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Antibody ratios against NS1 antigens of tick-borne encephalitis and West Nile viruses support differential flavivirus serology in dogs

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Abstract

Flavivirus diagnostics are complicated by substantial cross-reactivity of antibodies between different flavivirus species. This is of particular importance in regions with multiple endemic flaviviruses in co-circulation. Tick-borne encephalitis virus (TBEV) is the causative agent of tick-borne encephalitis, the most common infection of the central nervous system in endemic regions of Europe and Asia. Since 2018, the related West Nile virus (WNV) has spread to Germany where its geographic distribution overlaps with TBEV endemic regions. Besides humans, various animal species are susceptible to TBEV and WNV infection. To compare antibody responses against these flaviviruses and test for cross-reactivity, we developed a multi-species luciferase immunoprecipitation system antibody detection assay for several different antigens. We performed a serosurvey of 682 dogs from five different European countries to detect antibodies against TBEV and WNV. Twelve specimens were positive for TBEV NS1 only and seven for WNV NS1 only. Two specimens were reactive to both NS1 antigens and another two were equivocal for WNV NS1. Interestingly, 89.5% of positive specimens had TBEV/WNV or WNV/TBEV signal ratios of 10 to >300 between individual NS1 antigens, allowing for a clear distinction between the two viruses. The remaining 10.5% of reactive specimens showed a five- to 10-fold difference between the two viruses and included possible dual exposures to both viruses. In contrast, equivocal samples showed low signal ratios between the NS1 antigens, suggesting unspecific reactivity. Based on these data, we found the NS1 protein to be a suitable antigen to distinguish between TBEV- and WNV-specific antibodies in dogs with sensitivity and specificity similar to virus neutralization tests.

KEYWORDS
differential diagnostics, flaviviruses, serology, tick-borne encephalitis virus, West Nile virus

1 | INTRODUCTION

Tick-borne encephalitis virus (TBEV) is the most common tick-borne viral pathogen in Europe and Asia and the causative agent of tick-borne encephalitis (TBE). Despite available vaccines, the incidence of TBEV infections is increasing, making it a growing health concern in endemic countries (Bogovic & Strle, 2015). In Europe, the most affected regions are located in Austria, southern Germany, the Czech Republic, Slovenia, Baltic States and the southern part of Scandinavia. The number of endemic areas is rising with newly established endemicity regions in Switzerland, north-eastern France and northern Italy (Chrdle et al., 2016). In Germany, risk areas increased from 129 in 2007 to 161 in 2019 (Hellenbrand et al., 2019). The virus is mainly transmitted by ticks from infected animal reservoir hosts, such as rodents or insectivores (Michelitsch et al., 2019), to humans or other incidental large animal hosts. However, there are also human cases associated with the consumption of unpasteurized milk products from infected small ruminants (Mansfield et al., 2009). Besides humans, a number of wild and domestic animal species are susceptible to TBEV infection with variable clinical manifestations ranging from no symptoms to febrile illness in a first clinical phase and severe neurological complications like meningitis, meningoencephalitis or meningoencephalomyelitis in a fraction of infected individuals that enter a second phase of disease (Ruzek et al., 2019). Of the potential TBEV hosts, dogs are of special interest, because they have a high tick exposure while living close to humans. Although most TBEV-infected dogs remain free of disease, in rare cases, individuals can develop almost identical neurological manifestations (e.g. encephalomyelitis) as observed in human patients (Pfeffer & Dobler, 2011).

West Nile virus (WNV) is the most widespread cause of arboviral neurological disease in the world and persists on all continents except for Antarctica (Bai et al., 2019). The virus is transmitted by mosquitoes from infected animal reservoir hosts (mainly birds) to various hosts (Habarugira et al., 2020). The majority of WNV infections are related to the transmission by mosquito bites, but several different transmission routes have been shown or are hypothesized like infection via blood transfusions or organ transplants (Iwamoto et al., 2003; Pealer et al., 2003). A wide array of species are susceptible to WNV infection, including humans, horses and other vertebrates. The disease outcomes vary between species and individuals ranging from asymptomatic hosts to symptomatic hosts with mild febrile disease (West Nile fever) to West Nile neuroinvasive disease including meningitis, encephalitis and poliomyelitis (Byas & Ebel, 2020). Due to their clinical sensitivity to WNV infection, horses are often used as sentinels for WNV surveillance (Beck et al., 2017) and some studies suggest that dogs offer an alternative sentinel species to horses (Bowser & Anderson, 2018). Large outbreaks have been observed in Southern Europe and North America, as well as ongoing transmission in the Middle East, Africa and Asia (Chancey et al., 2015). Since 2018, the virus has spread to Germany and has recurred annually since then (Ziegler et al., 2019, 2020).

TBEV and WNV are single-stranded, positive-sense RNA viruses and belong to the genus *Flavivirus* in the family of *Flaviviridae*, which also includes other important arthropod-borne human pathogens, including yellow fever virus, Japanese encephalitis virus, Zika virus and dengue virus. Many of these viruses are expanding their geographic range and together they account for several hundred million infections globally each year (Collins & Metz, 2017). In many regions worldwide, multiple flaviviruses are co-circulating (Beck et al., 2013; Nikolay, 2015; Pierson & Diamond, 2020).

