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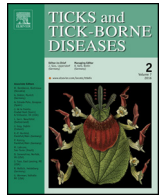
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# Prevalence estimation of tick-borne encephalitis virus (TBEV) antibodies in dogs from Finland using novel dog anti-TBEV IgG MAb-capture and IgG immunofluorescence assays based on recombinant TBEV subviral particles



Lev Levanov<sup>a,\*</sup>, Cristina Pérez Vera<sup>a,b,c</sup>, Olli Vapalahti<sup>a,d,e</sup>

<sup>a</sup> Department of Virology, University of Helsinki, Helsinki, Finland

<sup>b</sup> Department of Equine and Small Animal Medicine, University of Helsinki, Helsinki, Finland

<sup>c</sup> Department of Clinical Veterinary Studies, Vetsuisse Faculty, University of Bern, Bern, Switzerland

<sup>d</sup> Department of Virology and Immunology, Helsinki University Hospital, Helsinki, Finland

<sup>e</sup> Department of Veterinary Biosciences, University of Helsinki, Helsinki, Finland

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## ABSTRACT

Tick-borne encephalitis (TBE) is one of the most dangerous human neurological infections occurring in Europe and Northern parts of Asia with thousands of cases and millions vaccinated against it. The risk of TBE might be assessed through analyses of the samples taken from wildlife or from animals which are in close contact with humans. Dogs have been shown to be a good sentinel species for these studies. Serological assays for diagnosis of TBE in dogs are mainly based on purified and inactivated TBEV antigens. Here we describe novel dog anti-TBEV IgG monoclonal antibody (MAb)-capture assay which is based on TBEV prME subviral particles expressed in mammalian cells from Semliki Forest virus (SFV) replicon as well as IgG immunofluorescence assay (IFA) which is based on Vero E6 cells transfected with the same SFV replicon. We further demonstrate their use in a small-scale TBEV seroprevalence study of dogs representing different regions of Finland. Altogether, 148 dog serum samples were tested by novel assays and results were compared to those obtained with a commercial IgG enzyme immunoassay (EIA), hemagglutination inhibition test and IgG IFA with TBEV infected cells. Compared to reference tests, the sensitivities of the developed assays were 90–100% and the specificities of the two assays were 100%. Analysis of the dog serum samples showed a seroprevalence of 40% on Åland Islands and 6% on Southwestern archipelago of Finland. In conclusion, a specific and sensitive EIA and IFA for the detection of IgG antibodies in canine sera were developed. Based on these assays the seroprevalence of IgG antibodies in dogs from different regions of Finland was assessed and was shown to parallel the known human disease burden as the Southwestern archipelago and Åland Islands in particular had considerable dog TBEV antibody prevalence and represent areas with high risk of TBE for humans.

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## 1. Introduction

Tick-borne encephalitis virus (TBEV) is the most important pathogen of the mammalian tick-borne group of the genus *Flavivirus* within the family *Flaviviridae* (Gubler et al., 2007). TBEV has been reported to cause 3000 human cases of encephalitis in Europe and at worst, up to 10 000 cases in Russia annually (Gritsun et al., 2003; Lindquist and Vapalahti, 2008). The virus is transmit-

ted to mammals, birds, reptiles and amphibians by ixodid ticks (Farkas, 2002; Weissenböck et al., 1998). It has been shown that the distribution area of *Ixodes ricinus* ticks, which are the principal vector for TBEV in Europe, is increasing (Gilbert, 2010; Medlock et al., 2013; Jaenson et al., 2012) due to climate changes (Rizzoli et al., 2009; Jaenson and Lindgren, 2011; Knap and Avšič-Županc, 2013) and/or anthropogenic factors (Randolph, 2004, 2010). It is known that the distribution rate of ticks and risk of tick-borne encephalitis (TBE) might be evaluated through analyses of the samples taken from wildlife or from animals, which are in close contact with humans (Paillard et al., 2015; Lindhe et al., 2009; Roelandt et al., 2011). Due to the close relationship between humans and

\* Corresponding author.

