



Development and validation of a novel enzyme-linked immunosorbent assay for the differentiation of tick-borne encephalitis infections caused by different virus subtypes

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Abstract

Objectives Tick-borne encephalitis (TBE) is an infection caused by the tick-borne encephalitis virus (TBEV) that can lead to symptoms of central nervous system inflammation. There are five subtypes of TBEV, three of which – European, Siberian and Far Eastern – occur in Europe. As it is thought that different subtype infections exhibit varying clinical courses and outcomes, serological differentiation of the virus subtypes is clearly important. However, to date, this has proved difficult to achieve.

Methods An ELISA format was developed based on TBE virus NS1 antigen against the European, Siberian and Far Eastern subtype. The three NS1 antigens were biotechnologically produced in a human cell line and used for ELISA coating. Sera from German (European subtype) and Russian (Siberian and/or Far Eastern subtypes) TBE patients with positive TBEV IgG were used to test the reactivity against these three NS1 antigens.

Results Testing of 23 German and 32 Russian TBEV IgG-positive sera showed that the ELISA was able to differentiate between TBEV European subtype and TBEV Siberian and Far Eastern subtype infections.

Conclusions In geographical areas where two or more TBEV subtype infections can occur, the NS1-IgG ELISA developed here constitutes an important diagnostic tool to differentiate between European subtype infections and Siberian/Far Eastern subtype infections and to use the new assay for epidemiological studies to clarify the importance of particular subtype infections in an area. Consequently, it may help to better describe and anticipate the clinical courses and outcomes of particular TBEV subtype infections.

Keywords NS1 antigen · NS1 antibodies · ELISA · TBEV subtypes · Flavivirus

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Introduction

Tick-borne encephalitis (TBE) is a viral infectious disease of the central nervous system caused by the tick-borne encephalitis virus (TBEV) [1]. TBEV (according to the new ICTV taxonomy *Orthoflavivirus encephalitidis*) is predominantly transmitted to humans via the bite of an *Ixodes* tick. Less commonly, it can be transmitted by consumption of unpasteurised dairy products [1, 2]. TBE is endemic in Northern, Central and Eastern Europe as well as parts of Asia, where it is one of the most common causes of viral meningitis and encephalitis [1–3]. The clinical course of the disease can be unpredictable, with symptoms ranging from febrile illness and mild meningitis through to severe encephalitis and even death [1]. Currently, there is no specific treatment for TBE. However, highly effective vaccines against TBEV are available in Europe (FSME-Immun[®], Pfizer; Encepur[®], Bavarian Nordic) and recommended for people living in or travelling to TBE endemic regions [4].

TBEV is a single-stranded RNA virus belonging to the Flaviviridae family and genus *Orthoflavivirus*. Based on phylogenetic differences, TBEV can be divided into five different subtypes: European (TBEV-Eu), Siberian (TBEV-Sib), Far Eastern (TBEV-FE), Himalayan (TBEV-Him) and Baikalian (TBEV-Bkl). Three of the five subtypes are clinically relevant to humans, namely TBEV-Eu, TBEV-Sib and TBEV-FE [5]. At the amino acid level, the genetic difference can be up to 2% within a subtype and 5–6% between subtypes [6]. These subtypes are associated with different geographical distributions and clinical courses. The geographical distribution of each subtype mostly corresponds to the nominal region; however, there are some exceptions. For instance, the European subtype has been found in South Korea [7], Altai (Southern Siberia) and Irkutsk (Eastern Siberia). Furthermore, the Siberian subtype has been found to circulate in parts of Scandinavia [8], the Baltic states [9], Bosnia and Central Asia [10]. Additionally, the Far Eastern subtype has been detected in Southern Siberia, the Urals, the Baltic states [11] and Moldova [12]. Clinically, the European subtype is reported to be associated with a milder disease and lower case fatality rates (0.5–2%) compared to the Siberian and Far Eastern subtypes (case fatality rates between 5 and 20%) [13, 14].

The European Centre for Disease Prevention and Control's definition of a TBE case is as follows: a person with a laboratory-identified TBEV infection and symptoms of central nervous system inflammation. The laboratory diagnosis of TBE is mainly based on the detection of TBEV-specific IgM antibodies in cerebrospinal fluid (intrathecal production) and/or TBEV-specific IgM and IgG antibodies in serum [15]. Enzyme-linked immunosorbent assay (ELISA) is considered the standard method for the serological

diagnosis of TBE. However, the currently available ELISAs have the fundamental disadvantage of cross-reactivity with other flavivirus infections or vaccination [16]. Presently, the most specific assay for serological differentiation of flavivirus antibodies is the virus neutralisation test; however, this test is unable to distinguish between different TBEV subtype infections. Therefore, serological differentiation of TBEV subtype infections in geographical regions with co-existence of two or more TBEV subtypes is not possible at this time and consequently data collection on the incidence, prevalence and clinical course of particular TBEV subtype infections is majorly restricted.

