ORIGINAL ARTICLE

Virus Detection in Questing Ticks is not a Sensitive Indicator for Risk Assessment of Tick-Borne Encephalitis in Humans

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Impacts

- Collection of unengorged ticks and detection of tick-borne encephalitis (TBE) virus in the ticks was often undertaken in Poland and Germany to detect areas of increased TBE risk for humans.
- Experience gathered from 17 studies testing more than 65 000 ticks shows that the virus prevalence in ticks does not correlate with increased risk for humans.
- We conclude that other methods for objective assessment of TBE risk for humans should be identified, for example, systematic testing of wild or domestic animals.

Keywords:
Ixodes ricinus; tick-borne encephalitis virus; surveillance; Poland; Germany

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Received for publication September 10, 2011

doi: 10.1111/j.1863-2378.2012.01517.x

Summary

Tick-borne encephalitis virus (TBEV) is the most important tick-transmitted arbovirus causing human disease in Europe, but information on its endemic occurrence varies between countries because of differences in surveillance systems. Objective data are necessary to ascertain the disease risk for vaccination recommendations and other public health interventions. In two independent, separately planned projects, we used real-time RT-PCR to detect TBE virus in questing ticks. In Poland, 32 sampling sites were selected in 10 administrative districts located in regions where sporadic TBE cases were reported. In Germany, 18 sampling sites were selected in two districts located in a region with high TBE incidence. Altogether, >16 000 ticks were tested by real-time RT-PCR, with no sample testing positive for TBEV. A systematic search for published studies on TBEV prevalence in ticks in Poland and Germany also suggested that testing large numbers of collected ticks could not consistently assure virus detection in known endemic foci. Although assignment of results to administrative regions is essential for TBE risk mapping, this was possible in only 10 (investigating 22 417 ticks) of 15 published studies (>50 000 ticks) identified. We conclude that the collection and screening of ticks by real-time RT-PCR cannot be recommended for assessment of human TBE risk. Alternative methods of environmental TBEV monitoring should be considered, such as serological monitoring of rodents or other wildlife.

Introduction

The tick-borne encephalitis virus (TBEV) is transmitted through tick bites or consumption of unpasteurized milk from recently infected farm animals. The western subtype of TBE virus (TBEV) is focally distributed in Central and Eastern European countries (Oehme et al., 2002; Randolph, 2008; Dobler et al., 2011). Reliable surveillance of TBE is necessary to assure adequate protection of people living in or visiting endemic regions (Stefanoff et al., 2011). At such areas, the possible prophylactic interventions range from health education on personal protective measures and avoidance of consumption of unpasteurized dairy products to vaccination of residents and travellers.

As humans are incidental hosts and do not play a role in the natural transmission cycle of TBEV (Randolph, 2008), human surveillance alone is insufficient for effective monitoring of TBE virus (TBEV) circulation in endemic areas (Oehme et al., 2002; Süss et al., 2004). There are several reasons for limited usefulness of human TBE surveillance in assessing disease risk: (i) differences in surveillance performance and physician's approach to TBE diagnosis across European regions have not been quantified (Haglund et al., 2003; Stefanoff et al., 2011); (ii) high vaccination uptake in several European regions may lead to a false impression of decreased TBE infection risk, while it remains the same for visitors and susceptible inhabitants (Kunz, 2003); (iii) the variability in TBEV virulence (Golovljova et al., 2004; Dobler et al., 2011); (iv) dependence on human activities that are triggered by social, environmental and meteorological factors (Randolph, 2008, 2010; Dobler et al., 2011). The identification of further risk indicators conducive to efficient surveillance (Box 1) is therefore desirable.

Box 1. Attributes of Efficient Environmental Vector-Borne Disease Surveillance (Adapted from CDC 2001)

1. Simple and acceptable by system users.
2. Sufficiently flexible to easily implement new laboratory methods and recognize new/modified pathogens.
3. Characterized by high sensitivity and positive predictive value.
4. Representative of the monitored region, that is, performed measurements can be extrapolated to a defined administrative entity.
5. Timely in recognition of changes in disease risk or emergence of disease in previously non-endemic areas.
6. Sustainable, allowing repeated measurements in defined intervals.

