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The first detection of the tick-borne encephalitis virus (TBEV) RNA in *Dermacentor reticulatus* ticks collected from the lowland European bison (*Bison bonasus bonasus* L.)

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Abstract

Tick borne encephalitis virus (TBEV) (Flaviviridae, *Flavivirus*) is the causative agent of tick-borne encephalitis (TBE), a potentially fatal neurological infection. The disease is endemic in a large region in Eurasia, where is transmitted mainly by hard ticks: *Ixodes ricinus* and *I. persulcatus*. It is known that also *Dermacentor reticulatus* is involved in a circulation of TBEV, but the knowledge of its importance in the TBE epidemiology is still insufficient. The Białowieża Primeval Forest is located in eastern Poland and it is a well-known endemic focus of tick-borne encephalitis. The aim of this study was to identify the prevalence of tick-borne encephalitis virus (TBEV) in *Dermacentor reticulatus* ticks collected from European bison (*Bison bonasus bonasus*), an important host of hard ticks in the Białowieża Primeval Forest. In the years 2008-2009, a total of 114 adult *D. reticulatus* ticks were collected from 7 European bison and examined individually for the presence of TBEV RNA using nested RT-PCR assay. Positive results were noted in 18.42% of ticks. This is the first record of TBEV infection in ticks collected from European bison.

Keywords

Dermacentor reticulatus, ticks, European bison, *Bison bonasus bonasus*, TBEV, tick-borne encephalitis virus, Poland

Introduction

The Białowieża Primeval Forest is the last ancient primeval woodland and the best-preserved forest ecosystem of its kind on the European continent (Okołów *et al.* 2009). It is a well-known endemic focus of tick-borne diseases, including tick-borne encephalitis, Lyme borreliosis, rickettsioses and granulocytic anaplasmosis (Przesmycki *et al*. 1960; Stańczak *et al.* 1999; Grzeszczuk *et al.* 2004; Podsiadły *et al*. 2011). The European bison *Bison bonasus* (Linneaeus, 1758) (Artiodactyla, Bovidae) is the largest terrestrial herbivorous mammal in Europe and is protected by both international and national laws. Currently, the Białowieża Primeval Forest has the very large Lowland line (*Bison bonasus bonasus*) population over 900 bison (504 animals in the Polish part and 456 in the Belarusian) (Pucek *et al*. 2004; Raczyński 2013). Furthermore, the bison population in the Białowieża Primeval Forest has been culled regularly since the 1970s to reduce its growth and remove infirm animals (Krasińska and Krasiński 2007). It is known that diseases posing a serious threat to European bison populations have recently appeared in several herds (Pucek *et al.* 2004).

Tick borne encephalitis virus (TBEV) (Flaviviridae, *Flavivirus*) is the causative agent of tick-borne encephalitis (TBE), a potentially fatal neurological infection (Gritsun *et al.* 2003). TBE is the most important viral tick-transmitted disease in Europe and in most European countries TBE incidence increased 400% from 1974 to 2006 (Süss 2008). TBE is an endemic disease in a zone extending from central and eastern Europe to Siberia and Japan and in recent years it has been spreading to northern and western Europe (Gray 2008; Qviller *et al*. 2014). TBEV consists of three subtypes: the European

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(Western) (TBEV-Eu), the Far Eastern (TBEV-Fe) and the Siberian (TBEV-Sb) (Süss 2011).

