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Roe deer sera used for TBE surveillance in Austria

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ABSTRACT

A large majority of Austrian citizens are aware of tick-borne encephalitis (TBE), consequently reflected by a high vaccination rate of 85%. In return, risk assessment and disease mapping on human cases might be hampered due to high and inhomogeneous vaccination rates and travel habitats of humans. The roe deer was used to obtain a starting point for the integral view on the actual risk of TBE in Austria. The roe deer exhibits several attributes which makes it suitable as an indicator species: the roe deer has a restricted home range and it is known to be a heavy tick carrier. Furthermore it sero-converts after infection with TBE, but no outbreak occurs.

Sera from 945 roe deer were obtained from all over Austria and screened with IFAT for the antibodies against TBE. Twenty-two positive samples, 2.4%, and 17 samples at the borderline titre of 1:16 were identified. The majority of the positive samples, 70.6%, were located in known TBE areas based on human cases. Further research is needed to confirm or reject new endemic foci of TBE transmission.

Introduction

The *Flavivirus* responsible for causing tick-borne encephalitis (TBE) can be split into three subtypes: the Far-Eastern, the Siberian and the European subtype. The latter subtype can be predominantly found in Central and Northern Europe and Western parts of Russia (Heinz et al., 2013). This virus subtype is mainly transmitted by the vector tick *I. ricinus* (Labuda and Randolph, 1999), and can also be transmitted by raw milk and milk products originating from recently infected goats, sheep or cattle (Holzmann et al., 2009; Labuda et al., 2002).

Annually about 3000 human TBE cases are reported from European countries (Kiffner et al., 2012); estimations worldwide calculate more than 10,000 hospitalized people (WHO, 2011). Nowadays, in 2014, the immunization for TBE – with at least one dose once in their life – of Austrian citizens is about 85% of all inhabitants (GfK Healthcare, 2014). It is assumed that more than 4000 people were prevented from becoming infected with the TBE virus between 2000 and 2011 (Heinz et al., 2013). This high value of vaccinated people is responsible for a low number of TBE cases in Austria with an incidence below 1 per 100,000 inhabitants in 2009 with a vaccination rate in that period of 88% with at least one dose per life (Donoso Mantke et al., 2011; Heinz et al., 2013; Walder et al., 2008). In comparison the neighbouring country of the Czech Republic had a much higher incidence in the same year of 7.8 per 100,000, reflecting the low immunization rate of 16% of inhabitants. Among the non-vaccinated groups similar incidence rates are observed between Czech Republic and Austria (Heinz et al., 2013).

For many reasons such as children's health, tourism and animal health e.g. rare cases in dogs, further efforts in this field is obligatory and mapping of the occurrence of the virus is of major concern. But the areas, so called "foci of transmission", where the TBE virus (TBEV) circulate, are very restricted due to different requirements on vector and reservoir hosts, which in turn are influenced by habitat parameters such as climate and vegetation (Estrada-Peña and de la Fuente, 2014; Randolph, 2009). Therefore in middle Europe the occurrence of TBEV in ticks is supposed to be clustered in small areas. Consequently integral identification of risk areas is hampered due to the difficulty of discovering all these small foci. Based on data of patients, the current distribution map of TBE in Austria is built and constantly updated (Baxter Healthcare GmbH, Wien). The outcome of this map could be biased due to spatially inhomogeneous immunization rates, and the lack of data from the unsuspicious mild infections of human patients. To overcome these problems, additional strategies were developed.

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Questing ticks were sampled in various countries and screened for the occurrence of the virus (Dobler et al., 2011; Gäumann et al., 2010; Holbach and Oehme, 2002; Kupča et al., 2010; Rieille et al., 2014; Süss et al., 2004). These investigations delivered important results concerning proven virus distribution and gave the chance to obtain different virus isolates of different regions, ticks and time points. For epidemiological screening the limits in terms of this method are not negligible. First of all the occurrence of the virus is focussed on highly localized areas (Stefanoff et al., 2013), which needs a very small meshed sampling design. This in turn increases the costs of the surveillance, especially in low-risk areas (Süss et al., 2004). In some cases the virus load in questing ticks could be very low and only reach detection limit after replication, which takes place after attachment (Belova et al., 2012). Especially if the samples are pooled, a low virus load might remain undetected, reflecting a wrong picture of the actual virus distribution.