Detection of TBEV in ticks is widely held to be the 'gold standard' for confirmation of local TBEV circulation (Süss et al., 2004; Klaus et al., 2010). Thus, this method could be a very useful and independent source of information on TBE risk in Poland and Germany, where occurrence of TBE in humans is confined to

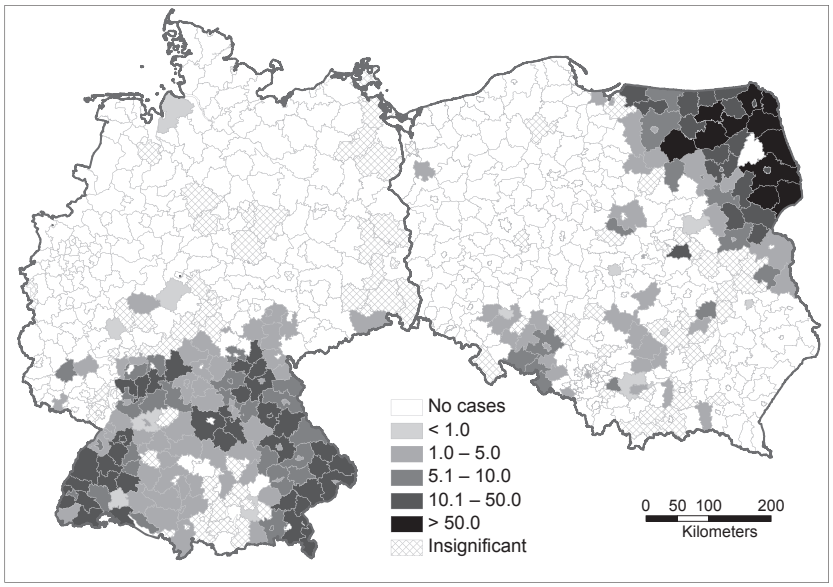

Fig. 1. Incidence of locally exposed tick-borne encephalitis (TBE) cases per 100 000 district inhabitants in Poland and Germany, 2005–2009. The 5-year TBE incidence in gridded districts was not statistically different from 0.1 case/100 000 inhabitants/5 years.

Table 1. Summary of methods used in Poland and Germany, 2008–2009

	Polish study	German study
Study aim	To extrapolate the results from TBEV detection in ticks into NUTS4 administrative areas (districts) in Poland	To confirm endemic TBEV circulation in an area indicated by patients as frequent place of exposure
Rationale for sampling sites location	Selection of districts from which only sporadic human TBE cases are reported, but indirect evidence from serologic surveys or animal studies was found	Selection of sites in known endemic area to increase the probability of finding the virus
Criteria for sampling site location		
Occurrence of human cases	Not criterion for selection of sampling sites	Sites selected in areas indicated by confirmed TBE cases
Environmental criteria	In each district distinct biotopes favourable to tick activity were selected as tick collection sites: animal paths, wide forest tracts in deciduous or mixed forests	At each site appropriate vegetation was identified: transition of forest to meadows, paths in the ground vegetation, mixed forests, with Norway spruce, beech, Scotch pine, common oak and black locust
Detailed selection procedure	1. Selection of districts with indirect evidence for TBEV circulation from serologic or animal studies; 2. Selection of 3–5 sites spatially 'representing' each administrative area (district); 3. Selection of specific locations on site	1. Selection of site locations based on patient interviews; 2. Selection of suitable tick habitats on site
Tick collection methods		
Frequency of flagging	≥2 flagging sessions at each site: one in Spring, one in Autumn	One flagging session every 2 months at each site, from March until September
Flagging area	Not restricted	Four transects 1, 50 m long (200 m^2)
Laboratory methods		
Tick pool size	Adults: 10, nymphs: 20	Adults: 1, nymphs: 10, larvae: 20
Homogenization method	Disruption of pooled samples with Tissue Lyser (Qiagen, Hameln, Germany) in presence of 5 mm stainless steel beads for adult ticks or 3 mm tungsten carbide beads for nymphs (Qiagen). After 30 s run, the samples were spun down. The tick powder was mixed with lysis buffer. For the additional homogenization the QIA shredder was used	Disruption of pooled samples with Bio101 Tissue Lyser (Qiagen) in presence of Minimal Essential Medium (Invitrogen, Karlsruhe, Germany), sterile sand and ceramic bullets (Lysing Matrix A; MP Biomedicals LLC, OH, USA). Between two runs for 20 s each, the tubes were cooled down for one minute in an ice water bath
RNA extraction	RNA was eluted in Rnase-free water using RNeasy mini kit (Qiagen) and added to RNA secure reagent (Applied Biosystems)	Extraction automate (MagnaPURE, Boehringer Mannheim) using the Viral NA Kit according to the instructions of the manufacturer
Method of RNA detection	Schwaiger and Cassinotti (2003). The reactions were performed using the Super Script One-Step RT-PCR in the Platinum Taq system (Invitrogen). The reverse transcription and the amplification steps were performed in the Rotor Gene 6000 (Qiagen). The positive control was the Neudörfl vaccine strain, and the negative control was Rnase-free water. 3% randomly selected tick pools were tested for inhibitors by amplifying genomic tick DNA	Schwaiger and Cassinotti (2003) with no internal control. Real-time RT-PCR was performed in a MX 3000 PCR instrument (Stratagene, Heidelberg, Germany). The positive control was 100 copies *in vitro*-transcribed RNA of TBEV strain Neudörfl and the negative control was Rnase-free water. 5% randomly selected tick pools were tested for inhibitors by amplifying genomic tick DNA

TBEV, tick-borne encephalitis virus.