A new approach has recently been applied to ELISA [17] and suspension multiplex immunoassay [18, 19] to detect antibodies against TBEV non-structural protein 1 (NS1). NS1 is a glycoprotein that is central for viral RNA replication. It also induces an immune response against the virus, which may even play a role in protection [20]. In contrast to TBEV envelope (E) protein (the antigen commonly used in ELISA), NS1 protein elicits an immune response exclusively after wild-type natural infection, which could possibly be distinguishable between TBEV subtypes. The currently available vaccines against TBEV, namely FSME-Immun[®] and Encepur[®], are highly purified, inactive and do not contain substantial amounts of NS1. Therefore, following vaccination, no TBEV replication occurs and generally there is no formation of NS1 protein nor development of NS1-specific antibodies. These aspects of TBE vaccination are extremely advantageous for the employment of NS1-IgG ELISA in TBE testing [17, 19].

The aim of this study was to develop and validate a diagnostic method to detect NS1-IgG antibodies against the three main TBEV subtypes (European, Siberian and Far Eastern). Furthermore, we wanted to establish whether our NS1-IgG ELISA could serologically differentiate infections caused by TBEV-Eu, TBEV-Sib and TBEV-FE, which to date has not been possible. This diagnostic capability of an ELISA is of major importance in order to more fully understand each TBEV subtype's epidemiology, pathogenicity, disease severity and vaccine effectiveness.

Methods

Ethics statement

The study was approved by the Rīga Stradiņš University Ethics Committee (No. 6–1/03/19; March 26, 2020). Only anonymous samples or sera for research purposes were used in the present analysis.

Table 1 Study population groups

Group	Characteristics
TBEV infection (SENSITIVITY analysis)	
Group 1	23 sera from acute TBEV-Eu cases, confirmed by specific IgM and IgG antibodies and TBEV-Eu-NS1-IgG ELISA
Group 2	32 sera from Russian patients with confirmed acute (IgG ELISA positive; neutralisation antibody positive) or past TBEV infection (non-TBEV-Eu)
No TBEV infection (SPECIFICITY analysis)	
Group 3	9 sera with confirmed dengue fever infection: 6 patients with acute secondary dengue antibody response; 1 with acute primary dengue infection; 2 with past dengue infection (diagnosed and titrated by indirect immunofluorescence using Euroimmun Flavivirus Microchip.
Group 4	7 sera from individuals with yellow fever vaccination and TBE vaccination.
Group 5	43 sera from blood donors with confirmed TBEV-Eu vaccination (TBEV-IgG ELISA; neutralisation test).
Group 6	45 sera from blood donors from TBE non-risk area negative for both infection and vaccination (negative TBEV-IgG ELISA; negative neutralisation test).

Study population and serum samples

For the validation of NS1 ELISA, we analysed a total of 159 serum samples which were divided into six groups (Table 1). All sera, except sera in Group 2, were from the German National Consulting Laboratory for TBE and were anonymized prior to analysis. The samples were from TBE patients and patients with other confirmed diseases (including other flavivirus infections). In addition, samples from TBE vaccine recipients and individuals vaccinated against other flaviviruses (yellow fever virus, Japanese encephalitis virus) were included for flavivirus diagnostics. Sera were stored at -80°C until use.

Group 3, consisting of nine sera from confirmed dengue fever cases, was included in the analysis to test the specificity of the assay (Table 2). Six sera (TM58, DEN-71, DEN-96, DEN-100, DEN-136, DEN-137) were from patients with an acute dengue fever infection (IgM positive) and a serological pattern of secondary dengue infection (i.e.

high level of IgG antibodies against all flaviviruses tested, including anti-TBEV-IgG). One serum (DEN-72) was from a patient with acute primary dengue infection (high level of IgG against dengue virus; no antibodies against other flaviviruses tested). Two sera were from patients with a past dengue infection, one (DEN-131) from a patient with a primary antibody response (high level of specific anti-dengue IgG, no other anti-flavivirus IgG) and one (DEN-84) from a patient with a past secondary dengue infection (IgG cross-reaction against all tested flaviviruses). All sera are coming from a study of travellers returning from tropics and were diagnosed and titrated by standard indirect immunofluorescence using the.

Development and performance of anti-TBEV NS1 IgG ELISA (NS1 ELISA)

Three recombinant TBEV NS1 proteins (European – strain Neudörfl, Siberian – strain MucAr DZIF 19/556 and Far-Eastern – strain Sofjin) were purchased from The Native Antigen Company (United Kingdom). Antigens were produced in HEK-293 human cell lines, highly purified and presented in a hexameric, native folding state.