Another study compared the occurrence of human cases with the findings in the ticks and concluded that tick surveillances alone do not deliver reliable data for prediction maps of the TBE virus (Stefanoff et al., 2013).

So, other efforts were conducted to identify indicator species to obtain spatial distribution data. It has been suggested to use wildlife or livestock animals (Stefanoff et al., 2013). The obvious animals therefore are the natural reservoir of the virus, the rodents. Some studies on these showed a sero-conversion of about 2.6% and virus infection 0 of up to 20% (Achazi et al., 2011; Radda et al., 1971; Tonteri et al., 2011). The time-consuming effort needed for the catching and sampling, including a small meshed sample design, is the reason for this study not to choose these species.

In this study the roe deer was chosen to look for the occurrence of TBE for several reasons. The roe deer is distributed all over Austria. It can be found in the lowlands up to a higher level, with an assumed reduction of habitat quality above 1600 m in summer (Reimoser et al., 2009). This species is known to remain in a rather small home range of about 0.16–0.81 km^2 (Jeppesen, 1990; Nosek et al., 1967; Radda et al., 1968b). Roe deer are known as heavy tick carriers (Kiffner et al., 2010; Vor et al., 2010), but symptomatic TBE in roe deer has never been described so far (Nosek et al., 1967; Radda et al., 1968a,b). Sampling can be done on a large scale by instructed hunters. Last but not least several studies on roe deer declared the animals suitable for use as sentinels (Gerth et al., 1995; Kiffner et al., 2012; Nosek et al., 1967; Radda et al., 1968a,b; Skarphédinsson et al., 2005). All these attributes make the roe deer suitable as an indicator species for TBE.

Therefore we designed and conducted a surveillance of Austrian roe deer for the occurrence of TBE all over Austria. The aim of this study was to deliver additional data as a starting point for an integral risk assessment of the virus distribution.

Materials and methods

A total of 2480 sample tubes sent in packages of five tubes were distributed – related to the size of each county – to the hunters with the help of the local hunting organizations. These packages consisted of pre-numbered tubes and form sheets questioning data on sex, estimated age and location of the roe deer. Additionally a prepaid, labelled and addressed envelope was provided in each package to ensure a higher return rate.

945 sera of male and female roe deer, shot between 1st September 2013 and 31st December 2013, were sent to the Institute of Parasitology. These were aliquoted and forwarded to Gernot Walder GmbH for further investigation.

For the production of the IFAs, 25 cm^2 flasks with monolayers of Vero B4 cells (no. ACC-33, DSMZ) were infected with TBE virus strain K306, Westnile virus (WNV) strain Milano 1 or Usutu strain Vienna and incubated at 36 °C by gently shaking the flasks every 10 min. After 1 h, Medium199 (Invitrogen GmbH, Darmstadt, Germany), supplemented with 5% inactivated foetal calf serum (Invitrogen GmbH, Darmstadt, Germany), was added to the cultures. When cytopathic effects were detected, the infected cultures were trypsinized, cells were adhered to IFA slides (GML) for 1 h at 37 °C and then fixed with ice-cold 1:1 acetone-methanol mixture. The percentage of infected cells was adjusted to 50% by adding a certain amount of uninfected cells.

The IFA cut-off titres for TBE were established by analysing 125 sera from roe deer which were shot at least 50 km from the next known focus or residence of a human case of TBE. Among this low-risk collective 20.8% were positive for IgG antibodies at a titre of 1:4, 8% at a titre of 1:8 and 0.8% were positive at 1:16. Thus, according to the criteria of WHO, the cut-off titre was set at 1:16, where at least 98% of negative sera or low-risk sera yield a negative result.