E-mail addresses: [lev.levanov@helsinki.fi](mailto:lev.levanov@helsinki.fi) (L. Levanov), [cristina.perezvera@helsinki.fi](mailto:cristina.perezvera@helsinki.fi) (C.P. Vera), [olli.vapalahti@helsinki.fi](mailto:olli.vapalahti@helsinki.fi) (O. Vapalahti).

dogs, dogs are a good sentinel species for these studies (Lindhe et al., 2009; Roelandt et al., 2011; Imhoff et al., 2015). For example, when the first five human cases of TBE were recognized in Norway, a 16% seroprevalence was detected in dogs in the same geographic area (Csango et al., 2004). In addition, clinical dog cases of TBE may also be diagnosed, leading to meningoencephalitis and death/euthanasia, although despite the high seroprevalence in many countries, verified dog TBE meningoencephalitis cases are rather infrequent (Pfeffer and Dobler, 2011). The diagnosis of TBE for dogs is usually performed serologically (Roelandt et al., 2011). Several different enzyme immunoassays (EIAs) have been developed over the last several years, including commercially available EIAs, which are mainly based on purified and inactivated TBEV antigens. In order to produce TBEV antigen for these EIAs, biosafety level 3 facilities and a specially trained staff are needed. To detect IgG antibodies in human sera we developed previously a specific and sensitive IgG monoclonal antibody (MAb)-capture immunoassay (Levanov et al., 2014) based on secreted recombinant TBEV prME subviral particles produced in mammalian cells from Semliki-Forest virus (SFV) replicon. Here we describe and evaluate the diagnostic potential of the modified SFV-prME immunoassay as well as IgG immunofluorescence assay (IFA) based on Vero E6 cells transfected with SFV replicon encoding TBEV prME subviral particles for the detection of IgG antibodies in dog sera. Furthermore, we used both assays to have the first estimate of the prevalence of TBEV antibodies in Finnish dogs as well as to potentially identify new TBEV risk areas in Finland.

## 2. Materials and methods

### 2.1. Serum samples

This study was approved by the Laboratory Animal Board of the Southern Finland Regional State Administrative Agency. 148 dog serum samples from different regions of Finland, described in a previous study (Perez Vera et al., 2014) were tested using a commercial Immunozytm FSME IgG All species test (Progen Biotechnik GmbH, Heidelberg, Germany) as well as for TBEV-specific hemagglutinating antibodies by an in-house hemagglutination inhibition test and for anti-TBEV IgG antibodies in immunofluorescence assay (IFA) as described below.

### 2.2. Reference tests

As an IgG reference test, we used Immunozytm FSME IgG All species test (Progen Biotechnik GmbH, Heidelberg, Germany) according to the manufacturer's instructions. This is a two-step EIA and based on EIA wells coated with inactivated TBEV. Briefly, diluted serum samples and subsequently the anti-dog IgG conjugate were incubated in the wells, and the bound conjugate was detected by incubation with a substrate; the reaction was stopped with sulfuric acid, and the optical density (OD) was measured at a wavelength of 450 nm. The cutoff values and border line zones were provided by the manufacturer in the test kits. Total antibodies to TBEV from serum were also determined by standard hemagglutination inhibition test (Vene et al., 1998).

### 2.3. Production of TBEV prME subviral particles

The expression of TBEV prME subviral particles was described recently (Levanov et al., 2014). Briefly, Vero E6 cells (10th or 11th passage) were grown to 75–80% confluence in a 100-mm plate and transfected with 19 µg CMV-SFV-2SG-prME Kumlinge A52 construct using FuGENE HD transfection reagent (Promega) in Opti-MEM (Invitrogen) without serum and antibiotics. The transfected cells were grown in Opti-MEM supplemented with 7.5%

fetal calf serum and antibiotics. Thirty hours posttransfection the supernatant (15 ml) was harvested and 75-fold concentrated using Amicon Ultra-15 30 K columns (Millipore), according to the manufacturer's instructions. The concentrated supernatant was stored at 4 °C and used as an antigen for the EIA.

### 2.4. TBEV IgG MAb-capture assay

The assay for TBEV-specific IgG was modified from a previously described assay (Levanov et al., 2014). The optimal conditions for the assay were determined by box titrations of all included reagents. Microtiter plates were coated with MAb 1418 (5 µg/ml) which is targeted against domain III of glycoprotein E of TBEV (Niedrig et al., 1994). Concentrated TBEV prME antigen diluted 1:8 in EIA buffer (PBS with 0.5% bovine serum albumin and 0.05% Tween 20) and negative control antigen (concentrated supernatant from Vero E6 transfected with CMV-SFV-2SG construct without insert) were incubated alone for 1 h and then with duplicates of studied and control dog sera, diluted 1:200 in EIA buffer, for 1 h. Specific antibody binding was detected with 1:2500 diluted peroxidase-conjugated goat anti-dog IgG (Santa Cruz Biotechnology), and tetramethylbenzidine substrate. The reaction was stopped with sulfuric acid, and the OD was measured at a wavelength of 450 nm. The mean values of the sample absorbances were adjusted to an average OD value in this experiment (0.6) by dividing the measured absorbance of a sample by this average OD. To control the specificity of the assay, the OD values from wells with negative control antigen were subtracted from the OD values of the wells containing TBEV antigen. To control the interplate variation, an acute-phase TBE dog serum sample was used as an internal standard in all plates; the control values between the plates were adjusted as described above.