Flavivirus diagnostics mostly rely on the detection of specific antibodies as viral RNA is only detectable during the first, often subclinical phase of an infection. During the severe second phase of disease, diagnostics rely on serological detection of flavivirus-specific antibodies. Current serological methods such as enzyme-linked immunosorbent assay (ELISA) or virus neutralization are restricted to the detection of antibodies against the major viral surface protein, the envelope (E) protein. Of particular interest is the domain III of the E protein (EDIII) as it contains the crucial amino acid residues for binding of a cellular receptor (Chu et al., 2005; Lee et al., 2006). Antibodies directed against this domain interfere with receptor engagement and typically lead to neutralization of viral particles (Crill & Roehrig, 2001; Kanai et al., 2006). However, serologic distinction of individual flavivirus infections is complicated by substantial cross-reactivity of antibodies between different flavivirus species (Beck et al., 2013; Reusken et al., 2019). The detection of neutralizing antibodies in virus neutralization tests (VNTs) yields the highest specificities. Unfortunately, neutralization assays are time-consuming and depend on high-containment laboratories. Therefore, new diagnostic tools are needed for differential serologic diagnostics of flavivirus infections in a simple and fast assay system. We developed a multi-species luciferase immunoprecipitation system (LIPS) antibody detection assay for several flavivirus antigens, based on the method of Burbelo et al. (2009, 2010). The use of a luciferase reporter allows the sensitive detection of specific antibodies without the need to express large amounts of antigens, which can present a bottleneck in other serologic test formats. Furthermore, as a bead-based method, the LIPS assays does not require any coating steps and reduces the time needed for assay performance. In this study, different viral antigens were tested in a modified LIPS assay to identify candidates that allow discrimination between infections with TBEV and WNV, which are both currently circulating in Germany. We describe the benefits of the LIPS assay in screening large numbers of sera from a mammalian species for the presence of virus and antigen-specific antibodies and compare the results from the antigen-specific LIPS assays with conventional serologic methods. In addition, we define criteria, under which a serologic distinction between TBEV- and WNV-specific antibody responses is feasible.

2 | MATERIAL AND METHODS

2.1 | Serum samples

Canine serum samples were originally collected in 2015 in the course of an unrelated study on *Leishmania* infections in European dogs (Wright et al., 2020). From this collection, 682 samples were included

in the present study based on the country of origin to be known as a potential TBEV or WNV risk area. These included 281 samples from Hungary, 218 samples from Romania, 92 samples from Germany, 10 samples from Serbia, 10 samples from Ukraine, seven samples from Poland, five samples from Slovakia, two samples from Czech Republic, one sample from Slovenia and one sample from Russia. Additionally, 54 samples from Portugal were included in the survey as negative controls. Serum from a confirmed local canine TBE case collected during the late acute phase (positive qPCR and histology, kindly provided by A. Tipold, Small Animal Clinic, University of Veterinary Medicine, Hannover) was included as a positive control in ELISA and LIPS assays.

2.2 | Cell culture

Cos-1 cells (African green monkey kidney cell line) and BHK-21 cells (Baby hamster kidney cell line) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal bovine serum, 1% penicillin, 1% streptomycin and 1% glutamine at 37°C and 5% CO_2 in a humidified atmosphere.

2.3 | Production of luciferase-antigen fusion proteins

The mammalian expression plasmid pcDNA3.1/Zeo was modified to express a secreted Nano luciferase (secNLuc) downstream of the IL6 signal peptide (cloned from pNL1.3.CMV[secNluc/CMV], Promega) and upstream of a multiple cloning site for in-frame insertion of genes of interest to generate N-terminal secNLuc fusion proteins. For TBEV strain Neudoerfl (GenBank # U27495.1), the sequences encoding capsid (C; amino acids (aa) 1–112 of the polyprotein), pre-membrane (prM; aa 113–280) as well as non-structural proteins 1 (NS1; aa 777–1128) and 4B (NS4B; aa 2260–2511) were cloned into the secNLuc vector. Furthermore, EDIII (aa 581–675 of the polyprotein) and the C-terminal helicase domain of TBEV non-structural protein 3 (NS3-DIII; aa 1857–1954) as well as the full-length NS1 (aa 777–1128 of the polyprotein) of WNV strain NY99 (GenBank # KC407666.1) were cloned into the secNLuc vector. The resulting plasmids were transfected into Cos-1 cells in 10-cm culture dishes using TransIT-2020 transfection reagent (Mirus) following the manufacturer's instructions. After 48 h, the supernatant was collected and centrifuged for 5 min at 500 × *g* to remove cell debris. The cell-free supernatant containing the luciferase-antigen fusion proteins was tested for NLuc activity using the Nano-Glo luciferase assay system (Promega), aliquoted and stored at −20°C before use in the LIPS assay. A plasmid expressing only secNLuc without any antigen fusion but with full enzymatic activity was expressed alongside and used as background control in the LIPS assay (see below).