20 μl of diluted sample was applied on the slides and the slides incubated for 40 min at 39 °C, then washed in PBS twice. For detection, 20 μl of FITC-labelled chicken anti-deer IgG antibodies (ACerIG-F, Gallus Immunotech Inc., Ontaria, Canada) were applied on the slide and incubated for 40 min at 39 °C, washed in PBS and covered with Glycerine/PBS 9:1.

Sera were rated positive when the fluorescence signal could be clearly distinguished from background at a 16-fold dilution. Sera were rated as borderline, when they gave a weak fluorescence signal at a 16-fold dilution. Positive and borderline sera were tested by two independent teams comprising of one technically and one microscopically working person each. Sera which were rated positive by both teams were marked as positive and diluted to the endpoint. Sera which were rated negative by at least one team were rated as negative. All other sera (e.g. positive/borderline or borderline/borderline) were rated as questionable. Only the samples tested positive and questionable for TBE, were tested further for Usutu- virus and WNV.

The positions of the roe deer were located on commune level in a map using the geographical information system “arcinfo” (ESRI®arcmap™ 10.0). Results of the IFAT were classified as negative (0), questionable (1) and positive (2). Maps using the inverse distance weighting function of arcinfo were used to draw a map. A catch distance of 10 km between the locations was chosen.

Maps of known TBE cases in humans were inserted in the maps as well to give an integral view (Baxter Healthcare GmbH, Wien). Calculations were performed in Excel® 2002 (Microsoft, Washington) and SPSS v. 20 (SPSS Inc., Chicago, USA). Differences between the groups were analyzed by using the Kruskal–Wallis test.

Results

Of the 945 sera, 22 were positive and 17 were questionable on the borderline of 1:16 titre. Latter questionable sera showed reaction at the cut of level and cannot be counted neither as positive nor as negative samples. All of the 22 positive roe deer sera originated from females (Table 1). The prevalence was 2.4%. Thirteen of the 17 borderline sera were also females. None of the 22 sera positive for TBE were positive for Usutu- or Westnile virus. One questionable serum was tested positive (1:32) for Usutu virus, therefore was left out for further investigations.

Fourteen of the 22 positive roe deer sera, 63.6%, are found in supposed human risk areas. Eight of the positive sera were found in areas where no human case has been reported so far. Of the questionable 16 sera at the borderline, 68.8% were found in areas with no human case (Fig. 1). Concerning the age composition there is a significant difference between males and females ($p < 0.01$), but there is no significant difference in terms of positive or questionable sera between males and females.

