Tick-Borne Encephalitis Virus Habitats in North East Germany: Reemergence of TBEV in Ticks after 15 Years of Inactivity

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1. Introduction

Tick-borne encephalitis (TBE) is the most widespread arthropod-borne viral disease in central Europe. The TBE virus belongs to the genus Flavivirus (Fam. Flaviviridae), which has three different subtypes: the European subtype, transmitted by Ixodes ricinus, and the Siberian subtype and the Far Eastern subtype, both transmitted by Ixodes persulcatus. The European subtype is found in all European countries except the Benelux and Great Britain [1, 2].

The incidence of TBE in Europe has risen dramatically since 1990 [3–5]. Comparing the periods from 1974 to 1983 and 1994 to 2003, the average increase in TBE infections in humans in ten European countries was 311% [5]. In Germany, the number of reported TBE cases increased from 254 in 2001 to a maximum of 546 in 2006 and the number of areas at risk for TBE transmission increased from 96 in 2005 to 137 in 2012 [6]. Epidemiological analyses of the two decades from 1991 to 2000 and 2001 to 2010 in Germany show a significant (P < 0.001) increase in morbidity to 199.4% [2]. 410 TBE infections were reported in 2013 [7]. In Bavaria and Baden-Württemberg, 0.5 to 2% of unfed ticks have been shown to be TBEV-infected. However, in fed ticks removed from humans, TBEV RNA was detected in 7 to 20% in endemic areas of high risk, indicating that the infection rate in fed ticks can be up to 21.5 times higher than in unfed ticks. On this basis it has been hypothesized that a blood meal leads to an increase in virus replication [5].

Over the last few years, the TBE virus has been documented to be spreading into regions where it was not previously endemic. Not only has it been detected in more northern areas such as Denmark and Norway, it has also been found at higher altitudes, including the Krkonose Mountains in the Czech Republic and the Austrian Alps [2, 4, 8–11].
2. Animals, Material, and Methods

Between February and May 2007, 300 *Ixodes ricinus* ticks were collected by flagging in the regions of Lake Woblitz (Neustrelitz), Boldekow near Anklam, and Thiessow on the island of Ruegen (Figure 1). 50 unfed nymphs from each region were processed immediately as described below or frozen separately at −80°C. Another 50 nymphs from each region were put to feed on mice.

Tick-feeding chambers were prepared as follows: 10 ml syringes (Becton-Dickinson, Franklin Lakes, NJ, USA) were cut to a length of 10 mm and fixed with gauze to the proximal back of a 12-week-old immunocompetent NMRI (Naval Medical Research Institute) mouse from conventional (open-caged) housing, with health status control. Supplier of the mice: Harlan Laboratories, Inc. (Rossdorf, Germany). Ten *Ixodes ricinus* nymphs were placed together in one feeding chamber for five days. The engorged ticks were then removed with pointed tweezers and used for RNA isolation as described below or frozen separately at −80°C. Only 100 of 150 fed ticks were engorged and alive when we removed the feeding chambers and were processed as described below.

Each tick (fed and unfed) was processed separately to avoid possible RNA dilution by pooling. Each tick was homogenized using a sterile microcystill (Eppendorf, Hamburg, Germany) and then mixed in 200 µl sterile 0.9% NaCl solution in a 1.5 ml tube. RNA and DNA isolation was performed using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions without adding RNase to the procedure. This method was chosen in order to copurify DNA from ticks for further studies. After isolation, a nested RT-PCR was performed as described [20], using the primers Pp1 (5'-GGC TTT GCT TCG GAC AGC ATT AGC-3') and Pnl1 (5'-GCG TCT TCG TTG CGG TCT CTT TCG-3') for the first PCR step and Pp2 (5'-TCG GAC AGC ATT AGC AGC GGT TGG-3') and Pm2 (5'-TGCG GTG CTC TTT CGA CAC TCG TCG-3') for the second PCR step. 5 µl of each PCR product was analyzed by electrophoresis on 1% tris acetate EDTA (TAE) gel. The positive PCR products were then excised from the gel and transferred separately to 1.5 ml tubes, where they were purified using the gel extraction kit (Qiagen, Hilden, Germany). The DNA concentration was measured using GeneQuant II (Pharmacia Biotech, Freiburg, Germany) and the PCR products were sequenced (MWG Biotech, Ebersberg, Germany).

A DNA sequence analysis was performed using BLAST version 2.2.18 (National Center of Biotechnology and Information; Bethesda, MD, USA) and MEGA 4.0 (Center for Evolutionary and Functional Genomics, NCBI, Temple, AZ, USA) to confirm TBEV subtypes and detect point mutations. As reference strains, the TBEV sequences Neudoerfl (U27495.1), Salem (FJ572210.1), Hypr (U39292.1), and Toro-2003 (DQ401140.2) were used.

A phylogenetic analysis was carried out using the program CLC main workbench version 5.0 (CLC bio, Aarhus, Denmark). A phylogenetic tree was created using the neighbour joining method (1000 replicates).
Statistical analysis was performed with SPSS 11.0 (SPSS Inc., Chicago, IL, USA). TBEV prevalence among fed and unfed nymphs from each region was compared using Fisher’s exact test.