The main vector of TBEV-Eu, which is present in central and western Europe, is the tick *Ixodes ricinus*, whereas TBEV-Fe and TBEV-Sb are transmitted mostly by *I. persulcatus*. All TBE viruses from Poland have been classified as the European subtype (Wójcik-Fatla *et al*. 2011; Biernat *et al*. 2014a; Biernat *et al*. 2014b). *D. reticulatus* is also considered as an occasional and competent TBE vector (Georgiev *et al*. 1971; Kožuch and Nosek 1971; Randolph *et al.* 1996). TBE viruses are usually focally distributed in forest ecosystems, where they circulate between tick populations and their vertebrate hosts (Korenberg 1994). A tick can be infected in any active stage of development and due to transstadial and transovarial transmission, every stage may transmit infections to mammals. Humans are accidental viral hosts, with the main hosts being small rodents (Randolph and Rogers 2000). A study carried out in Eastern Poland by Cisak *et al.* (2012) suggests that wild boar, instead of roe deer, plays the most important role in the prevalence of TBE in endemic areas. The cases of infections with TBE virus in bison have not been recorded so far (Biernat and Karbowiak 2014), although 4.1% of cattle in eastern Poland reacted positively to TBEV antigen (Cisak *et al.* 2012). In Poland, adult *D. reticulatus* attack mostly wild Cervidae including elks (*Alces alces*), whereas in the Białowieża Primeval Forest also European bison, on whose bodies they can overwinter (Karbowiak *et al*. 2003). In free ranging bison herd from Białowieża Primeval Forest, the presence of two tick species: *I. ricinus* and *D. reticulatus* has been recorded several times (Kadulski 1977; Izdebska 1998, 2000, 2001, 2004; Karbowiak *et al*. 2003, 2014a). The latter species was the most common, even though until the nineties it has been found on these hosts only occasionally. At the beginning of the nineties, only one specimen of *D. reticulatus* was collected from 61 bison in summer (Kadulski *et al*. 1996). Since 1994, however, regular presence of this species on Białowieża Primeval Forest bison has been detected in winter (Izdebska 1998), although it is noted sporadically among bison from other regions in Poland (Izdebska and Cydzik 2010). Similar increase of infection prevalence is observed also in the case of many another bison parasites (Karbowiak *et al*. 2014b). Over the past decades, TBE has become a growing public health concern in Europe and Asia and is the most important viral tick-borne disease in Europe (Süss 2011). Cases reported in Podlaskie voivodeship, where the Białowieża Primeval Forest is situated and where the first cases of TBE in humans were detected in Poland (Demiaszkiewicz 1952), constitute almost a half of reported cases in Poland, notably 43% and 46% in years 1998 and 2008, respectively. The research conducted in this area showed a significant increase in the incidence of TBE during the last 20 years (Czupryna *et al*. 2011). The aim of the present study was to identify the viral prevalence (TBEV) in *Dermacentor reticulatus* ticks collected from European bison – natural hosts of these ticks in Białowieża Primeval Forest – the endemic focus of TBEV.

Materials and Methods

Study area and tick collecting

The study was conducted in the Białowieża Primeval Forest located on the border of Poland and Belarus, between 23°21′ – 24°21′E and 52°29′ – 52°37′N (Sokołowski 2004). The western part of this complex is located in Podlaskie voivodeship (Poland) and covers 650 km^2. Ticks (n = 114) were removed from ears of seven lowland European bison (*Bison bonasus bonasus*) eliminated from free-roaming herd in Białowieża Primeval Forest during the winter 2008 and 2009. Bison shooting was carried out according to a decision made by the Director General of Environment Protection. Ticks were identified according to the developmental stage and species using the key presented in a monographic work of Siuda (1993), placed individually in plastic vials and frozen in – 80°C until RNA extraction.

Molecular detection of TBE virus in *D. reticulatus* ticks RNA extraction

Ticks were homogenized individually in Fenozol (A&A Biotechnology, Poland) with glass pearls using Ultra Turrax Tube Disperser (IKA, Germany). Total RNA extraction was carried out by the phenol-chloroform method according to the A&A Biotechnology protocol and the obtained templates were kept frozen in –80°C until use. The reverse transcription reaction and nested PCR were carried out according to Huang *et al*. (2001) and Ramelow *et al.* (1993) using procedures described in details earlier (Biernat *et al*. 2014a). PCR products were visualized with Midori Green stain (Nippon Genetics Europe GmbH) following electrophoresis in 1.5% gels. The presence of the specific products of 128 base pairs (bp) was considered as positive result.

Amplicons of randomly selected positive samples were removed from the gel under UV exposure and purified with the Gel Out purification kit (A&A Biotechnology). DNA sequencing reactions were performed with the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA) with a standard procedure described by the manufacturer. The nucleotide sequences were assembled using CLC Main Workbench 7.1 Software. Sequence similarity search was performed by NCBI BLAST (Altschul *et al*. 1997).