2.4 | LIPS assay

For antibody testing, fusion proteins were normalized to similar input levels between 0.5 and 1 million relative light units (RLU). Fusion proteins were incubated with serum at a 1:100 dilution for 1.5 h at room temperature under light orbital shaking. Protein A beads (Thermo Fisher) were washed and resuspended in PBS as a 30% slurry. Ten microliters of the slurry were transferred to a filter plate (MultiScreen$_{HTS}$ BV Filter Plate, Millipore) along with the antigen–serum mix and incubated for an additional hour. Unbound luciferase-tagged antigen was removed from the microtiter filter plate by aspiration in a 96-well vacuum manifold followed by five wash steps with PBS. Finally, luciferase activities were measured in a microplate reader (Tecan) using the Nano luciferase substrate system (Promega). All samples were tested in duplicates. A secreted NLuc without any antigen fusion but with full enzymatic activity was incubated with each serum to determine unspecific background signals. Luminescence values higher than the average of 10 presumed negative samples plus five times the standard deviation (SD) are considered positive, whereas values above the average of 10 presumed negative samples plus three times SD are considered inconclusive.

2.5 | TBEV ELISA

All samples reactive for TBEV in the LIPS assay were additionally tested in a commercial TBEV antibody ELISA (Immunozym® FSME [TBE] IgG All Species, Progen). The ELISA was performed according to the manufacturer's instructions. In the last step, colour intensities were quantified in a microplate reader (Tecan) and correlated to concentration of anti-TBEV IgG (Vienna Units, VIEU) in the serum by using reference curve optical densities (OD) at 450 nm. Samples with less than 63 VIEU/ml are specified as negative and samples with values higher than 126 VIEU/ml are specified as positive. All values in between are defined as equivocal.

2.6 | Virus neutralization test

All samples with reactivity in the LIPS assay for either virus were additionally tested in VNTs. Heat-inactivated sera diluted in tissue culture medium were incubated for 1 h at 37°C with 100 tissue culture infectious doses (TCID50) and applied to cell monolayers. Fresh cell culture media was added after 1 h of incubation at 37°C, and cytopathic effects were evaluated after 6–7 days. The neutralizing antibody titre (ND50) was determined as the reciprocal of the serum dilution that inhibited cytopathic effect in ≥50% of replicates. Neutralization effects at 1:10 or greater dilutions were regarded positive. All tests were performed with WNV strain Germany (lineage 2, GenBank accession no. MH924836) and TBEV strain Neudoerfl (kindly provided by

G. Dobler, Bundeswehr Institute of Microbiology, Munich, Germany, GenBank accession no. U27495) following the identical protocols. The mouse monoclonal antibody 19/1786 with neutralizing activity against TBEV (kindly provided by M. Niedrig) (Fuzik et al., 2018; Niedrig et al., 1994) was used at 1:1000 dilution as a positive control.

3 | RESULTS

3.1 | Antibody screen for multiple TBEV antigens reveals 30 samples with potential reactivity

We further developed the LIPS assay originally described by Burbelo et al. (2009, 2010) to use secreted antigens fused to nano-luciferase to increase the sensitivity and reduce background signals in the detection of TBEV-specific antibodies in serum samples. Using this assay, 682 canine sera were screened for anti-TBEV antibodies by LIPS assay against six viral antigens: C, prM, EDIII, NS1, NS3-DIII and NS4B. Cut-offs for the primary screen were determined by calculating the average luciferase signal across 10 presumed negative samples. Signals above the average + 3 standard deviations (SDs) were considered equivocal, whereas signals above the average + 5 SDs were considered positive. Following these guidelines, none of the samples displayed any reactivity against the C and NS4B proteins. Three samples were found equivocal and one low positive for antibodies against NS3-DIII, while one equivocal and three low-positive samples were detected for antibodies against prM (Figure 1). In contrast, six samples tested equivocal and 13 positive for antibodies against EDIII. For TBEV NS1, two samples tested equivocal, while 25 samples yielded positive signals in the primary screen (Figure 1). The highest positive samples yielded luciferase signals $>10^5$ RLUs with background signals of $\leq$500 RLU. While the highest signals for NS1-specific antibodies were found in the 13 samples with positive EDIII result, additional samples with low reactivities against TBEV-NS1 were identified in the screen. Interestingly, antibodies against prM could only be detected in the four samples with the highest signals for EDIII and NS1 (*Ago*, *Porthos*, *Caesar* and *Buster*), suggesting the presence of non-dominant epitopes in prM (Figure 1). The low-level detection of antibodies against NS3-DIII occurred in samples with otherwise low signal intensities and likely represents non-specific binding events.