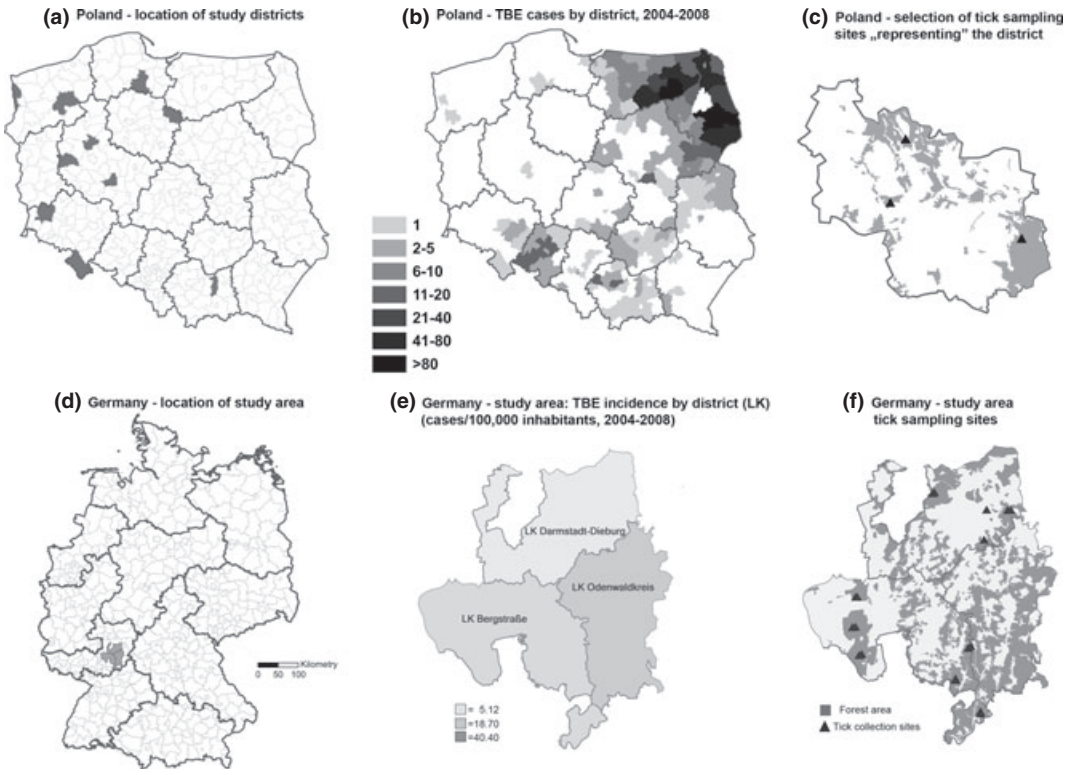

Fig. 2. Illustration of study site selection in Poland and Germany during 2008–2009.

approximately one-third of their territories (Fig. 1). Our aim was to investigate whether TBEV detection in ticks could supplement routine TBE surveillance systems to aid in identifying TBE endemic areas. During recent years, various methods of TBEV detection were used in our countries. In this paper, we describe results of studies on TBEV detection in ticks we performed in Poland and Germany in 2008–2009 and review similar studies previously undertaken in these two countries. Strengths and weaknesses of this approach for supplementing routine human TBE surveillance are discussed.

Material and Methods

The two studies performed in Poland and Germany in 2008–2009 were designed and implemented independently of each other and had different aims. The methods used for selection of study sites, tick sampling and laboratory investigations in both countries are summarized in Table 1.

Selection of study areas

In Poland, 32 sampling sites located in 10 districts (Fig. 2) with very low reported TBE incidence were selected with the aim of extrapolating TBEV prevalence in ticks to the administrative district (NUTS4), which is the level at which TBE risk areas are defined in Poland based on human TBE surveillance. In contrast, the German group focused on an area spanning two districts in southern Hesse adjacent to Baden-Württemberg, in which 18 sampling points were selected that had been indicated as probable places of exposure by TBE patients over the previous decade (Fig. 2f). The overall rationale for the German study was to investigate whether TBEV prevalence in ticks would reflect the high incidence observed in humans. Overall 18 sampling sites located in the Odenwald, a low mountain range, and in the adjacent Rhine Valley, were selected for the study (Fig. 2d–f).