TBEV-Sib-NS1-IgG ELISA and TBEV-FE-NS1-IgG ELISA were prepared as previously described for TBEV-Eu-NS1-IgG ELISA [17] with some modifications. Polystyrene plates (96-well) (Nunc Immuno MaxiSorp, Thermo Fisher Scientific, Waltham, Massachusetts, USA) were coated overnight at 4°C with $100\ \mu\text{l}$ of the three recombinant TBEV NS1 antigens at a concentration of $1,000\ \text{pg/ml}$ in carbonate buffer ($0.6\ \text{M}$, $\text{pH}\ 9.6$) to test the optimal coating concentration. We used the concentration as described earlier [17] and tested one 2fold dilution lower and higher to check if the resulting ODs were more discriminative. Wells were blocked with gelatine (PanReac AppliChem, Darmstadt, Germany) in phosphate-buffered saline (PBS) for 1 h at room temperature, after which antigen plates were stored at -80°C until use. Sera were tested in triplicate against

Table 2 IgG titers of sera in Group 3 against different flaviviruses

No.	ID	Den-IgM	Den-IgG	YF-IgG	WN-IgG	TBE-IgG
1	TM58	80	20,480	10,240	2,560	5,120
2	DEN-71	80	1,280	640	640	640
3	DEN-72	40	320	< 10	< 10	< 10
4	DEN-84	< 10	640	160	160	640
5	DEN-96	160	10,000	> 20,000	20,000	> 20,000
6	DEN-100	20	2,560	2,560	1,280	640
7	DEN-131	< 10	320	< 10	< 10	< 10
8	DEN-136	40	2,560	2,560	2,560	2,560
9	DEN-137	40	5,120	5,120	10,000	5,120

Dengue fever; YF, yellow fever; WN, West Nile fever; TBE, tick-borne encephalitis

The results shown in Table 2 were produced using indirect immunofluorescence according to standard procedures using the Flavivirus Microchip 1 (Euroimmun, Lübeck, Germany; FI 2661-1005-1 G, (IgG), FI 2661-1005-1 M (IgM)

all three subtypes in parallel and average optical densities (ODs) of the different antigens were compared.

For the screening of sera, TBE-IgM ELISA and TBE-IgG ELISA (both from Euroimmun, Lübeck, Germany) were used according to the instructions of the manufacturer.

Each serum sample was diluted 1:100 and applied in triplicate to wells of each of the three TBEV subtypes (TBEV-Eu, TBEV-Sib and TBEV-FE), a total of nine wells. After incubation for 1 h at 37 °C, the ELISA plates were washed three times. Then, 100 µl of horseradish peroxidase (HRP)-conjugated detection antibody (polyclonal rabbit anti-human IgG-HRP, Dako, Jena, Germany) was added to each well and incubated for 1 h at 37 °C. Following three washes with Phosphate Buffered Saline with Tween (PBS-T), 100 µl of substrate tetramethylbenzidine (TMB; Substrate-Chromogen ready to use, Dako) was added for 6 min at room temperature. The reaction was terminated by adding 50 µl of 0.5 M sulphuric acid. The OD was measured in an ELISA microplate reader (Infinite F50, Tecan, Männedorf, Switzerland) at 450 nm, 620 nm reference.

Data analysis

Positive and negative controls consisted of pooled serum samples with known anti-TBEV IgG antibody levels, tested by indirect immunofluorescence test (IIFT; Flavivirus Mosaic 1, Euroimmun, AG, Luebeck, Germany). The cut-off was calculated as three times the mean NS1 ELISA OD of the negative serum against all three TBEV-NS1-IgG antigens. The mean cut-off OD + 1 standard deviation (SD; 0.1) was defined as the negative threshold. The mean cut-off OD + 3 SD was defined as the positive threshold. Samples with an OD lower than the negative threshold were considered negative. Samples with an OD higher than the positive threshold were considered positive. Samples with an OD in between the mean negative and positive thresholds were considered borderline.

Calculation of sensitivity and specificity

Sensitivity was calculated as the proportion of patients acutely ill with TBE (Group 1, Group 2) who were correctly identified as positive by the assay. Specificity was calculated as the proportion of patients without a current or previous TBEV infection (Groups 3–6) that tested negative in the assay. Sensitivity and specificity analyses were also conducted with respect to the NS1 antigen subgroups used, i.e. TBEV-Eu, TBEV-Sib and TBEV-FE. All statistical analyses were conducted with Excel software, IBM SPSS Statistics version 22 and GraphPad Prism V 6 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Evaluation of NS1 ELISA outcome against the actual subtype of TBEV

Group 1: TBEV-Eu-infected sera

This group consisted of 23 serum samples from acutely ill patients with a proven TBEV-Eu infection. All sera had previously demonstrated a positive reaction in an anti-TBEV IgM-ELISA and anti-TBEV IgG-ELISA and had also shown reactivity against anti-TBEV NS1-IgG in the assay described by Girl et al. [17]. We found that all sera reacted against TBEV-Eu NS1-IgG; however, all sera also reacted against TBEV-Sib NS1-IgG and TBEV-FE NS1-IgG. However, a comparison showed that each of the sera exhibited a significantly higher OD against TBEV-Eu NS1-IgG than against TBEV-Sib NS1-IgG and TBEV-FE NS1-IgG (Figs. 1 and 2). Thus, using the newly developed subtype-specific NS1-IgG assay, it was possible to clearly differentiate the TBE-Eu cases infected with TBEV-Eu subtype. See also results in supplementary material: Table S1 (group 1) and Table S2 (group 2).