Results

Altogether 114 adult *D. reticulatus* ticks (4 females and 110 males) were found on 7 European bison (4 bulls and 3 cows) (Tab. I.). The average number of ticks per one bison was 5.42 and a maximum of 40 ticks were removed from a 5.5 year-old male (animal no 833). RT-PCR-positive results were obtained from ticks collected from 5 bison's carcasses. All specimens were individually tested for the presence of TBEV RNA. Of them 21

Table I. Prevalence of tick-borne encephalitis virus RNA in *Dermacentor reticulatus* ticks collected from European bison

Number of bison	Sex (F – cow; M – bull) and age (year)	Date of elimination	*Dermacentor reticulatus* infected/examined (% infected)		Total infected/examined (% infected)
			males	females	
833	M; 5.5	Feb 4–6.2008	9/40 (22.5)		9/40 (22.5)
851	M; 2.5	Dec 16 2008	3/19 (15.78)		3/19 (15.78)
852	M; >2	Dec 16 2008	0/2 (0)		2 (0)
853	F; 26	Dec 16 2008	2/11 (18.18)		2/11 (18.18)
854	M; 15	Jan 20 2009	2/7 (28.57)		2/7 (28.57)
856	F; 0.4	Feb 4 2009	0/2 (0)	0/2 (0)	4 (0)
863	F; 0.8	Mar 3 2009	4/29 (13.79)	1/2 (50.0)	5/31 (16.12)
	TOTAL		20/110 (18.18)	1/4 (25.0)	21/114 (18.42)

Table II. TBEV sequences from *Dermacentor reticulatus* ticks collected from European bison

TBEV isolate abbreviation	Female (F) or Male (M)	Date of tick collection	Number of bison
Dr504-3	M	4.02.2008	833
Dr505-3	M	16.12.2008	851
Dr513-3	M	16.12.2008	851
Dr545-3	M	16.12.2008	853
Dr547-3	F	3.03.2009	863
Dr549-3	M	16.12.2008	853
Dr551-3	M	20.01.2009	854
Dr552-3	M	20.01.2009	854

(18.42%) were positive in the nRT-PCR assay (Tab. I.). The overall infection rate of ticks with TBEV was calculated as 18.42%, ranging from 18.18% (males) to 25.0% (females) (Tab. I.).

Sequencing of PCR products was performed on 8 randomly selected positive samples (Tab. II.). Single strand sequencing with primer 3 gave identical results for all analyzed samples, 82 bases with ambiguous base at the position 77 of the sequence G or A (Fig. 1). NCBI BLAST search performed with sequence containing G at 77th position of the sequence revealed 99% level of the sequence similarity to the following European subtype tick-borne encephalitis virus sequences from GenBank KJ000002.1 (strain Absettarov, Russia), KC835595.1 (114, Slovakia), KF151173.1 (A104, Austria), JQ654701.1 (Ljubljana, Slovenia), GU183380.1 (Kumlinge A52, Finland), U27495.1 (Neudoerfl, Austria), and ENA database record LK934689 (Poland, Podlaskie). NCBI BLAST search performed with sequence containing A at 77th position of the sequence revealed 98% level of the sequence similarity to the above mentioned sequences (Fig. 1). Lower level of similarity was recorded for a number of TBEV sequences (Fig. 1).

Discussion

TBE, characterized with increasing morbidity in humans is a notifiable disease in Poland and Podlaskie voivodeship, where