Tick collection

Taking into consideration the low TBEV prevalence found in ticks in previous studies (Table 2), both studies aimed at collecting as many ticks as possible. In Poland, each site was flagged at least once in spring and once in autumn. A lower limit of 200 ticks per sampling site was adopted, and thus, flagging was continued at least until this quota was achieved. In Germany, flagging was performed in defined areas of 200 m^2 every 2 months during two consecutive seasons (Table 1). In both studies, we used pooling of ticks to increase cost-efficiency of micro-

Table 2. Prevalence in ticks established in studies from Poland and Germany, permitting ascertainment of results to concrete districts and compare with reported incidence, 1990–2010

Author	Study period	Rationale for study	Pooling	Laboratory method	District	TBE incidence[a]	Ticks examined	TBE pools positive (MIR) (%)
Present study (PL)	2008–2009	To extrapolate the results from TBEV detection in ticks into NUTS4 administrative districts in Poland	10 adults 20 nymphs	Real-time RT-PCR (Schwaiger and Cassinotti, 2003)	Boleslawiecki	0	418	0
					Brodnicki	0	1047	0
					Brzeski	2.7	683	0
					Drawski	0	794	0
					Jarocinski	0	1173	0
					Klodzki	0.2	592	0
					Nowotomyski	0	701	0
					Obornicki	0	794	0
					Policki	0	625	0
					Starogardzki	0	609	0
Present study (DE)	2008–2009	Confirmation of endemic TBEV circulation in an area indicated by patients as place of exposure, 3 districts in Hesse, Germany	1 adults 10 nymphs 20 larvae	Real-time RT-PCR (Schwaiger and Cassinotti, 2003)	Bergstrasse	3.1	7191	0
					Darmstadt-Dieburg	0.8	1616	0
Wójcik-Fatla et al. (2011)	2008–2009	Confirmation of TBEV circulation in a region where consecutive seroprevalence studies indicated TBEV presence, 11 sites in 6 districts, Lubelskie province, Poland	10 adults, 30 nymphs	Nested RT-PCR (Schrader and Süss, 1999)	Krasnostawski	0	140	1 (0.7)
					Lubartowski	0.4	31	0
					Lubelski	0.4	448	8 (1.7)
					Parczewski	1.6	11	0
					Włodawski	1.5	247	19 (7.6)
					Zamojski	0	146	2 (1.4)
Dobler et al. (2011)	2005–2009	Temporal and spatial dynamics of TBEV in high-endemic focus, molecular characterization of the virus, 1 site in Bavaria, Germany	5 adults 10 nymphs, and larvae	Real-time RT-PCR (Schwaiger and Cassinotti); isolation in cell culture	Amberg-Sulzbach	5.6	4079	14 (0.3)
Kupča et al. (2010)	2005–2008	Temporal and spatial dynamics of TBEV in high-endemic focus, molecular characterization of the virus, 1 district in Bavaria, Germany	1 adults 5 nymphs and larvae	Real-time RT-PCR (Schwaiger and Cassinotti, 2003)	Amberg-Sulzbach	5.6	2150	5 (0.2)
Frimmel et al. (2010)	Feb–May 2007	Confirmation of TBEV circulation in 3 non-endemic areas where single cases occurred, Mecklenburg-Western-Pomerania, Germany	No pooling	Nested RT-PCR (Schrader and Süss, 1999)	Rügen	0	89	2 (2.2)
					Ostvorpommern	0	84	0

Table 2. (Continued)