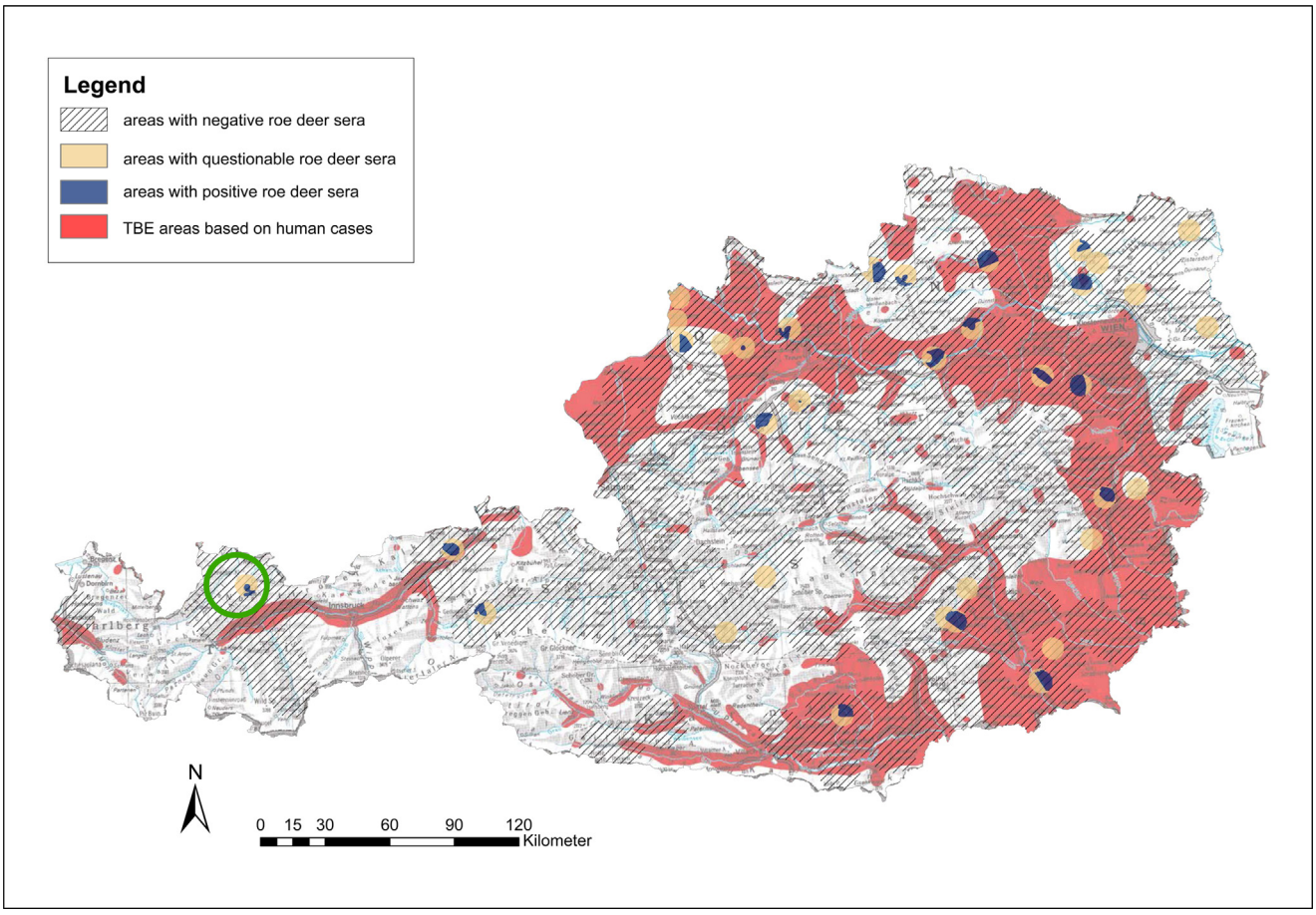

Fig. 1. Map of Austria showing TBE risk areas (Baxter Healthcare GmbH, Wien) and found potential transmission foci based on the roe deer samples. The coloured areas for positive and questionable (at the borderline titre) roe deer sera reflect an estimation based on interpolation of the roe deer sera by inverse distance weighting. The green circle indicates the area of Berwang, where a human seroconverted during the study in a supposed TBE-free region. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Age (estimated by the local hunter), sex, positive and questionable (at the borderline titre) roe deer sera. "pos" = TBE antibody positive.

Age	Females/pos/questionable	Males/pos/questionable	Sex unspecified
1	33/2/–	16/–/–	1
2	149/3/2	16/–/–	1
3	211/8/2	16/–/–	2
4	145/5/2	12/–/–	1
5	102/2/2	18/–/3	2
6	60/–/3	8/–/1	–
7	68/–/1	2/–/–	–
8	44/–/–	1/–/–	1
9	6/–/–	–/–/–	–
10	11/–/–	–/–/–	–
11	1/–/–	–/–/–	–
Unspecified	14/2/–	2/–/–	2
Total	**844/22/12**	**91/–/4**	**10**
%	90.3/2.4/1.4	9.7/–/0.4	1.07
Mean age	4.1/3.1/4.4	3.4/–/5.3	3.88
95% CI	[4.0–4.3]/[2.6–3.6]/[3.4–5.5]	[3.1–3.8]/–/[4.4–6.0]	[2.1–5.7]