Group 2: TBEV-Sib/-FE-infected sera

32 sera from Russian patients with a confirmed acute or past TBE infection (positive TBEV-IgG ELISA utilizing a TBEV-Eu antigen) were tested for reactivity against the NS1 antigens of TBEV-Eu, TBEV-Sib and TBEV-FE. 31/32 sera (for one serum no material was available) were also tested against TBEV-IgM using an ELISA with TBEV-Eu. Four of the 31 sera were found negative and one serum was in the borderline range of the assay. Of the 32 serum samples, 27 showed a positive or borderline result against any of the three NS1 antigens used in the assay. The five remaining sera which had previously shown reactivity in the TBEV-IgG ELISA and TBEV-IgM-ELISA, but were negative in the NS1-IgG assay against all three subtype NS1 antigens, showed positive neutralisation against TBEV strains of the European subtype (K23) and Siberian subtype (Baltic clade) and were subsequently confirmed to be TBEV-IgM positive. These results may indicate early serum sample testing and consequently undetectable levels of TBEV NS1 antibodies in the sera.

None of the tested sera showed a higher OD against TBEV-Eu NS1 in comparison to TBEV-Sib NS1 and/or TBEV-FE NS1 (Figs. 1 and 2, see also Table S2 in supplementary materials). Indeed, for 26 of the 27 reactive sera, the OD of TBEV-Eu was significantly lower than the ODs of TBEV-Sib and TBEV-FE; two samples were actually negative against TBEV-Eu NS1. Therefore, these 26 sera

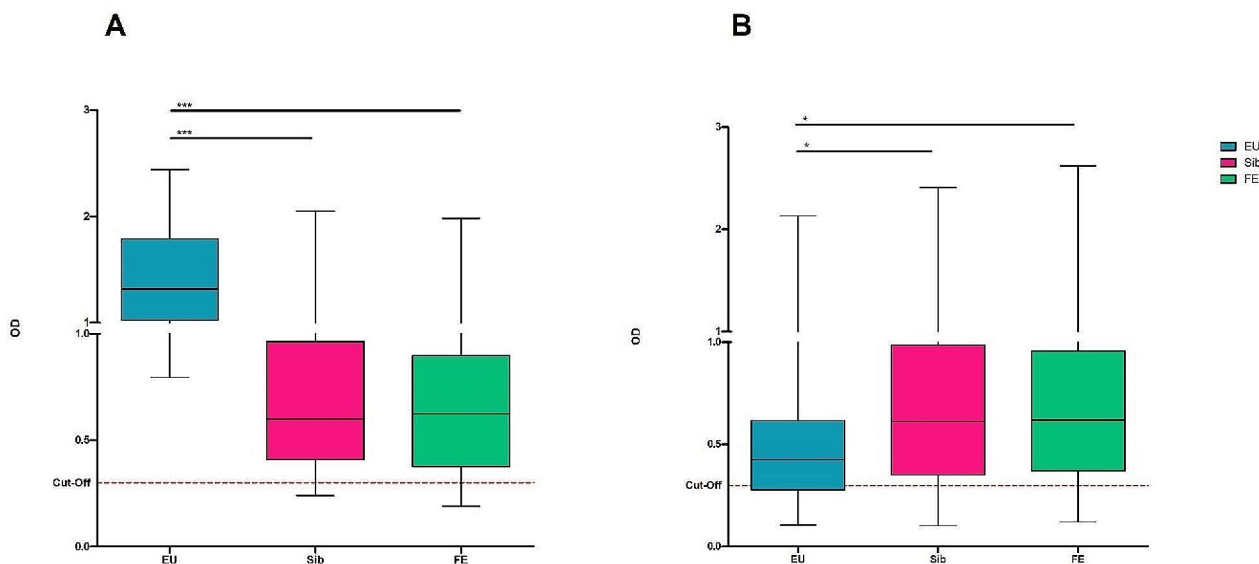


Fig. 1 A: Mean ODs of TBEV-EU sera (group 1, $n=23$) tested against TBEV-EU NS1, TBEV-Sib NS1, and TBEV-FE NS1 (***) highly significant; Mann–Whitney test, $p < 0.0001$). B: ODs of Russian sera (group 2, $n=32$) tested against TBEV-EU NS1, TBEV-Sib NS1, and TBEV-FE NS1 (* significant; Mann–Whitney test, $p < 0.05$). The test has a dynamic cut-off, the absolute value of which can vary depending

on the ELISA plate. The cut-off line shown in the figure is for orientation and represents an “average cut-off” between “borderline” and “positive”. The boxplot diagrams show the median (line in the box), upper and lower quartiles (upper and lower limits of the box) as well as the minimum and maximum (ends of the whiskers)

could be clearly classified as non-TBEV-Eu. In the case of the remaining reactive serum, we could not differentiate between TBEV-Eu and TBEV-Sib/TBEV-FE as it showed a low, non-discriminating OD against all three subtypes.