3.2 | Sensitivities and specificities of LIPS assays are comparable to VNTs

Thirty samples originating from five countries (Germany, Hungary, Czech Republic, Romania and Portugal) were identified in the primary screen to yield luminescence signals above the average + 3 SDs for one or more TBEV antigens. When individual values were plotted for both assays, the data separated into two distinct populations—one with values below 3×10^3 RLU and one with values above 5×10^3 RLU with most values in this population above 1×10^4 RLU. This led us to adapt the initial cut-offs of the LIPS-based primary screen to more stringent parameters and define signals below 3×10^3 RLU as negative, signals between 3 and 5×10^3 RLU as equivocal, signals between 0.5 and 1×10^4 RLU as low positive and any signal $>1 \times 10^4$ RLU as positive or high positive. The 30 samples identified in the primary screen were tested with a commercial all-species TBEV ELISA (Progen) based on inactivated whole viral particles to compare assay reactivity with the results obtained in the LIPS assay for TBEV-EDIII (Figure 2a). While the ELISA detected 10 positive and five equivocal samples, the EDIII-based LIPS assay found 12 positive and one equivocal sample following the more stringent cut-off definition (Table 1). The sample with equivocal results by EDIII-based LIPS tested negative in ELISA. Among the 12 EDIII-LIPS-positive samples were two samples with equivocal results in ELISA. In contrast, the other three samples with equivocal results in the ELISA tested negative in the LIPS assay for TBEV-EDIII (Table 1). To investigate the deviations between both serologic assays targeting the TBEV E protein, we performed VNTs with live TBEV for confirmation of our previous results. Thirteen samples tested positive in VNT with titres between 60 and 1280 (Table 1). Besides all 12 LIPS-EDIII high-positive samples, these also included the one LIPS-EDIII low-positive sample (*Cereja*) that was not detected by ELISA. Furthermore, the three samples that tested equivocal by ELISA, but negative in LIPS-EDIII, did not show any neutralizing activities. This suggests a lower specificity of the ELISA compared to LIPS-EDIII or VNT. To corroborate these findings and exclude any false-negative results due to the minimal design of the LIPS-EDIII and VNT with focus on crucial domains of the E protein, we included the TBEV NS1 protein in the analysis. The 13 samples with positive results in the VNT were also found to contain NS1-specific antibodies (Table 1). Additionally, one sample (*Cami*) with equivocal results in ELISA but negative VNT and LIPS-EDIII showed low reactivity against the TBEV NS1 protein. When compared to the VNT as the 'gold standard' of serologic assays, both LIPS assays for the detection of antibodies targeting either TBEV EDIII or NS1 showed comparable sensitivities and specificities in this small sample set of canine sera. The additional 11 low-positive and two equivocal samples identified for NS1 in the primary screen (Figure 1) showed no reactivities in any of the other assays and likely represent unspecific signals, suggesting the need for a higher assay cut-off for the classification of positive and equivocal results, respectively. A sample obtained from a confirmed canine TBE case during the late acute phase of disease was tested alongside in ELISA and LIPS, but was not included in VNT due to low sample volume. While this sample was highly positive in the TBEV ELISA with 308 VIEU/ml, LIPS signals for TBEV EDIII and NS1 resulted in 35,933 and 51,007 RLU, respectively (Table 1). In contrast, the signal obtained for WNV NS1 was 8462 RLU.

3.3 | Correlation of data from different TBEV antibody detection assays

Three samples with equivocal TBEV ELISA and negative TBEV-EDIII LIPS results (*Lady*, *Cami* and *Drazse*) deviated most strongly from the proposed regression line in the data correlation for the two assays (Figure 2a). This may indicate cross-reactive or non-specific signals.