Discussion

Risk assessment of TBE remains a topic of major concern in terms of public health. Although a good proportion of Austria's citizens are aware of the topic and vaccination rates – rate of people who received at least one dose per life – are incomparably high, these rates seem to have decreased in the recent past from 88% in 2005 to 85% in 2011, to 82% in 2013 and slightly increased to 85% in 2014 again (GfK Healthcare, 2014; Heinz et al., 2013). One important tool for prevention is to provide distribution or risk maps. Nevertheless risk maps for TBE based on human cases can be biased due to the travel behaviour of humans, different vaccination rates and disproportionate exposure risk among vaccinated and non-vaccinated people (Heinz et al., 2013; Kiffner et al., 2012), and also possibly due to a different pathogenicity of different virus isolates in various areas (Dobler et al., 2009; Stefanoff et al., 2013).

In terms of TBE a countrywide surveillance system based on tick sampling seems to be ineffective and time- and cost-consuming, and thus not an ideal method to substitute human risk maps (Stefanoff et al., 2013; Süss et al., 2004). Furthermore it does not reflect the actual risk onto humans, so other surveillance methods such as wildlife or farm animal investigations have been suggested (Stefanoff et al., 2013).

Roe deer represent very good sentinel animals, which can help to substitute risk maps (Gerth et al., 1995; Kiffner et al., 2012; Nosek et al., 1967; Radda et al., 1968a, 1968b). Furthermore it is believed to be one of the driving forces of tick distribution, spreading and sustaining the population (Carpi et al., 2008; Knap and Avšič-Županc, 2013; Labuda and Randolph, 1999; Medlock et al., 2013), therefore might be responsible for he spreading of pathogens as well (Kiffner et al., 2012). In terms of TBE this is discussed controversially: the viraemia in roe deer is supposed to be too low to have an impact on spreading the pathogen itself (Labuda et al., 2002; Nosek et al., 1967). Large ungulates are believed to harbour mainly adult ticks. The vertical transmission of the virus to

the progeny seems negligible, presumably not delivering enough infected ticks to implement a new transmission foci (Estrada-Peña and de la Fuente, 2014). Large mammals contribute to the transmission by feeding adult ticks and thus maintaining the tick population (Labuda and Randolph, 1999). Additionally on roe deer all three life stages can be found, feeding (Skarphédinsson et al., 2005) and potential co-feeding between nymphs and females and larvae and nymphs was predicted due to substantial overlapping of attachment sites on the host (Kiffner et al., 2011).

Quite intriguing is the low number of total seroconverted animals compared to other studies, where 15% to up to 50% positive roe deer in an area were detected (Kiffner et al., 2012; Radda et al., 1968b). We tried to achieve sera from many different places to cover more areas. In contrast to the study of Kiffner and colleagues (2012), for example, a much larger area was screened, thus including many areas with no TBEV foci. So, if the "mesh size" of the investigated roe deer samples had been smaller, we would have obtained more animals in and around the positive foci, and consequently the overall prevalence would have increased.

Although there has been a trend of TBE shifting to higher altitudes over the last ten years, natural foci of TBE are usually recorded below 1400 m of altitude, yielding a relatively small percentage of potentially affected areas in the alpine parts of the country (Walder et al., 2008).

Additionally the sex might have an impact on the result. Interestingly in one study females were less positive concerning antibodies than male roe deer, for which the authors cannot give an explanation (Gerth et al., 1995). Although there are no differences in the home range of the sexes, this might be reflected by the habit of movement of the animals. Males have bigger activity patterns in springtime, probably as a result of territorial behaviour (Jeppesen, 1990). So, males might have an increased likelihood of achieving a positive focus at a time of the year with ongoing tick activity. In our data set we could not confirm a higher risk for males of becoming infected.

A third explanation for the low number of positive roe deer sera could lie in the material itself. The blood of the roe deer often had a haemolytic appearance due to the logistical challenges, so antibodies might have denatured as well. Although this cannot be ruled out, the antibodies are known to be very stable and we do not expect this to have a large impact.