Mecklenburg-Strelitz	0.2	77	4 (5.2)					
Klaus et al. (2010)	Jun 2005–Jul 2006	Confirmation of TBEV circulation in risk, non-risk and former risk areas in Thuringia (TH, 1 district), Saxony-Anhalt (S-A, 4 districts), Mecklenburg-Western-Pomerania (MV, 2 districts)	5 adults, 10 nymphs, 20 larvae	Real-time RT-PCR (Schwaiger and Cassinotti, 2003)	Unstrut-Hainich, TH	0	1075	0
					Börde, S-A	0.1	917	0
					Altmarkkreis Salzwedel, S-A	0	387	0
					Stendal, S-A	0	462	0
					Magdeburg, S-A	0	436	0
					Rügen, MV	0.3	570	0
					Mecklenburg-Strelitz, MV	0.2	81	0
Klaus et al. (2010)	Jun 2005–Jul 2006	Engorged ticks removed from volunteers from 8 districts in Bavaria (BY)	No pooling	Real-time RT-PCR (Schwaiger and Cassinotti, 2003)	Rosenheim, BY	1.2	135	1 (0.7)
					Miesbach, BY	0.8	42	0
					Mühldorf, BY	2.6	11	2 (18.2)
					Berchtesgardener Land, BY	2.7	18	0
					Ebersberg, BY	0.1	7	0
					Traunstein, BY	3.0	17	0
					Munich, BY	0	4	0
					Altötting, BY	3.4	5	0
Süss et al. (2006)	Feb–Jun 2002	Comparison of TBEV prevalence in ticks removed from humans, compared with previous studies of free-feeding tick from 17 districts in Bavaria, Germany	3 adults	Nested RT-PCR (Schrader and Süss, 1999)	Landshut	5.5	60	4 (6.7)
					Passau	15.5	132	6 (4.5)
					Cham	6.8	51	6 (11.8)
					Regen	1.2	36	6 (16.7)
					Freyung-Grafenau	6.1	33	5 (15.1)
					Rottal-Inn	2.2	72	5 (6.9)
					Ansbach	0	36	4 (11.1)
					Straubing-Bogen	0.4	27	2 (7.4)
					Schwandorf	2.6	3	0
					Dingolfing-Landau	1.7	21	1 (4.7)
					Deggendorf	1.9	30	3 (10)
					Kelheim	0.5	6	0
					Neustadt ad. Waldnaab	2.0	18	0
					Regensburg	1.8	27	3 (11.1)
					Mühldorf am Inn	1.1	3	0
					Amberg-Sulzbach	1.8	3	0
					Tirschenreuth	0	3	0
Holbach and Oehme (2002)	2000–2001	Confirmation of TBEV circulation in known endemic area in Baden-Württemberg, Germany	5 adults, 10 nymphs	PCR (amplification of 5′ non-coding region)	Main-Spessart	1.1	1657	2 (0.1)
Pietsch et al. (2002)	2000	Confirmation of TBEV circulation in known endemic foci in Rhineland -Palatinate, Germany	10 adults 20 nymphs	Nested RT-PCR (Schrader and Süss, 1999).	Birkenfeld	0.9	998	0

Table 2. (Continued)

Oehme et al. (2002)	1998–2000	Comparison of surveillance reports, serological survey, and TBEV prevalence in ticks from same period, in Baden-Württemberg, Germany	10 adults and nymphs	Nested RT-PCR (Schrader and Süss, 1999)	Emmendingen	3.0	740	16 (2.2)
					Ortenau	5.1	1405	20 (1.4)
					Breisgau-Hoch-Schwarzwald	1.6	445	9 (2.0)
					Bodensee	1.0	2057	37 (1.8)
					Pforzheim	1.2	1054	2 (0.2)
					Stuttgart	0.1	868	4 (0.5)
					Ludwigsburg	0.1	2620	14 (0.5)
Cisak et al. (2002)	2000–2001	Confirmation of TBEV circulation in known endemic foci in Lubelskie province, Poland	1 pool per site	Inoculation of mice, passaging in cell culture	Radzyński	0	24	1 (4.2)
					Lubartowski	0.2	14	0
					Lubelski	0.1	19	0

[a]TBE incidence expressed as the annual mean number of reported cases per 100 000 inhabitants in a 5-year period including the study period (see *Material and Methods*).
MIR, Minimum infection rate; TBEV, Tick-borne encephalitis virus.

biological investigation of collected ticks. Pooling of max. 20 ticks per pool has been shown to assure adequate precision of estimates for rare pathogens (Abel et al., 1999).

Laboratory investigation of collected ticks

In both studies, similar methods were used for identification of TBEV RNA in ticks (Table 1). Ticks collected in plastic tubes were transferred to the laboratory alive, where they were sorted according to species (identified morphologically), developmental stage and sampling place. RNA detection in both studies was performed using real-time RT-PCR (Schwaiger and Cassinotti, 2003). Three per cent of all samples collected in Poland and 5% of samples from Germany were selected at random to assess the quality of RNA extraction and check the absence of inhibitory substances using an RT-PCR targeting the 16S rRNA of *Ixodes ricinus*. Sequences for the primers and probe were identical to those proposed by Schwaiger and Cassinotti (2003), and the same protocol as for TBEV detection was used. TBE virus RNA from strains Neudörfl was routinely included at concentrations of about 100 copies/reaction (as tested using an artificial *in vitro* RNA transcript) to guarantee a constantly high detection limit of each PCR run. Furthermore, ticks from known German endemic regions were simultaneously tested in the same RT-PCR runs with positive results as expected.

Statistical analysis

The minimum infection rate (MIR) was calculated by dividing the total number of positive pools by the total number of collected ticks. We calculated binomial confidence intervals for overall and site-specific MIR estimates. As no positive ticks were identified, pooling was not taken into account in the statistical analyses.