3. Results

A total of 250 Ixodes ricinus nymphs were processed. RNA was isolated from 50 unfed ticks from each region and from those ticks which were alive and intact after feeding (27 from Lake Woblitz, 39 from Thiessow, and 34 from Boldekow).

A total of six ticks (2.4%) were tested positive for TBEV: three of 50 unfed (6%) and one of 27 fed nymphs (3.7%) from Lake Woblitz, along with one of 50 unfed (2%) and one of 39 fed nymphs (2.6%) from Thiessow. Neither fed nor unfed nymphs from Boldekow were TBEV-RNA positive. The difference between the infection rates in fed and unfed ticks was not significant (P > 0.05).

The RNA sequences detected were broadly homologous to described TBEV strains, as the phylogenetic analysis shows (Figure 2). The sequence Wu 01 (tick from Lake Woblitz, unfed) was shown to be closely related to the tick-borne encephalitis virus isolates Neudoerfl (U27495.1), Salem (FJ572210.1), Hypr (U39292.1), and Toro-2003 (DQ401140.2) (92% homology in every case).

Wu 09 (Lake Woblitz, unfed) showed 100% homology to the TBEV isolates Neudoerfl, Salem, Hypr, and Toro-2003.

Wu 10 (Lake Woblitz, unfed) showed 98% homology to the TBEV isolates Neudoerfl, Salem, Hypr, and Toro-2003.

Wf 5.5 (Lake Woblitz, fed) showed 100% homology to the TBEV isolates Neudoerfl, Salem, Hypr, and Toro-2003.

Tu 30 (Thiessow, unfed) was 100% homologous to TBEV Hypr and TBEV Toro-2003 and 99% homologous to TBEV Neudoerfl and TBEV Salem.

Tf 16.2 (Thiessow, fed) showed 100% homology to the tick-borne encephalitis virus isolates Neudoerfl, Salem, Hypr, and Toro-2003.

The phylogenetic analysis revealed a close relationship of all six positive sequences and a TBEV sequence (IZ11/92) from Mecklenburg-East Pomerania obtained in 1992 (Süss 1997). The results of the phylogenetic analysis of the sequence data are shown in Figure 2.

4. Discussion

Six of 250 ticks (2.4%) were TBE virus-positive in the nested RT-PCR. This is the first proof of natural TBE foci in Mecklenburg-West Pomerania since 1992. In 1992, Süss et al. analyzed 18,760 unengorged ticks subdivided into 260 pools using n-RT-PCR and southern blot hybridization. Two tick pools from the Darss peninsula (near the villages Ahrensloho and Müggenburg) and three tick pools from the island of Usedom (the villages Ahlbeck, Schmollensee, and Koserow) were found to be TBEV-positive, and one sequence was published (IZ-11/92) [14, 16, 21] (Figure 1). In the aftermath of this study over 16,000 ticks were collected between 1993 and 2003 but none was found to be TBEV-positive, leading to the assumption that natural foci were extinct or only present at an extremely low level of activity (Health
Department of the State of Mecklenburg-West Pomerania, unpublished data) [17].

In 2004, a clinically proven case of TBE infection was reported from a campsite near Groß Quassow on Lake Woblitz [15], and in 2007 we collected and analyzed ticks from this region. Four of 77 (5.2%) ticks were TBEV-positive with nested RT-PCR. In Thiessow in the south-east of the island of Ruegen, where another autochthonous case occurred in 2005, two out of 89 (2.2%) ticks were TBEV-positive with nested RT-PCR. In Boldekow near Anklam where the third autochthonous case was reported, all 84 ticks we investigated tested negative for TBEV.

The TBEV sequences obtained displayed a high level of homology with the European prototype strain Neudoerfl and distinct western TBEV subtype isolates (Figure 2). The close relationship between the sequences we found and the single published sequence from Usedom from the year 1992 (IZ-11/1992) may indicate that the TBEV-foci in Mecklenburg-West Pomerania persisted over the years at low levels of activity for years. This is exemplified by the area around Lake Woblitz, near Neustrelitz, which was a focus of TBE between 1960 and 1985, during which four clinical cases were reported, after which the next case did not occur until 2004 [2, 14, 15]. The close relationship between the sequences reported in this study and a TBEV strain found on the island of Usedom in 1992 supports this hypothesis. Another possible scenario is that TBEV may have reemerged in extinct natural foci through the agency of migrating birds. This hypothesis could also apply to the emergence of TBEV in Thiessow on the island of Ruegen. Together with other coastal areas of Mecklenburg-West Pomerania, Ruegen is a rest stop for more than 27 species of water bird and over five million migratory birds annually along the Atlantic flyway [26]. The theory that migrating birds act as hosts and transport media for ticks could also explain TBE in another setting in South West Germany, where at a monkey park in 2006 a closely related TBEV strain was isolated from the brain tissue of an infected, naturally exposed monkey (Macaca sylvanus) [27, 28].

Further studies using unpooled ticks from Mecklenburg-West Pomerania are needed if we are to obtain complete prevalence data for this region and detect possible new risk areas for TBE infection early. Examining TBEV reservoirs, such as mice, goats, and sheep, which can function as sentinels, and returning to explore the natural foci described in 1992 may generate results that would serve as a useful addition to the present data [29].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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