Regarding the differentiation of TBEV-Sib and TBEV-FE, seven of the 27 reactive sera did not show a significant difference in the ODs against the two NS1 antigens. Ten showed a significantly higher OD against the NS1 of TBEV-Sib than the NS1 of TBEV-FE, while ten showed a significantly higher OD against the NS1 of TBEV-FE compared to the NS1 of TBEV-Sib.

Group 3: Flavivirus-positive, TBEV-negative sera

Nine sera from patients with acute or past dengue infection were used to test for cross-reactions for NS1-IgG against other flaviviruses. Although all except two sera showed high cross-reacting antibody titers against TBEV in indirect immunofluorescence (up to $> 1:20,000$), 8/9 sera did not show any positive reactivity against any of the three NS1 antigens (Fig. 3). One of these eight sera did show a higher OD than the threshold for a negative result; however, the OD value was still in the upper negative range. This might be due to a former yellow fever vaccination causing somewhat higher ODs against all the TBEV NS1 antigens. The remaining serum (DEN-71) reacted against all three NS1 antigens, with the highest positive OD against TBEV-Eu.

We therefore assume that this patient had an unknown prior TBEV infection and during their acute dengue infection they developed a serological secondary-type response due to this former TBEV infection.

Group 4: TBEV-Eu-vaccinated, yellow fever-vaccinated sera

Seven serum samples from individuals with TBEV-Eu vaccination and yellow fever vaccination were tested. All sera showed a somewhat higher OD than serum samples from individuals with TBEV-Eu vaccination only (Group 5). However, none of the ODs reached the calculated cut-off (Fig. 3). Therefore, none of the sera showed a positive or borderline reactivity against any of the three tested TBEV subtype NS1 antigens.

Group 5: TBEV-Eu-vaccinated, non-TBEV-infected sera

43 sera from vaccinated and non-infected individuals, as determined by IgG-ELISA, neutralisation test and TBEV-Eu-NS1-IgG, were tested against the NS1-IgG of all three subtypes. All sera showed negative reactivity (Fig. 3).

Group 6: Non-TBEV-Eu-vaccinated, non-TBEV-infected sera

45 sera from non-vaccinated and non-infected individuals, as determined by a negative IgG-ELISA, were tested. All