Literature review of studies on TBEV detection in ticks in Poland and Germany

To place the discussion of our results regarding their usefulness for public health surveillance in a broader context, we performed a systematic search for published studies of TBEV detection in ticks, applied in Poland and Germany during 1990–2010. We reviewed the literature by searching PubMed for relevant articles [search string: TBEV and (Poland or Germany)], looked through reference lists of retrieved publications, and abstract books of international conferences on vector-borne diseases. We systematically abstracted the study location including human TBE incidence, sample size, size of pools, laboratory methods, TBEV prevalence detected in ticks and the rationale for the study. The mean annual incidence of human cases in

studied districts was calculated by dividing the mean annual number of cases that occurred during 5 years by the mean population size of the district from the respective 5-year period. The period for the above-mentioned calculation was chosen to overlap with the study, for example, if the study was limited to 1 year, we computed the mean incidence from a period including 2 years preceding, and 2 years following the study.

Results

TBEV detection in ticks from Poland

During the study period from 2008 to 2009, we collected 7436 *I. ricinus* ticks, of which 4451 were collected in spring and 2985 in autumn sessions (median number of ticks collected per site: 235; range, 78–394). Nymphs clearly predominated over adult female and male ticks (6338, 561, 537, respectively). In total, 523 pools were prepared for laboratory processing, the mean pool size was 14.1. The internal control was amplifiable in all samples examined, confirming the absence of polymerase inhibitors. All samples proved to be negative for TBEV RNA. The exact binomial 95% confidence interval (CI) for the null prevalence estimate was 0–0.05% overall, but much wider at the individual sampling sites: 0–4.62% at the site with the fewest collected ticks and 0–0.93% at the site with the largest number of collected ticks. All tested randomly selected samples were positive for tick DNA, indicating that no PCR inhibition occurred.

TBEV detection in ticks from Germany

A total of 8807 ticks of the species *I. ricinus* (50 female adults, 59 male adults, 2000 nymphs, and 6698 larvae) and 90 ticks of the species *Dermacentor reticulatus*, was collected in the German study sites. The median number of ticks collected per site was 380 (range: 62–1459). A total of 2289 pools were prepared and tested by real-time RT-PCR, the mean pool size was 3.8. None yielded a positive result. The exact binomial 95% confidence interval (CI) for the null prevalence estimate was 0–0.04% overall, but much wider at the individual sampling sites: 0–5.78% at the site with the fewest collected ticks and 0–0.25% at the site with the largest number of collected ticks.

Literature review of studies on TBEV detection in ticks in Poland and Germany

Our literature review identified 15 full articles, and six abstracts published only in conference proceedings. As two full papers and four abstracts repeated data published elsewhere, 15 studies were included in the present overview (Ramelow et al., 1993; Süss et al., 1996, 2004, 2006; Cisak et al., 2002; Holbach and Oehme, 2002; Oehme et al., 2002; Pietsch et al., 2002; Frimmel et al., 2010; Klaus et al., 2010; Kondrusik et al., 2010; Kubica-Biernat et al., 2010; Kupča et al., 2010; Dobler et al., 2011; Wójcik-Fatla et al., 2011). Over 50 000 ticks were tested in the identified studies. Of the 15 studies, however, only 10 permitted definitive assignment of results (including number of collected ticks and number of positive pools) to specific districts. A total of 22 417 ticks collected from 49 districts in both countries was tested in these 10 studies. The median number of ticks collected per district during 1990–2010 was 77 (range 3–6232). The mean pool sizes ranged from 1 to 18.5 ticks per pool. The results of TBEV prevalence in collected ticks varied greatly, from no positive pools in 23 districts to 2/18 (18.2%) engorged ticks in a Bavarian district, as reported by Klaus et al. (2010). TBEV was not consistently demonstrated in ticks collected in areas with high human TBE incidence. TBEV prevalence in ticks tended to be higher in studies using nested RT-PCR than real-time PCR method for viral RNA detection and in studies testing engorged ticks removed from humans compared with studies testing questing ticks (Table 2).

Discussion

Different approaches to determine the TBEV prevalence in ticks for assessment of TBE risk were independently attempted in Poland and Germany. In Poland, a multistage sampling scheme was applied for selection of tick sampling sites in regions with very-low TBE incidence and thus uncertain endemicity. In Germany, 18 sampling sites were selected in a known endemic area with high incidence of human disease, with the aim of confirming local TBEV circulation. We collected 7436 ticks in Poland and 8807 ticks in Germany, and all were found negative for TBEV RNA. While the number of ticks was large overall, the relatively small number of ticks at each sampling site led to wide confidence intervals, meaning that the circulation of TBEV in ticks could not be ruled out with certainty. Calculation of sample size needed for obtaining valid estimates of TBEV prevalence in collected ticks is complicated by marked variability of TBEV prevalence in time and space, depending on several mechanisms, including seasonal synchrony of larvae and nymphs (Randolph, 2004; Nonaka et al., 2010). By increasing the number of sampling sites in Poland, we increased the probability of identifying TBEV-infected tick pools. On the other hand, the small sample sizes achieved in some sampling sites decreased the likelihood of detection of a pathogen typically found in 0.02–2% of ticks (Table 2). The approach chosen in Germany, with somewhat larger tick samples collected in a closer-knit

