20% of tested sheep and goat milk samples, and in more than 11% of tested cow milk samples [16]. Studies on the detection of TBEV RNA by molecular methods of nRT-PCR were also conducted in the Provinces of Wielkopolskie, Zachodniopomorskie and Pomorskie [17]. Relatively high MIR was noted in the Podlaskie (1.29%) and Warmińsko-Mazurskie (1.55%) Provinces which for many years have been considered to be highly endemic [14]. In Pomorskie Province, the minimum infection rate is currently 0.73% (Tab. 1). In 2006 – 2010, there were 10 clinical cases of human TBE recorded in this region, 7 of which were noted in Nowy Dwór district [18]. In the presented study, 55 ticks were collected in this district; however, all of them

were TBEV-negative. No new cases of TBE were recorded at this time in Starogard Gdański district where Makówka *et al.* [17] showed that 7.9% of tick pools were positive; the MIR, however, was not calculated. In the current study, the MIR of ticks from this region was 1.12%, and 8.82% of tick pools were positive. Also, research conducted in recreational areas within the administrative boundaries of the Tri-city (Gdańsk: CF33, 43; Sopot: CF43; Gdynia: CF34) (Fig. 1) did not show any TBEV-infected ticks, even though about 28% of the whole ticks collection was tested; furthermore, no new cases of human TBE were recorded at this time [18]. In Podlaskie Province, where almost half of the Polish human cases are registered, the MIR was 1.29%. On the other hand, the study by Kondrusik *et al.* [19] concerning the same Province and involving the use of real-time PCR as the detection method, showed the MIR at 0.16%.

Analysing the results of the investigation of ticks for the presence of TBEV, Stefanoff *et al.* [20] showed that the prevalence of TBEV in ticks tends to be higher in studies using nested RT-PCR than real time PCR as the method for viral RNA detection. In the countries neighbouring with Poland, studies on the presence of TBEV in ticks were most widely conducted in Germany. In the State of Baden-Württenberg, south-west Germany, the percentage of infected ticks was 0% – 2.3%, as determined by nested RT-PCR [21]. A similar result was obtained in the neighbouring State of Bavaria where the percentage of infected ticks was 0.5% – 2.0% [22] and in Mecklenburg, northern Germany – 2.2% [23]. The study of *I. ricinus* from the Krkonoše Mountains in the Czech Republic, involving the use of real-time PCR, indicated the presence of TBEV RNA in 0.36 % of the ticks [24]. In the central part of Lithuania, 3,234 ticks were tested for the presence of TBEV RNA by nested RT-PCR. The prevalence of infected ticks was only 0.2%, but the collection sites were selected at random, not focusing on known TBEV foci [25].

CONCLUSIONS

Despite the fact that the prevalence of infected ticks may change in a given region depending on the season as well as over the years [26], the presented study shows the presence of TBEV RNA in ticks from the areas where human TBE cases were recorded, and the presence of this pathogen was detected in the vector. The prevalence of viral infection in ticks can be a useful indicator of TBE virus circulation, and may be used for risk assessment of the degree of natural focus activity and the risk to contract TBE in a particular natural habitat. Precise designation of foci in the squares of the UTM system is an important contribution for the determination of the distribution of infected vectors.