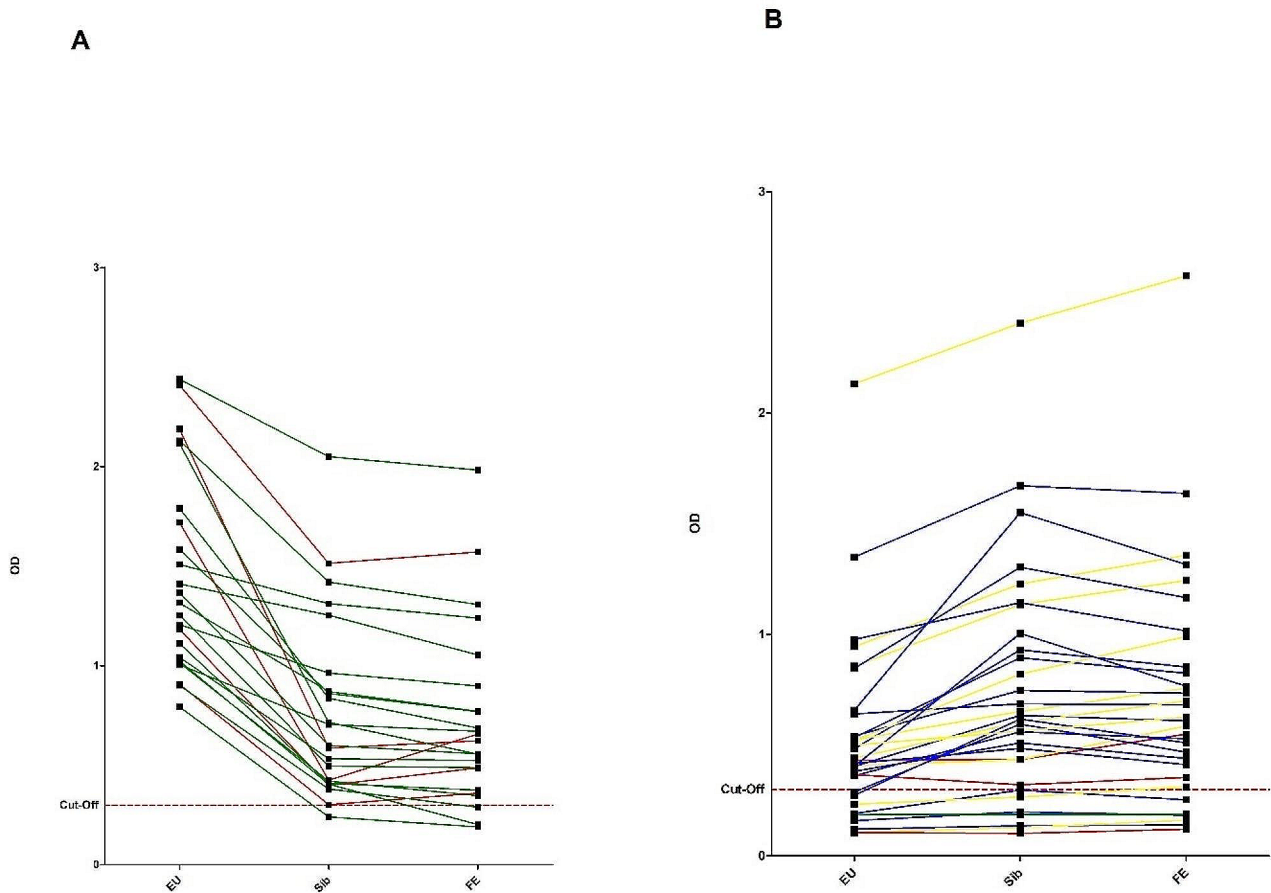


Fig. 2 **A:** Comparison of individual serum ODs of TBEV-EU sera (group 1, $n=23$) against NS1 antigen of TBEV-EU, TBEV-Sib and TBEV-FE. **B:** Comparison of individual serum ODs of Russian sera (group 2, $n=32$) against NS1 antigen of TBEV-EU, TBEV-Sib and TBEV-FE. Green lines: OD drop between TBEV-EU and Sib and

between Sib and FE; Red lines: OD drop between EU and Sib, with increase between Sib and FE; Blue lines: Increase in OD between EU and Sib and drop between Sib and FE; Yellow lines: Increase in OD between EU and Sib and increase between Sib and FE.

sera showed negative reactivity against the NS1-IgG of all three subtypes (Fig. 3).

Overall and subtype-specific sensitivity

Sensitivity was evaluated using 55 serum samples from patients acutely ill with TBE (group 1 and group 2). Of these serum samples, 23/23 (100%) of TBEV-Eu infection samples tested positive for TBEV-Eu NS1-specific IgG antibodies and 27/32 (84%) samples from Russian patients tested positive for TBEV-Sib or TBEV-FE NS1-specific IgG antibodies, resulting in an overall sensitivity of 91% (50/55). However, the five samples from Russian patients that tested negative subsequently tested positive when analysed by TBEV-IgM ELISA, indicating an acute infection. It is well established from studies of TBEV-Eu-NS1 IgG in acute TBE patients that TBEV-NS1 is detectable only between 5 and 7 days after the onset of neurological symptoms. It is

therefore plausible to assume that these five serum samples that tested negative in our NS1-IgG assay were taken too early during the symptomatic course of TBE illness to react positive. By omitting these five sera from the analysis, our assay detected 50/50 sera correctly, thus yielding an overall sensitivity of 100%. Regarding subtype-specific sensitivity, TBEV-Eu subtype differentiation sensitivity was 100% (23/23 correctly identified as TBEV-Eu) and TBEV-Sib/-FE differentiation sensitivity was 96% (26/27 correctly identified as non-TBEV-Eu).

Overall and subtype-specific specificity

104 serum samples from patients/individuals without TBE infection were included in the study. 43 sera from individuals with a complete TBE vaccination schedule tested negative against each of the three subtype NS1 antigens. Another 45 sera from individuals residing in non-endemic areas and