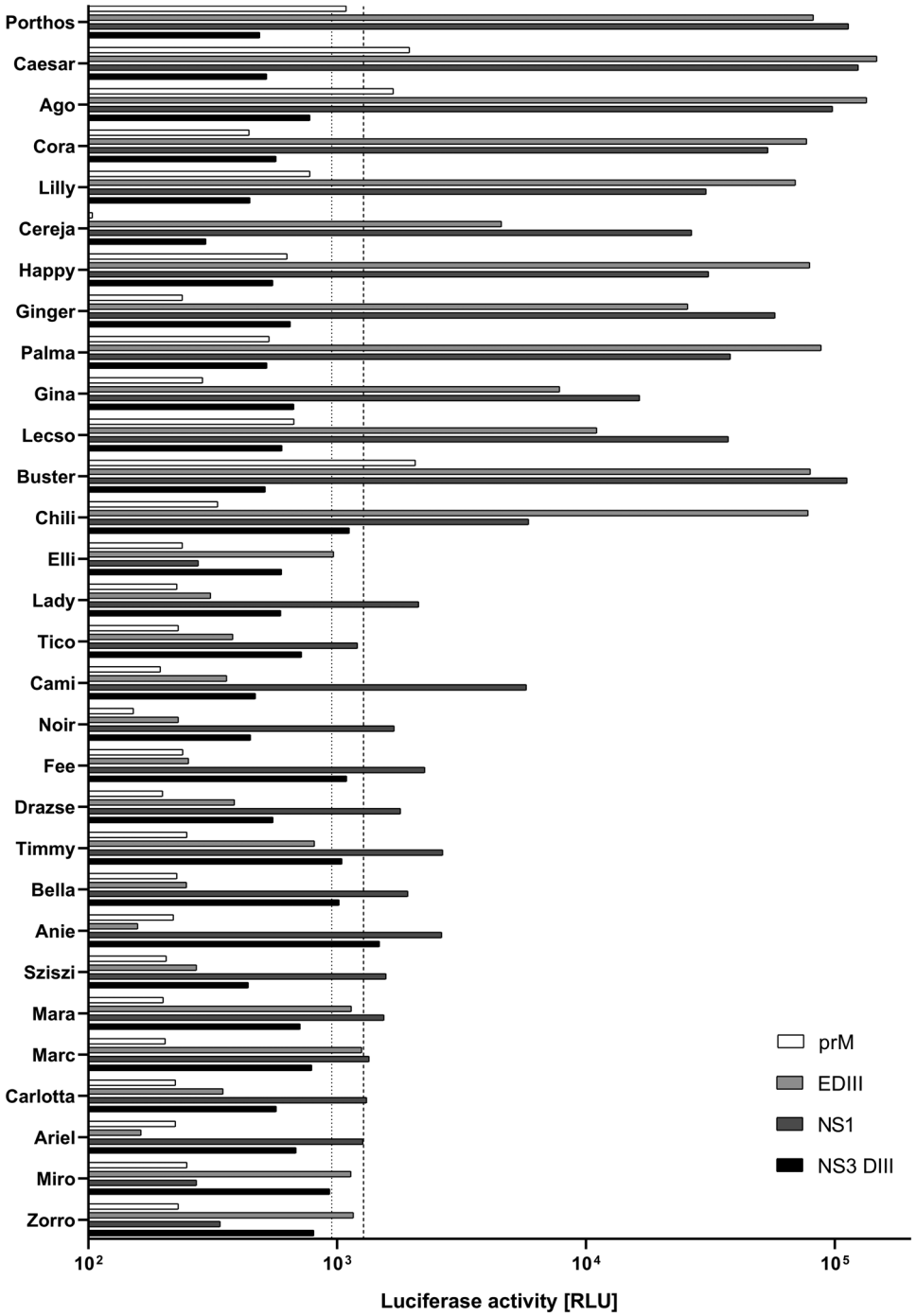

FIGURE 1 Luciferase immunoprecipitation system-based screen of 30 canine serum samples for antibodies against the TBEV proteins pre-membrane (prM), envelope domain III (EDIII), non-structural protein 1 and non-structural protein 3 domain III (NS3-DIII). Initial cut-offs calculated as mean + 3 standard deviations (equivocal) and mean + 5 standard deviations (positive) are indicated by the dotted and dashed lines, respectively. RLU, relative light units

Other values that fell outside the confidence intervals in the upper part of the regression curve included sera with high antibody signals in both assays (*Ago*, *Caesar*, *Happy* and *Palma*). This suggests an increased average distance between points and curve and a reduced 'goodness of fit' for higher signals (Table 1). Taken together, 10 samples were confirmed positive for anti-TBEV antibodies in both assays, resulting in a good overall correlation of the two assay systems with a Pearson's correlation coefficient r of .9474 with $p < .0001$ (Figure 2a). While the comparison of two different TBEV antigens (EDIII and NS1) in the LIPS assay yielded similar results (Table 1), the data correlation was less clear (Figure 2b). While some TBEV antibody-positive samples produced higher signals for EDIII than for NS1 (e.g. *Ago*, *Lilly*, *Happy* and *Chili*), others generated opposite responses (e.g. *Porthos*, *Ginger* and *Buster*). Similarly, data generated in the TBEV ELISA correlated less