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                      1st
seq 1 AGCTGTCTTTTTCTCA-CACGTTCACGACTGGTAGAAACAAAAGAATATCTCTTTCAAAC
      |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
ref   AGCTGTCTTTTTCTCAACACGTTCACGACTGGTAGAAACAAAAGAATATCTCTTTCAAAC
                2nd      3rd
seq CAACCGCTGCTAATGCTRTCCGA 82
    |||||||||| |||||| |||||
ref CAACCGCTGCtaAATGCTgaTCCGA
```

Fig. 1. Tick-borne encephalitis virus sequence similarity (Dr504-3, Dr505-3, Dr 513-3, Dr 545-3, Dr547-3, Dr 549-3, Dr551-3, Dr-552-3)

the Białowieża Primeval Forest is situated. In years 2008 – 2009, TBE morbidity in Podlaskie voivodeship was 8.14 and 11.67 per 100.000 residents, respectively, while the national average was 0.53 and 0.92, respectively, and this trend is continuing until today (Czarkowski *et al*. 2009, 2010; Czupryna *et al.* 2011). Intensity of *D. reticulatus* invasion in bison from Białowieża Primeval Forest found in this study was lower (5.42 individuals) than average from 12 years of research (1992 – 2004) on 202 bison (15 individuals) (Izdebska 2004). Males were prevailing (94.49%) among the ticks collected from wintering bison. Overwintering ticks are localized on ears of bison where they form clusters of up to over a dozen of individuals. Usually, several females and several dozens of males can be found on one animal (Karbowiak 2009). *D. reticulatus* males remain on the host longer than females, which may explain their lower numbers found on vegetation in spring (Bartosik *et al.* 2011). It is difficult to explain environmental reasons of the dominance of male specimens in *D. reticulatus* population overwintering on bison. Perhaps this is a result of the dominance of males among questing adult ticks during their autumn activity (Szymański 1987). This phenomenon has been observed in winter also in other hosts, such as dogs (Karbowiak *et al*. 2003), elk and foxes (according to Karbowiak, unpublished data). Higher incidence of infection was detected in *D. reticulatus* collected from bison (18.42%) than from vegetation in the same region (Białowieża, 1.58%) as well as in the whole Podlaskie voivodeship (2.91%) (Biernat *et al*. 2014a). Research conducted in eastern Poland (Lubelskie voivodeship) also showed lower incidence TBEV-infected ticks of the same species collected from vegetation 10.8% (Wójcik-Fatla *et al*. 2011). Higher virus prevalence in ticks collected from animals and humans than in ticks collected from vegetation in nature was observed earlier (Süss *et al*. 2004; Belova *et al*. 2012). Infected ticks under TBEV influence become more active and aggressive therefore they can be found more often on humans and animals. Moreover, questing ticks contain TBEV at an undetectable level. Virus replicates and reaches detectable titers during tick feeding (Belova *et al*. 2012).

European bison shares the same habitat as the main TBEV reservoirs, small mammals: rodents and insectivores. The Białowieża Primeval Forest has been the endemic focus of TBEV for many years; also population free-ranging bison is the highest in that region. Even though research on TBEV presence in bison from the Białowieża Primeval Forest in years 2005–2009 did not yield any positive results (Biernat and Karbowiak 2014), bison can be an important element in TBEV circulation. Studies on transmission of TBEV between co-feeding ticks showed that vertebrate hosts play an important role in TBEV transmission in the absence of detectable level of viraemia (Labuda *et al.* 1993). It is known that natural host with antibodies against TBE virus (and no detectable viraemia) can still support virus transmission between infected and uninfected ticks feeding closely together on the same animals (Labuda *et al.* 1997). Some importance in spreading of TBEV can have dispersion of bison. Most European bison live in Poland and their population in the Białowieża Primeval Forest is the largest in Poland. It plays an important role in restitution and protection of this species and in the last years, many of the bison have been transported to other locations in Poland, as well as other European countries (Denmark, Germany, Spain, Sweden) to create new herds (Bauer 2009; Kelterborn *et al.* 2009; Moran 2013; Svensson 2013). Also increasing use of open habitats outside of the Białowieża Primeval Forest by bison (Kowalczyk *et al*. 2013), and consequently local migrations of these animals, may result in spreading of TBEV-infected ticks. Possibly, the simultaneous increase of population number of deer and elk in Poland and utilization of this same habitat by these species cause the increase of infection prevalence with ectoparasites and arthropod-borne pathogens, as in the case of other bison's parasites (Karbowiak *et al*. 2014b). These observations have important epidemiological implications relating to the survival of TBE virus in nature.

Our study describes for the first time the prevalence of TBEV infection in ticks collected from European bison. In the detected TBEV DNA sequences a deletion was present (Fig. 1) which was absent in other sequences deposited in GenBank and ENA, including one sequence from *D. reticulatus* ticks collected in Poland, Podlaskie – LK934689 (Biernat *et al*. 2014a). In each of the tested samples, there were two variants of the sequence present, one with G and another with A at the position 77 (Fig. 1). The polymorphism within this site were described for a number of sequences originating from samples collected in Europe. Also the residue in the position 70 was one of the previously detected polymorphic sites (Fig. 1). The highest similarity level of the detected sequences to the ones deposited in GenBank was 99% and 98% for the sequences with T residue at the 70th position and G or A at 77th position, respectively (Fig. 1).

The obtained results lead to the necessity of further research on TBEV circulation – first of all serological studies of TBE infection in bison, as well as in wider spectrum of *D. reticulatus* hosts.

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