REFERENCES

1. Pfeffer M, Dobler G. Emergence of zoonotic arboviruses by animal trade and migration. Parasites & Vectors 2010; 3: 35.
2. Labuda M, Kožuch O, Lysy J. Tick-borne Encephalitis virus natural foci in Slovac: tick, rodents, and goats. Tick-borne Encephalitis and Lyme Borreliosis. Süss J, Kahl O. (eds.). Proc. 4th International Potsdam Symposium on Tick-borne. Pabst Science Publishers, Lengerich, 1997.
3. Anonymous. The Vector-borne Human Infections of Europe. World Health Organization Europe 2004; 1–37.
4. Dampis V, Crook D, Oksi J. Tick-borne encephalitis. Clin Infect Dis. 1999; 28: 882–890.
5. Süss J. Tick-borne encephalitis in Europe and beyond the epidemiological situation as of 2007. Euro Surveill. 2008; 13(26): pii=18916.
6. Charrel RN, Attoui H, Butenko AM, Clegg JC, Deubel V, Frolova TV, et al. Tick-borne virus diseases of human interest in Europe. Clin Microbiol Infect. 2004; 10(12): 1040–1055.
7. Süss J. Tick borne encephalitis 2010: epidemiology, risk areas and virus strains in Europe and Asia – an overview. Ticks Tick Borne Dis. 2011; 2(1): 2–15.
8. Demiaszkiewicz W. Wiosenno-letnie kleszczowe zapalenie mózgu w Puszczy Białowieskiej. Pol Tyg Lek. 1952; 7: 799–801 (in Polish).
9. Stefanoff P, Rosińska M, Zieliński A. Epidemiologia chorób przenoszonych przez kleszcze w Polsce. Przegl Epidemiol. 2006; 60(1): 151–159 (in Polish).
10. Huang C, Slater B, Campbell W. Detection of arboviral RNA directly from mosquito homogenates by reverse-transcription-polymerase chain reaction. J Virol Methods. 2001; 94: 121–128.
11. Ramelow Ch, Süss J, Berndt D, Roggendorf M, Schreier E. Detection of tick-borne encephalitis virus RNA in ticks (*Ixodes ricinus*) by the polymerase chain reaction. J Virol Methods. 1993; 45: 115–119.
12. Sparagano OA, Allsopp MT, Mank RA, Rijpkema SG, Figueroa JV, Jongejan F. Molecular detection of pathogen DNA in ticks (Acari: Ixodidae): a review. Exp Appl Acarol. 1999; 23(12): 929–960.
13. Stefanoff P, Siennicka J, Kaba J, Nowicki M, Ferenczi E, Gut W. Identification of new endemic tick-borne encephalitis foci in Poland – a pilot seroprevalence study in selected regions. Int J Med Microbiol. 2008; S1(298): 102–107.
14. Cisak E, Chmielewska-Badora J, Rajtar B, Zwoliński J, Jabłoński L, Dutkiewicz J. Study on the occurrence of Borrelia burgdorferi sensu lato and tick-borne encephalitis virus (TBEV) in ticks collected in Lublin region (eastern Poland). Ann Agric Environ Med. 2002; 9: 105–110.
15. Wójcik-Fatla A, Cisak E, Zając V, Zwoliński J, Dutkiewicz J. Prevalence of tick-borne encephalitis virus in Ixodes ricinus and Dermacentor reticulatus ticks collected from the Lublin region (eastern Poland). Ticks Tick Borne Dis. 2011; 2: 16–19.
16. Cisak E, Wójcik-Fatla A, Zając V, Sroka J, Buczek A, Dutkiewicz J. Prevalence of tick-borne encephalitis virus (TBEV) in samples of raw milk taken randomly from cows, goats and sheep in eastern Poland. Ann Agric Environ Med. 2010; 17: 283–286.
17. Makówka A, Gut W, Stefanoff P. Obecność RNA wirusa kleszczowego zapalenia mózgu w kleszczach Ixodes ricinus jako narzędzie oceny zasięgu obszarów endemicznych i czułości nadzoru nad zachorowaniami na kzm. Przegl Epidemiol. 2009; 63: 377–380 (in Polish).
18. Mucha D, Zielazny P, Karakiewicz B. Choroby przenoszone przez kleszcze – sytuacja epidemiologiczna w województwie pomorskim. Med Og Nauk Zdr. 2012; 18(2): 93–99 (in Polish).
19. Kondrusik M, Golovjova I, Zajkowska J. Genetic characterization of TBE virus obtained from Ixodes ricinus and Dermacentor reticulatus ticks. Proceedings of the International Conference EDEN 2010 Emerging Vector-borne diseases in changing environment, May 10–12 2010, Montepellier, France, 2010.
20. Stefanoff P, Pfeffer M, Hellenbrand W, Rogalska J, Rühe F, Makówka A, Michalik J, Wodecka B, Rymaszewska A, Kiewra D, Baumann-Popczyk A, Dobler G. Virus detection in questing ticks is not a sensitive indicator for risk assessment of tick-borne encephalitis in humans. Zoonoses Public Health 2012; 6: 215-226.
21. Oehme R, Hartelt K, Backe H, Brockmann S, Kimmig P. Foci of tick-borne diseases in southwest Germany. Int J Med Microbiol. 2002; 291(suppl. 33): 22–29.
22. Süss J, Schrader C, Falk U, Wohanka N. Tick-borne encephalitis (TBE) in Germany – epidemiological data, development of risk areas and virus prevalence in field-collected ticks and ticks removed from humans. Int J Microbiol. 2004; 293(suppl. 37): 69–79.
23. Frimmel S, Krienke A, Riebold D, Löbermann M, Littmann M, Fiedler K, Süss J, Reisinger EC. Tick-borne encephalitis virus in humans and ticks in Northeastern Germany. Dtsch Med Wochenschr. 2010; 135: 1393–1396.
24. Danielovà V, Schvarzovà L, Materna J, Daniel M, Metelka L, Holubovà J, Kříž B. Tick-borne encephalitis virus expansion to higher altitudes correlated with climate warming. Int J Med Microbiol. 2008; S1(298): 68–72.
25. Han X, Juceviciene A, Uzcategui NY, Brummer-Korvenkontio H, Zygutiene M, Jääskeläinen A, Leinikki P, Vapalahti O. Molecular epidemiology of tick-borne encephalitis virus in Ixodes ricinus ticks in Lithuania. J Med Virol. 2005; 77: 249–256.
26. Süss J, Schrader Ch, Abel U, Voigt WP, Schosser R. Annual and seasonal variation of tick-borne encephalitis virus (TBEV) prevalence in ticks in selected hot spot areas in Germany using a nRT-PCR: results from 1997 and 1998. Zentr Bakteriol. 1999; 289: 564–678.