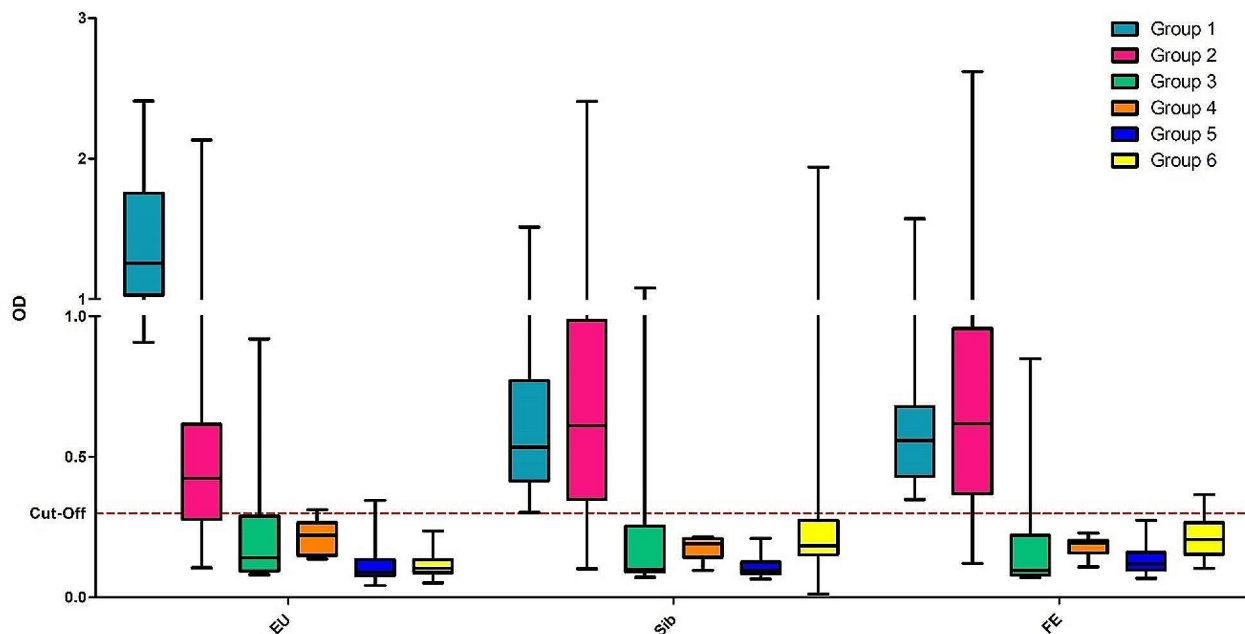


Fig. 3 Mean ODs of sera of all groups tested against NS1 antigen of TBEV-EU, TBEV-Sib, and TBEV-FE. The boxplot diagrams show the median (line in the box), upper and lower quartiles (upper and lower limits of the box) as well as the minimum and maximum (ends of the whiskers)

with no history of TBE vaccination also tested negative against each of the three subtype NS1 antigens. Additionally, eight out of nine sera from patients with primary or secondary serological reactivity against dengue infection due to acute or past dengue infection tested negative. However, one serum sample (DEN-71) in this group showed a clearly positive reactivity against all three NS1 antigens, with the highest OD against the TBEV-Eu NS1 antigen. We assume that this patient with an acute dengue infection had an unknown past TBEV-Eu infection and therefore developed a serological secondary-type response during their acute dengue infection.

An earlier validation of TBEV-Eu NS1-IgG revealed a weak cross-reactivity with sera from yellow fever-vaccinated individuals [17]. Therefore, we also included seven sera from individuals with known TBE vaccination and yellow fever vaccination. All seven sera were negative according to the calculated cut-off of the assay. However, they showed a somewhat higher OD than sera from individuals with TBE vaccination only.

In summary, the overall specificity of the tested 104 sera was 99% (103/104). By omitting the serum from the patient in Group 3 assumed to have had an unknown past TBEV-Eu infection from the analysis, the overall specificity would be 100% (104/104). This degree of specificity was found against all three subtype antigens.

Discussion

Since the introduction of sequencing of viral genes and genomes it has been recognized that TBEV can be divided into at least five subtypes (TBEV-Eu, TBEV-Sib, TBEV-FE, TBEV-Baikal, TBEV-Himal). To date, it has not been possible to ascertain TBEV subtype-specific information in a clinical setting from serum samples drawn from patients with TBE infection. However, the ascertainment of this information is important as different virus subtype infections are reported to exhibit different clinical courses and outcomes. This is especially important for many European TBE-endemic countries, where more than one virus subtype is in circulation. Also, a serological subtyping of TBEV infections will facilitate the monitoring of the emergency of TBEV subtypes into new areas and the overall extend of the subtype distribution which has been depending on virus detection and characterization so far, either in patients or in the vectors or mammalian hosts. In the present study, we describe for the first time the development and validation of an anti-TBEV NS1 IgG ELISA that can differentiate subtype-specific anti-TBEV IgG against three clinically relevant TBEV subtypes, namely European, Siberian and Far Eastern.

The development of our new immunoassay was based on previous work by two of the authors (Gerhard Dobler and Philipp Groll) which culminated in an anti-TBEV NS1

IgG ELISA based on the European subtype NS1 antigen [17]. This assay has a high sensitivity (94%) and specificity (93%) for the detection of wild-type TBEV infections in TBEV-Eu subtype-circulating areas. However, the assay may have a more limited use in European countries where TBEV-Sib and TBEV-FE infections are also prevalent as it could prove discriminatory to a certain extent and consequently have a lower sensitivity for the detection of non-TBEV-Eu infections.