grid of sampling points in a smaller endemic area with high human TBE incidence (Fig. 2), had higher statistical power. Nonetheless, we failed to detect the virus, suggesting that TBE foci in the German study region – and likely in other areas as well – may be highly localized. Thus, it is possible that many more sites would have to be sampled to ensure reliable detection of TBEV circulation in a given administrative region. Given that at least 1822–18 754 ticks would need to be tested to detect the expected prevalences of 0.5 ± 0.1%–5 ± 1% (not taking into account the effects of pooled testing); this would require enormous financial and personnel resources (sample size calculation using EPI-INFO, Centers for Disease Control and Prevention, Atlanta, GA, USA).

Only a few of the studies on TBEV detection in ticks identified in our literature search had the explicit aim of using results to supplement human TBE surveillance (present Polish study, Oehme et al., 2002; Süss et al., 2004; Frimmel et al., 2010). However, particularly positive results from any such studies could potentially provide useful information for human TBE surveillance if appropriately reported. In our systematic review of the literature, 10/15 studies provided information that allowed assignment of results to administrative districts, including the sampling sites, number of tested ticks and the number of positive pools (Cisak et al., 2002; Holbach and Oehme, 2002; Oehme et al., 2002; Pietsch et al., 2002; Süss et al., 2006; Frimmel et al., 2010; Klaus et al., 2010; Kupča et al., 2010; Dobler et al., 2011; Wójcik-Fatla et al., 2011). The remaining studies (that together tested >30 000 ticks) did not allow assignment of results to administrative areas, and results are therefore of limited use for assessment of human risk (Ramelow et al., 1993; Süss et al., 1996, 2004; Kondrusik et al., 2010; Kubica-Biernat et al., 2010). Thus, to assure the usefulness of TBEV RNA detection in ticks for surveillance purposes, published results should include information on the period and exact location of tick collection, number of ticks collected at each site, number of ticks positive and laboratory method used. However, in all such studies, failure to detect TBEV can only be interpreted as absence of TBEV circulation when extremely large numbers of ticks are collected.

Comparability of the results of reviewed studies is also limited by changes in laboratory methods for TBEV RNA detection in ticks from 1990 to 2010. The earliest two reviewed studies used the RT-PCR in combination with the Southern blot hybridization proposed by Ramelow et al. (1993) (Süss et al., 1996). Six studies used the nested RT-PCR procedure proposed by Schrader and Süss (1999) (Oehme et al., 2002; Pietsch et al., 2002; Süss et al., 2004, 2006; Frimmel et al., 2010; Wójcik-Fatla et al., 2011). The majority of recent studies used the real-time PCR method developed by Schwaiger and Cassinotti (2003) (Klaus et al., 2010; Kupča et al., 2010; Dobler et al., 2011; Kondrusik et al., 2010; present studies in Poland and Germany). In-house modifications of the nested RT-PCR method were applied in two studies (Holbach and Oehme, 2002; Kubica-Biernat et al., 2010), and inoculation of mice was used in another study (Cisak et al., 2002). Most authors provided a limited description of the laboratory methods used, especially in terms of RNA extraction, reverse transcription and its validation details, most commonly referring the details to articles describing the method. As only few articles were published after the issuance of MIQE guidelines for publication of quantitative real-time PCR experiments (Bustin et al., 2009), we did not systematically review the papers for inclusion essential information on real-time PCR methods used. Prevalence estimates of studies using nested RT-PCR tended to be approximately one order of magnitude higher than those studies investigating similar areas and using real-time PCR method for viral RNA detection (Table 2). Straightforward comparisons of those studies are therefore not possible.

To be useful for public health surveillance, environmental TBEV monitoring should be reliable and valid enough to draw conclusions regarding TBE human risk with reference to time and space. Following the adapted CDC attributes for efficient surveillance systems (Box 1), the method should firstly be simple and acceptable to its users. Tick flagging is straightforward and could be applied within the national or regional public health systems using trained public health officers or forestry services. Research groups and public health officers in both countries have gained extensive experience in organization and performance of such studies. Nonetheless, in our studies, it was not always possible to collect a large number of ticks in all sampling points – in Poland even despite several field trips.