The majority of positive roe deer sera (63.6%) were from the areas of known or assumed TBE risk based on human cases. This can be seen not only as confirmation of the already existing map, but also as additional information giving an integral view on possible risk in the already known areas. Due to the fact that roe deer do not migrate like humans do in the form of tourism and the like, positive foci could be identified more accurately. With the help of this information, attempts can be undertaken to localize transmission foci.

Much more important are the areas which were identified being potential risk areas based on the roe deer sera data, but have not shown any human cases in the past. These areas might have been neglected in terms of human infections, possibly due to fewer human visits based on lower "attractiveness" for humans (Estrada-Peña and de la Fuente, 2014) such as uninviting landscape, dense vegetation etc. Humans might not have reached these foci until now, whereas roe deer are living there, "sampling" positive ticks. If these areas are not attractive for human use, the actual risk coming from these areas can be discussed. Due to the increasing popularity of outdoor activities of people expanding to new and undiscovered places, any of these new potential foci should be considered in terms of potential TBE refuges. Similar to the other foci, these localized areas help to get an integral view on possible infection sites.

Notable is the fact that there is at least one potential focus based on the roe deer data in Berwang (marked with a green circle in Fig. 1), which overlaps geographical with a serologically confirmed human case, who has probably been acquired about 10–15 km away in the neighbouring community of Ehrwald during the course of this study (Walder, unpublished). This area had hitherto not been known to host TBE.

But caution has to be exercised with these data. Similarly to human case data, the roe deer data can be biased. Normally the movement of roe deer is supposed to be restricted to a certain area and during a study of tagged roe deer fawns, 64.7% stayed within 500 m of their birthplace. About 2.1% moved more than 20 km, but some individuals might migrate up to 64 km (Reimoser et al., 1999). Especially young deer migrate some distance, until they find their habitat. But TBE antibodies are believed to persist a lifetime (Stanek and Hofmann, 1994). So it cannot be excluded that young roe deer become infected in a certain area, then moved to another and settled there. This might draw a wrong picture of non-existing transmission foci. Therefore the location of one positive roe deer serum is not equal to one positive infection focus. Even more, there might be possibility concerning cross-reactions to other flavivirus such as Usutu- and Westnile virus. In the cases of the positive TBE sera found in this study, we could exclude this for the aforementioned viruses. One questionable serum was positive for Usutu, therefore the borderline TBE titre might reflect a wrong positive result for TBE. Nevertheless the others did not reveal a positive result neither for Usutu- nor for Westnile virus. So, even if we exclude the serum tested positive for Usutu from all questionable sera in unknown risk areas, there is still a high amount of 68.8% of these sera remaining. This might be a hint for a low infection pressure in these cases. It is generally assumed that roe deer become re-infected every now and then if situated near a transmission foci, consequently the antibody titre remains at a high level (Nosek et al., 1967). The questionable titre might be caused by a lack of re-infections due to an inability to reach positive ticks as a consequence of migration to a TBE-free region. Yet there are no long term data on antibody persistence in roe deer after TBE infection, therefore further investigations on other roe deer, rodents and ticks have to be made to confirm a positive site. Additionally the data reflect a snapshot and do not claim to deliver all positive foci of transmission in Austria, meaning that areas with confirmed negative roe deer do not necessary exclude the occurrence of any TBE foci in that area.

Concerning the age of the roe deer, it is assumed that it does not have a big impact on the results, because if situated near to a focus, the animals become infected quite early. If there is no infection foci, the roe deer will not be exposed during their entire life (Gerth et al., 1995). This is in concordance with this data. The animals with a positive titre have a lower mean age than the whole group, indicating a very early infection time point.

In conclusion roe deer represent a very good indicator species due to the easiness of obtaining samples, the overall distribution of the roe deer and the almost restricted home range of the animals. Especially in areas of high vaccination rates of inhabitants, such as in Austria, this method should be considered as additional data on the distribution of the virus. Only a combination of all available direct and indirect data e.g. from humans, ticks, rodents and wild ungulates is able to give an integral view on the actual distribution of the virus.

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