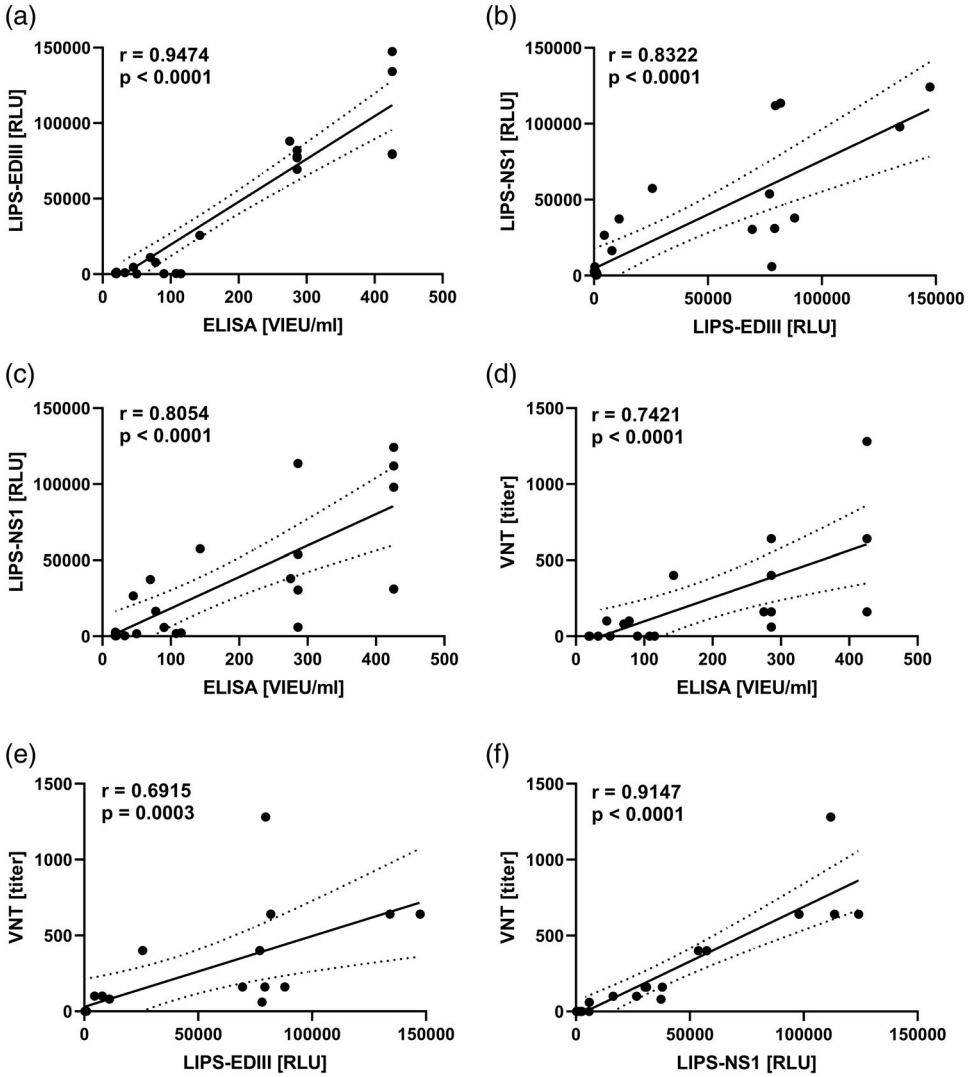

FIGURE 2 Pearson correlation and linear regression of TBEV antibody detection data obtained with different assay formats. (a) Commercial TBEV ELISA versus LIPS assay for TBEV EDIII, (b) LIPS assays for two different TBEV antigens, NS1 and EDIII, (c) commercial TBEV ELISA versus LIPS assay for TBEV NS1, (d) commercial TBEV ELISA versus VNT, (e) LIPS assay for TBEV EDIII versus VNT and (f) LIPS assay for TBEV NS1 versus VNT. Confidence intervals are shown as dashed lines, Pearson's correlation coefficient *r* gives a measure of linear correlation and *p*-values indicate significance. TBEV, tick-borne encephalitis virus; ELISA, enzyme-linked immunosorbent assay; LIPS, luciferase immunoprecipitation system; EDIII, envelope protein domain III; NS1, non-structural protein 1; VNT, virus neutralization test

strongly with data produced in the TBEV-NS1 LIPS assay (Figure 2c), suggesting that antibody titres against different TBEV antigens do not share a linear relationship in all individuals. Interestingly, neither the TBEV ELISA nor the LIPS assay for TBEV-EDIII produced data that correlated strongly with results from the VNT (Figure 2d,e), despite targeting similar antigenic structures of the viral envelope protein. In contrast, data generated with the TBEV-NS1 LIPS assay correlated well with neutralization titres measured by VNT, resulting in a Pearson's correlation coefficient *r* of .9147 with $p < .0001$ (Figure 2f). Only two samples fell outside the confidence intervals: *Cami*, scoring low positive in TBEV-NS1 LIPS and negative in VNT, and *Buster*, scoring disproportionally high in the VNT with the highest neutralization titre measured for any sample in this study. While the linear correlation was strongest for TBEV ELISA versus TBEV-EDIII LIPS (Figure 2a) and TBEV-NS1 LIPS versus VNT (Figure 2f), all assay pairs yielded significant data correlation and identical test results (*positive*, *negative*, *equivocal*) for the majority of tested samples (Table 1).

3.4 | Signal ratios for anti-NS1 antibodies allow serologic distinction between TBEV and WNV

To determine if the low-positive detection of antibodies against the TBEV NS1 protein in a number of samples that tested negative in the other assays could be due to antibody cross-reactivity, we performed LIPS assays for the NS1 protein of WNV for the previously tested 30 samples. For WNV NS1, the assay detected 15 samples with above-average luciferase signals. Of the above-average samples, eight were

TABLE 1 Summary of results for 30 canine serum samples identified in the initial serological screen for TBEV-specific antibodies (+ one confirmed control TBE sample) and data obtained from four different serologic tests for TBEV (ELISA and LIPS for EDIII; LIPS for NS1 and VNT) and two serologic tests for WNV (LIPS for NS1 and VNT)

		Tick-borne encephalitis virus (TBEV)				West Nile virus (WNV)				
			LIPS		VNT	LIPS	VNT	NS1 signal ratios		
ID	Country	ELISA (VIEU/ml)	EDIII (RLU)	NS1 (RLU)	(ND_{50} titre)	NS1 (RLU)	(ND_{50} titre)	TBEV: WNV	WNV: TBEV	Result
Confirmed TBE	Germany	308	35,933	51,007	n.t.	8462	n.t.	6.03	0.16	TBEV
Porthos	Germany	348	81,908	113,528	640	337	n.t.	337.38	0.00	
Caesar	Czech Republic	>426	147,428	124,161	640	403	n.t.	308.47	0.00	
Ago	Germany	>426	134,206	97,953	640	390	n.t.	251.16	0.00	
Cora	Germany	294	77,031	53,779	400	387	n.t.	138.96	0.01	
Lilly	Germany	286	69,494	30,394	160	350	n.t.	86.84	0.01	
Cereja	Portugal	45	4571	26,591	100	411	n.t.	64.78	0.02	
Happy	Germany	>426	79,299	31,052	160	565	n.t.	54.96	0.02	
Ginger	Hungary	142.5	25,652	57,479	400	1271	n.t.	45.22	0.02	
Palma	Hungary	275	88,009	37,996	160	912	n.t.	41.69	0.02	
Gina	Hungary	77.5	7836	16,409	100	924	n.t.	17.77	0.06	
Lecso	Hungary	70	11,041	37,344	80	2199	<10	16.99	0.06	
Buster	Hungary	>426	79,665	111,875	1280	15,051	60	7.43	0.13	TBEV + WNV
Chili	Hungary	344	78,034	5876	60	100,062	80	0.06	17.03	
Elli	Romania	32.5	967	277	<10	24,385	20	0.01	88.03	WNV
Lady	Hungary	115	310	2126	<10	66,295	160	0.03	31.18	
Tico	Hungary	<19	381	1207	<10	26,651	30	0.05	22.07	
Cami	Hungary	90	360	5754	<10	81,638	240	0.07	14.19	
Noir	Romania	50	230	1695	<10	19,715	20	0.09	11.63	
Fee	Romania	20	253	2248	<10	13,172	10	0.17	5.86	
Drazse	Hungary	107.5	387	1798	<10	4243	15	0.42	2.36	
Timmy	Czech Republic	<19	811	2658	<10	5818	<10	0.46	2.19	Equivocal/negative
Bella	Romania	<19	248	1923	<10	2789	<10	0.69	1.45	
Anie	Romania	<19	158	2630	<10	4410	<10	0.60	1.68	
Sziszi	Hungary	<19	272	1573	n.t.	324	n.t.	4.86	0.21	
Mara	Hungary	<19	1140	1542	n.t.	613	n.t.	2.52	0.40	
Marc	Romania	20	1255	1343	n.t.	690	n.t.	1.95	0.51	
Carlotta	Germany	<19	348	1314	n.t.	458	n.t.	2.87	0.35	
Ariel	Portugal	<19	163	1269	n.t.	690	n.t.	1.84	0.54	
Miro	Romania	<19	1137	272	n.t.	1581	n.t.	0.17	5.82	
Zorro	Romania	20	1163	339	n.t.	608	n.t.	0.56	1.79	