For the validation of our new anti-TBEV NS1 IgG ELISA coated with all three subtype antigens, serum samples from TBEV-infected patients and other flavivirus-infected patients were tested for broad and cross-reactive antibodies, and the subtyping results were analysed with regard to risk group and geographical origin (samples from Europe and Russia). Our new NS1-IgG ELISA against all three subtypes showed an overall sensitivity of 91%. However, based on IgM ELISA results, five serum samples may have been taken too early during the symptomatic course of TBE illness to react positive. By omitting these five sera from the analysis, the overall sensitivity of the assay would increase to 100%. Furthermore, the expected subtype of TBEV that infected a patient showed the highest OD value in comparison to the other subtypes; TBEV-Eu subtype differentiation sensitivity was 100% and TBEV-Sib/-Fe differentiation sensitivity was 96%. Therefore, the testing of TBEV IgG-positive sera from German and Russian patients showed that the new NS1-IgG ELISA was able to differentiate between TBEV European subtype and non-European (TBEV Siberian and Far Eastern) subtype infections. We are aware that due to relatively small sample size, it was acceptable to access new methods feasibility, optimize protocol and gather preliminary data, however, larger, more comprehensive “in field” studies are necessary to strengthen our data.

The unambiguous discrimination of TBEV-Sib and TBEV-FE was difficult and not possible for many of the sera of group 2. Among them, one serum showed highest reactivity against the TBEV-Eu NS1 antigen. Unfortunately, any information from the patients nor on their clinical form or subtype of TBEV infection was missing. In a very recent publication [21] the authors showed that in the Kemerovo region only the TBEV-Sib subtype could be detected. But there are also reports that in the Siberian region TBEV of the European subtypes have been detected by Russian researchers [22]. In our established assay a Baltic TBEV-Sib NS1 antigen was used for coating. There is at least one amino acid exchange from the Baltic NS1 to the Siberian NS1 sequences from Russia which might cause some change in binding of Russian sera to this antigen. Testing of TBE patients from the Baltics or from Finland with respective subtype infections would give valuable information further,

whether they can be unambiguously differentiated by using homologous NS1 antigen.

For the specificity analysis, we tested 104 serum samples from individuals with no prior TBEV infection history. These samples were comprised of nine sera from confirmed dengue fever cases, seven sera from individuals with yellow fever vaccination and TBE vaccination, 43 sera from individuals with a complete TBE vaccination schedule and 45 sera from individuals with no TBE infection nor TBE vaccination history. The overall specificity of the tested 104 sera was 99%. One serum showed a clearly positive reactivity against all three NS1 antigens, with the highest OD against the TBEV-Eu NS1 antigen; however, it is plausible that this patient may have had an unknown past TBEV-Eu infection and thus developed a serological secondary-type response during their acute dengue infection. By omitting this serum from the analysis, the overall specificity of the assay would increase to 100%. This degree of specificity was found against all three subtype antigens.

The sensitivity and specificity analyses demonstrate that the new NS1-IgG ELISA against all three subtypes is suitable for a range of practical and scientific purposes. From a practical point of view, the data presented here have proven NS1-IgG ELISA to be an appropriate tool to diagnose TBEV infections in both the acute and convalescence phases, alongside pre-existing common diagnostic methods such as standard ELISA and neutralisation test. However, due to the somewhat delayed antibody kinetics against NS1, commercially available ELISA kits based on the detection of specific antibodies against the whole TBE virus – particularly against the structural E protein – are more suitable for early acute TBE diagnosis. Conceivably, the NS1-IgG ELISA could play more of a supportive role in the early disease stage, particularly in challenging serological situations (differentiation of infection from vaccination) and for differential diagnosis purposes between other flavivirus infections and replace the complex neutralization test, or for monitoring the emergence of TBEV subtypes for surveillance and prevention by vaccination.

The specificity results of our NS1-IgG ELISA are consistent with other published results [17, 19], i.e. the assay is exclusively indicative for virus replication in natural infections and differentiates TBEV infection-induced specific antibodies from vaccine-induced antibodies. All 40 sera from TBEV-vaccinated individuals reacted negative against all three subtype NS1-IgG. Therefore, the NS1-IgG ELISA can be used as a valuable tool to accurately detect TBE vaccine failures or interpret serological patterns in people vaccinated in close proximity to the onset of the disease. Due to minimal cross-reaction with other flavivirus infections (e.g. West Nile fever virus, yellow fever virus and dengue fever virus), as well as vaccinated individuals (e.g. yellow fever

vaccination), TBEV-Sib NS1-IgG and TBEV-FE NS1-IgG can also be used for TBE differential diagnosis between other flaviviruses, as previously described for TBEV-Eu NS1-IgG [17].

In conclusion, our newly developed NS1-IgG ELISA provides a very important tool for use in TBEV diagnostics. It has the capability to differentiate three TBEV subtypes (TBEV-Eu, TBEV-Sib, TBEV-FE) and to detect wild-type TBE infection. Furthermore, it has the potential to be used in surveillance studies, epidemiological studies and vaccine safety studies, especially in TBE endemic regions with high vaccination rates, where TBEV subtypes overlap.

Future studies need to evaluate the sensitivity and specificity of NS1-IgG ELISA when used in populations/countries in which all three TBEV subtypes co-circulate. Additionally, a better understanding of NS1 protein and its role in immune protection in TBEV-infected individuals and vaccinees is urgently required.

Transparency declaration

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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