Secondly, a tick-based surveillance system should be fairly flexible. This criterion could also be fulfilled, as adaptation of laboratory methods or extension of surveillance to other tick-borne pathogens would in principle be easily possible. Some of the reviewed studies provided examples of tick investigations for simultaneous detection of TBEV and the *Borrelia* spirochaete (Cisak et al., 2002; Holbach and Oehme, 2002), in one study – additionally for *Anaplasma* spp. (Oehme et al., 2002).

The third attribute – high sensitivity and positive predictive value of a potential tick surveillance system – is a much more complex issue and difficult to achieve. As discussed earlier, because TBEV prevalence in ticks is low and TBEV seems to exhibit highly patchy distribution at tick-infested areas, large numbers of ticks must be collected at multiple sample sites to ensure valid results. The

fourth attribute, representativeness with ability to extrapolate surveillance results to the level of administrative areas (district, provinces and/or countries), is related to the previous attribute. Because of TBEV circulation in microfoci, geographical representativeness would require performance of measurements in dense grids of sampling points, located throughout entire regions. Representative selection of only a few sampling sites would be unlikely to ensure valid extrapolation of results to the level of an administrative district. Limiting the tick investigations to areas with known high TBE incidence (present German study, Ramelow et al., 1993; Süss et al., 1996; Klaus et al., 2010; Kupča et al., 2010; Kondrusik et al., 2010; Dobler et al., 2011) might be useful in regions with high vaccination coverage, but even in such regions, more sampling sites and larger sample sizes than achieved in the discussed studies appear to be required. Furthermore, environmental monitoring method should also enable confirmation of the lack of risk for humans in non-endemic areas to be useful for public health surveillance, that is, have a high negative predictive value. As shown by the wide confidence intervals of the null prevalence estimates obtained in our study, this also requires very large sample sizes.

The fifth attribute, timeliness of a tick-based surveillance system, could be ensured through collection and testing of ticks at regular intervals. This would permit monitoring of changes in TBE endemicity over time and space, thus enabling recognition of new endemic areas or genetic modifications of pathogens. The sixth and final proposed attribute for an efficient vector-borne disease surveillance system – sustainability – would necessitate political commitment and major long-term funding.

Thus, while a tick-based surveillance system could in principle be simple, acceptable and flexible, the criteria of high sensitivity, representativeness, timeliness and sustainability would entail enormous logistical challenges and could only be achieved at considerable cost. In our experience, a further important requirement for tick-based surveillance was collaboration between researchers with very diverse backgrounds – epidemiologists, microbiologists, medical entomologists and wildlife ecologists – reflected by the qualifications of the present paper co-authors.

Thus, based on these considerations stemming from numerous studies performed to date, we conclude that the determination of TBEV prevalence in ticks is not an ideal method to complement human TBE surveillance. Nonetheless, valid results of existing studies may be considered for mapping TBE risk for public health purposes if information on sampling sites, numbers of sampled ticks and laboratory methods are adequately documented. To supplement human TBE surveillance, however, other approaches should be considered, such as seroprevalence studies in wildlife or farm animals, as a correlation between TBE seroprevalence and human TBE incidence has been shown in several smaller studies. For instance, cattle herds showed a larger increase in seroprevalence after summer pasture in regions with higher, compared with lower, human TBE incidence in south-western Germany (Rieger et al., 1997). Correlation with human TBE incidence was found for TBE seroprevalence in TBE seroprevalence in roe deer in Germany and Denmark (Gerth et al., 1995; Skarphédinsson et al., 2005), in foxes in Germany (Rieger et al., 1999; Wurm et al., 2000), in goats in Northern Italy (Rizzoli et al., 2007) and to some extent in sheep in various regions in Germany (Klaus et al., 2012). A further possibility for sentinel monitoring of TBE is PCR testing for the TBE virus in small rodents, in which TBE infection becomes chronic (Tonteri et al., 2010; Achazi et al., 2011), but in which antibodies could not be reliably detected in a Finnish study (Tonteri et al., 2010). However, methodology for serological testing or direct detection of TBEV in animals (e.g. in which organs) remains to be fully standardized. In addition, as the strength and duration of the antibody response to TBE infection may vary among animal species, further background research is desirable for reliable interpretation of serological investigations in animals. Nonetheless, the authors believe these approaches to be more feasible and to have a higher validity than investigation of ticks for complementing human TBE surveillance, particularly in areas with high vaccination coverage.

Acknowledgements

The Polish study was partly supported by the Med-Vet-Net network of excellence funded by European Commission DG Research (FOOD-CT2004-506122). The German study was partly supported by the Federal Ministry of Education and Research (BMBF) grant 01KI 0712 to MP, GD, and FR. We thank Philippe de Mendonça for his assistance in adopting the real-time PCR method in the Warsaw laboratory, Harald Weber for his excellent technical assistance in crushing the tick and Astrid Thomas for performing the real-time RT-PCR in Germany.

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