Note: Reactive samples in either test are marked in dark grey for positive and light grey for equivocal based on the stringent cut-off criterion definition described in the text.

Abbreviations: EDIII, envelope protein domain III; ELISA, enzyme-linked immunosorbent assay; LIPS, luciferase immunoprecipitation system; NS1, non-structural protein 1; RLU, relative light units; n.t., not tested; TBEV, tick-borne encephalitis virus; VIEU/ml, Vienna units per milliliter; VNT, virus neutralization test; WNV, West Nile virus.

high positive with signals $>10^4$ RLU, two low positive with signals between 0.5 and 1×10^4 RLU (including the confirmed control TBE sample) and two equivocal with signals between 3 and 5×10^3 RLU. The remaining three samples had above-average signals for WNV NS1, but below the stringent cut-off of 3×10^3 RLU, and were classified as negative. Of the positive samples, nine could be confirmed to contain neutralizing antibodies for WNV by VNT, including the eight high-positive samples and one equivocal sample (*Drazse*) with an LIPS signal for WNV NS1 of 4243 RLU. Additionally, ratios for the LIPS signals of TBEV-NS1 to WNV-NS1 as well as WNV-NS1 to TBEV-NS1 were calculated for all samples (Table 1). Interestingly, there was a more than 10-fold difference between the two signals for anti-NS1 antibodies for most samples, allowing for a clear distinction between TBEV and WNV antibody-positive samples. For two samples, the ratios fell between 5 and 10. One of these (*Fee*) still indicated the correct low-level detection of WNV-specific antibodies with a WNV:TBEV ratio of 5.86 and VNT titre of 10. The other sample (*Buster*) exhibited antibody reactivity against both viruses, with the highest neutralization titre for TBEV and a TBEV:WNV ratio of 7.43. Since the antibody titres against both viruses differ strongly (1280 for TBEV vs. 60 for WNV), this likely indicates cross-reactivity for WNV caused by very high titres against TBEV. One sample (*Drazse*) did not score high enough (2.36) to be called correctly by the NS1 signal ratio approach, but was equivocal in the LIPS assay for WNV-NS1 antibody and had a low WNV neutralizing antibody titre of 15. One sample (*Chili*) identified as WNV specific by the signal ratio approach with a value of 17.03 exhibited antibody reactivity against both viruses. Interestingly, this sample showed high signal intensities in LIPS for TBEV-EDIII and WNV-NS1, but only low reactivity for TBEV-NS1. Neutralization titres for both viruses were low with 60 for TBEV and 80 for WNV. Finally, three of the five equivocal samples in the TBEV ELISA (*Lady*, *Cami* and *Drazse*) were confirmed to be WNV reactive. The other two (*Lecso* and *Gina*) were positive for anti-TBEV antibodies with LIPS signals between 0.7 and 3.8×10^4 RLU and neutralization titres of 80 and 100, respectively.