### 2.5. Precision

The precision of the IgG MAb-capture SFV-prME assay was measured as a coefficient of variation (%) from the mean value of the positive control (3 replicates on each plate). Both intra-assay precision (the reproducibility between wells within an assay) and interassay precision (the reproducibility between assays) were estimated.

### 2.6. Linearity of dilution

To assess the dilution linearity of the standard curves of the developed EIA, the positive dog serum samples were initially diluted 1:20 and then serially titrated in EIA wells in the IgG MAb-capture SFV-prME assay. The linear range of the curve was estimated following this step.

### 2.7. TBEV IgG IFA

The Vero E6 cells transfected with the CMV-SFV4-2SG-prME Kumlinge A52 construct and the cells transfected with the empty CMV-SFV4-2SG plasmid were fixed onto microscope slides with acetone for 7 min and stored at 4 °C until use. Dog serum samples diluted 1:10, 1:40, 1:160, 1:640, and 1:2560 in PBS were incubated for 1 h for the IgG IFA at 37 °C. Fluorescein isothiocyanate-conjugated anti-dog IgG (Jackson Immuno Research, West Grove, PA), diluted 1:30 in PBS, were incubated for 1 h at 37 °C. Unbound antibodies and anti-dog IgG were washed away with PBS and distilled water. The slides were covered with mounting medium and coverslips and read using a ×20 objective of fluorescence microscope Olympus IX71 (Japan). IFA with the cells infected with TBEV (strain Kumlinge A52) was done in the same way.

**Table 1**

Comparison of the results obtained in commercial EIA and EIA using recombinant antigen with the results obtained in IgG IFAs and hemagglutination inhibition test for positive serum samples.

Sample ID	Geographical location	EIA tests		IFA tests		HI titer
		IgG SFV-prME A52 test	Immunozyt FSME IgG All Species	Vero E6 cells transfected with SFV-prME A52	Vero E6 cells infected with TBEV A52	
117	Åland Islands	0.79	1.81	+++	+++	80
126	-//-	0.83	1.87	+++	+++	320
128	-//-	1.82	1.19	+++	+++	160
129	-//-	0.67	0.58	+	+	<20
136	-//-	0.92	1.6	+++	+++	80
142	-//-	1.15	1.69	+++	+++	320
146	-//-	1.69	1.42	+++	+++	<10
147	-//-	0.5	1.2	+	+	40
152	Kemiönsaari	0.72	1.99	+++	+++	160
154	Taalintehdas	0.17	0.74	-----	-----	<10

### 3. Results

In order to test canine serum samples in IgG MAb-capture assay, the optimal conditions for the assay were determined by box titrations of all included reagents (see Section 2) and the dilution linearity of the standard curves, the intra- and interassay precision were estimated. It was shown that the linear range of the curves extended from 0.25 to at least 2.3 optical densities for the IgG MAb-capture SFV-prME assay (data not shown). Coefficient of variation was <10% for intra and interassay precision. After this evaluation and optimization, 148 dog serum samples from Finland, including Åland Islands (20 samples), Southwestern archipelago (17 samples), Southern Finland (98 samples), Eastern Finland (3 samples), Oulu (2 samples) and Lapland (9 samples) were tested in IgG MAb-capture SFV-prME EIA with TBEV prME subviral particles of European subtype. The results were compared to those obtained with a commercially available IgG ELISA. One hundred negative-control dog serum samples were first used to adjust the cutoff value of the assay, and the mean absorbance plus three standard deviations was  $0.104 + 3 \times 0.0366 \approx 0.213$ . In order to show the gray zone and to increase the specificity of the assay after the initial evaluations, the cutoff was adjusted to a 25% higher value, to 0.266. Of the 10 serum samples shown to be positive by the commercial IgG ELISA, 9 were positive and one sample (#154) was negative in the IgG MAb-capture SFV-prME assay (Table 1), for a calculated sensitivity of 90%. However, sample #154 was also negative in IgG IFA with TBEV infected cells and in hemagglutination inhibition test. If we take these data into account and consider the sample #154 a true negative, the resolved sensitivity of the IgG MAb-capture SFV-prME test was 9/9 (100%), although the low amount of positives found does not allow the sensitivity to be thoroughly assessed. All 138 dog serum samples that were negative by the commercial IgG ELISA were also negative in the IgG MAb-capture assay, yielding a calculated specificity of 100%. It should be noted that sample #146 which was positive in both IgG EIAs and IgG IFA with TBEV infected cells was negative in hemagglutination inhibition test, and was considered positive (Table 1).