4 | DISCUSSION

We screened 682 canine sera from 11 European countries for the presence of TBEV- and WNV-specific antibodies. Antibodies against TBEV were detected in five samples from Germany, one from Czech Republic, six from Hungary and one from Portugal. This is in line with the known geographical distribution of the virus (Beck et al., 2013) with the exception of one Portuguese sample. However, in the absence of additional epidemiologic data, a travel history of the animal to TBEV endemic countries cannot be excluded. Interestingly, this sample showed a rather untypical serological profile with no or low reactivity in ELISA or LIPS against TBEV-EDIII, respectively. However, antibodies against TBEV-NS1 and neutralization activity were readily detectable, suggesting a specific antibody response against the virus. Our data result in an overall TBEV seroprevalence of 1.9% in European dogs, with 5.4% in Germany and 2.1% in Hungary. These rates are in the expected range for untargeted screening of healthy dogs in regions with low to moderate risk of TBEV infection (Balling et al., 2015; Klimeš et al., 2001). For WNV, nine samples tested positive for antibodies against the NS1 protein or neutralizing activity, six of which originated from Hungary and three from Romania. This leads to an overall WNV seroprevalence of 1.3% in European dogs, with 2.1% in Hungary and 1.4% in Romania. Recent studies have found similar rates in healthy dogs from other European countries (Garcia-Bocanegra et al., 2018; Knap et al., 2020). Ten canine sera from Hungary contained flavivirus-specific antibodies, including four with specificity for TBEV and four with specificity for WNV. The other two samples were reactive with both viruses, suggesting either the presence of cross-reactive antibodies or a dual exposure. One of these samples (*Buster*) showed much higher signals against TBEV than WNV in LIPS and the highest TBEV neutralizing titre among the samples tested in this study. A strong antibody response against TBEV in this animal may favour cross-reactivity with WNV. Substantial cross-reactivity with dengue virus in humans with previous TBEV infection was observed in samples containing the highest anti-TBEV antibody titres (Allwinn et al., 2002). Based on the NS1 signal ratio of 7.43 for TBEV:WNV, we concluded this animal to be more likely to be TBEV positive, although with lower confidence. NS1-specific LIPS signals for the confirmed control TBE sample yielded a TBEV:WNV ratio of 6.03 (Table 1), placing it in the low confidence category as well. In the absence of VNT data, this could be explained as cross-reactivity induced by high sample reactivity for TBEV (similar to *Buster*) as evidenced by the highly positive ELISA data (Table 1). Alternatively, the early time point of sample collection during the late acute phase of the disease may point towards a less mature antibody response with enhanced cross-reactivity. The other sample with reactivity against both viruses (*Chili*) had similar neutralizing titres for both viruses, but much stronger reactivity with WNV-NS1 compared to TBEV-NS1, despite a strong signal for TBEV-EDIII and positive TBEV ELISA test. In our 'signal ratio approach', this led to a categorization as WNV positive. However, the positive ELISA and LIPS-EDIII tests suggest a dual exposure may be more likely in this case, especially as both flaviviruses are known to co-circulate in Hungary and Eastern Austria (de Heus et al., 2021; Nagy et al., 2022).

While classic flavivirus serology has focused on the envelope protein as the main antigen, for example in whole-particle ELISA, the focus is shifting towards the use of NS1-based assays for the differential serology of multiple flaviviruses (Mora-Cardenas et al., 2020; Thao et al., 2021; Tyson et al., 2019). While substantial cross-reactivity with TBEV-positive samples was reported for a commercial WNV competitive ELISA (Klaus et al., 2014), we observed limited cross-reactivity with WNV-positive samples from Hungary that produced equivocal results in the all-species TBEV ELISA. Furthermore, our data indicated a lower sensitivity of the ELISA when compared to LIPS or VNT, as described previously by others (Girl et al., 2021; Klaus et al., 2011). In contrast, our NS1-based LIPS data for both viruses correlated well with the respective VNT. While we have no explanation for this observation, there is evidence that both, neutralizing responses as well as

NS1-specific antibodies, are important correlates of protection against flavivirus infection (Bailey et al., 2018; Li et al., 2012). The strong correlation between VNT and LIPS-NS1 data may be an indirect effect of robust protective immune responses in those individuals that include neutralizing and anti-NS1 antibodies. Interestingly, similar observations were recently made in a human ZIKV cohort in Thailand (Sornjai et al., 2019). Our study presents additional evidence for the suitability of the flavivirus NS1 protein as an antigen not only for the detection of flavivirus-specific antibodies per se, but for differential serologic testing against TBEV and WNV in regions with co-circulation of both viral species. The antibody levels detected in our newly established LIPS assay suggest a similar immunogenicity of the NS1 protein compared to EDIII. Furthermore, the use of NS1 as a single antigen or in combination with the E protein allows the differentiation between natural infection and vaccination-induced antibody responses (Girl et al., 2020). The NS1-based LIPS assay reached similar sensitivities than VNT and allowed a serologic distinction between the two viruses, even in samples with some observed cross-reactivity, by calculating the signal ratios between both viruses. For samples with signals $>3\times10^3$ RLU and signal ratios >10, a clear distinction was possible in most cases with the exception of cases with possible dual exposure. The method was less accurate for very high or low signals. This is not unexpected as very high antibody titres are known to cause more cross-reactivity and low signals may represent false-positive results. It is important to note that in the absence of well-defined positive and negative control sera, a full validation of the assay could not be performed in this study. Given the relatively small number of samples included in this study, more data are needed to confirm our observations. However, we speculate that this method would yield similar results with sera from other host species, including humans. Whether the 'signal ratio approach' is applicable to other combinations of flavivirus NS1 proteins remains to be investigated. A possible drawback of this method is a reported lack of longevity of anti-NS1 antibodies, as described in Zika virus-infected individuals (Moreira-Soto et al., 2020). Our results suggest that distinct epitopes exist in TBEV and WNV NS1 proteins that allow differential serology in regions with co-circulation of both viruses. Future studies could be aimed at identifying species-specific epitopes and developing multiplex assays for simultaneous detection of antibodies against multiple flaviviruses.

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ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no data sets were generated or analysed during the current study.

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