The IgG IFA with the cells transfected with the plasmid CMV-SFV-2SG-prME Kumlinge A52 and canine serum samples was performed as described in Section 2. The results were compared to those obtained with IgG IFA based on the cells infected with TBEV as well as with the commercial IgG EIA and hemagglutination inhibition test. Of those 10 samples indicated as positive in the commercial ELISA, 9 were positive in developed IgG IFA, yielding a calculated sensitivity of 90%. The inconclusive sample #154 which was considered above as a true negative, was also negative in developed IgG IFA. If we take these data into account, the resolved sensitivity of the developed IgG IFA was 9/9 (100%). All 138 samples

that were determined previously as negative, were also negative in generated IgG IFA, yielding a calculated specificity of 100%.

Of the 9 true positive ELISA samples, 8 were from Åland Islands and one was from Kemiönsaari, Southwestern archipelago (Table 1). Analysis of these ELISA samples showed a seroprevalence of 40% on Åland Islands and 6% on Southwestern archipelago (Table 1).

### 4. Discussion

In this work, we described the design of the IgG MAb-capture SFV-prME EIA based on TBEV prME subviral particles expressed from SFV replicon in mammalian cells as well as IgG IFA based on Vero E6 cells transfected with the same SFV construct for the detection of IgG antibodies in canine sera. The developed assays identified 9 positive serum samples out of 10 samples indicated to be positive by the commercial IgG ELISA. It was shown later that the one inconclusive serum sample was probably a false positive in a commercial IgG ELISA, because it was also negative by the hemagglutination test and the IgG IFA with TBEV infected cells (Table 1). All of the 138 serum samples which were negative in the commercial test were also negative in developed assays. Therefore, the resolved sensitivities of created assays were 90–100%, and the specificities of the two assays were 100%. These data indicate that the developed tests might be useful tools for the detection of IgG antibodies against TBEV in canine sera.

The ELISA and IFA results obtained in this study demonstrated that Southwestern archipelago (seroprevalence of 6%) and especially Åland Islands (seroprevalence of 40%) are known areas with high risk of TBE for animals and humans. Similar ELISA results (seroprevalence of 30.4%) were obtained for dogs from Bornholm, Danish Island in Baltic Sea (Lindhe et al., 2009). The high percentage of seropositive dogs is likely explained by situation of most important foci in the archipelago regions of the country due to an optimal ratio of temperature and humidity allowing tick co-feeding, as compared to continental parts of Finland (Tonteri et al., 2015). However, there is evidence of new foci accumulating, and is likely that if the mean annual temperatures continue to increase, as predicted, it is likely that both the population of ticks and TBE foci will continue to spread northward (Perez Vera et al., 2014). Therefore the amount of seropositive dogs as well as the amount of TBE human cases might also increase. Other factors can also influence on the seroprevalence such as urbanization. The urban dogs have fewer chances to be bitten by ticks compare to dogs living countryside. The few samples collected from Eastern Finland and Lapland were negative, and more samples from these regions are needed in order to estimate TBEV seroprevalence in dogs as we know that TBEV strains are circulating in these areas (Jääskeläinen et al., 2006, 2010).

## 5. Conclusion

In conclusion, specific and sensitive EIA and IFA for the detection of IgG antibodies in canine sera were developed. Based on these assays the seroprevalence of IgG antibodies in dogs from different regions of Finland was assessed. The Åland Islands, where incidence has been highest in Finland but case numbers have now diminished due to vaccination, was shown to represent a major TBEV risk area in Finland also using this approach, which further shows that dog TBEV antibody screening is one useful mean in the toolbox for estimating TBEV risk to humans.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

LL and OV designed the study, LL drafted the first version of the manuscript and finalized it. CP collected the serum samples from the dogs. LL and CP performed the serological tests. All authors read and approved the final version of the manuscript.

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