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Multiplex Serology for Sensitive and Specific Flavivirus IgG Detection: Addition of Envelope Protein Domain III to NS1 Increases Sensitivity for Tick-Borne Encephalitis Virus IgG Detection

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Abstract: Tick-borne encephalitis is a vaccine-preventable disease of concern for public health in large parts of Europe, with EU notification rates increasing since 2018. It is caused by the orthoflavivirus tick-borne encephalitis virus (TBEV) and a diagnosis of infection is mainly based on serology due to its short viremic phase, often before symptom onset. The interpretation of TBEV serology is hampered by a history of orthoflavivirus vaccination and by previous infections with related orthoflaviviruses. Here, we sought to improve TBEV sero-diagnostics using an antigen combination of in-house expressed NS1 and EDIII in a multiplex, low-specimen-volume set-up for the detection of immune responses to TBEV and other clinically important orthoflaviviruses (i.e., West Nile virus, dengue virus, Japanese encephalitis virus, Usutu virus and Zika virus). We show that the combined use of NS1 and EDIII results in both a specific and sensitive test for the detection of TBEV IgG for patient diagnostics, vaccination responses and in seroprevalence studies. This novel approach potentially allows for a low volume-based, simultaneous analysis of IgG responses to a range of orthoflaviviruses with overlapping geographic circulations and clinical manifestations.

Keywords: multiplex protein array; tick-borne encephalitis; flavivirus; NS1; EDIII; serology



Citation: Valle, C.; Shrestha, S.; Godeke, G.-J.; Hoogerwerf, M.N.; Reimerink, J.; Eggink, D.; Reusken, C. Multiplex Serology for Sensitive and Specific Flavivirus IgG Detection: Addition of Envelope Protein Domain III to NS1 Increases Sensitivity for Tick-Borne Encephalitis Virus IgG Detection. *Viruses* **2024**, *16*, 286. <https://doi.org/10.3390/v16020286>

Academic Editor: Yannick Simonin

Received: 9 November 2023

Revised: 31 January 2024

Accepted: 7 February 2024

Published: 13 February 2024



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1. Introduction

Over the past decades, the geographic distribution of viruses transmitted by arthropods like mosquitos and ticks, grouped under the term “Arboviruses”, has expanded, and the burden of associated disease is increasing worldwide. Tick-borne encephalitis virus (TBEV) is an important arbovirus for public health, affecting the human central nervous system (CNS), and it is now widespread in Europe and Asia [1]. Between 2000 and 4000 TBEV infections are reported every year in Europe, and TBEV is of concern for a significant part of Europe [2]. Autochthonous cases of tick-borne encephalitis (TBE, the disease caused by TBEV), were identified for the first time in the Netherlands in 2016. Since then, 16 autochthonous cases have been identified [3,4].

TBEV is transmitted to humans by bites from infected ticks. *Ixodes ricinus* is the main vector for TBEV-EU in Europe, while a role for *Dermacentor reticulatus* has been suggested [5–7]. TBEV-EU infection is often monophasic and starts with an influenza-like prodromal period, followed in 25% of cases by a severe neurological disorder [8,9]. There is no specific treatment and up to 50% of patients who develop neurological complaints and survive TBEV infection can develop long-term sequelae [9]. Despite the availability of a TBEV vaccine, clinical TBEV infection can still occur in vaccinated people [10]. TBEV infections are typically not diagnosed based on a direct detection of TBEV RNA in blood

samples, as the viremic phase occurs at the very beginning of disease when symptoms are non-specific and mild. Sampling typically takes place when there are more severe disease manifestations when the patient is in the second phase. Therefore, TBE diagnosis is typically based on the detection of specific antibodies directed against TBEV in the serum or in cerebrospinal fluid of infected individuals [11].

TBEV belongs to the orthoflavivirus genus within the Flaviviridae family, which includes several other human pathogens such as West Nile virus (WNV), dengue virus (DENV), Zika virus (ZIKV), Japanese encephalitis virus (JEV) and Usutu virus (USUV) [8]. Three genetic subtypes of TBEV are described, the European (TBEV-Eu), Siberian (TBEV-Sib) and Far Eastern (TBEV-FE) subtypes [8], and two others have been recently proposed: Baikalian (TBEV-Bkl) and Himalayan (Him-TBEV) [12,13]. The molecular organization of orthoflaviviruses is conserved. They are enveloped viruses containing a positive-sense RNA genome of approximately 11,000 bases in length. The genome encodes a single polyprotein cleaved by cellular and viral proteases into three structural proteins: the capsid (C), the precursor membrane (prM) and the envelope (E) proteins; and seven non-structural proteins (NS): NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5 [14]. NS1 is a dimer protein, with a molecular weight between 46 and 55 kDa depending on its glycosylation pattern [15,16]. NS1 plays a role in viral replication and immune evasion, and can be used as an antigen for the diagnosis of orthoflavivirus infections in serological assays [17–20]. The orthoflavivirus E protein is organized in three domains: EDI, EDII and EDIII [21–24]. EDIII has been proposed to be the receptor-binding domain (RBD) and undergoes important repositioning during virus–membrane fusion [21,22]. The protruding position of EDIII suggests that it can be a potent antigen for a serological diagnosis of TBEV infection. Indeed, several studies have shown that EDIII is a major target for neutralizing antibodies (NAbs) [25–30] and that it contains virus-specific epitopes [31,32].

The standard test for TBEV-antibody detection, such as an enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay (IFA), typically use the whole virus or the complete E protein as antigens [33]. However, the detection of TBEV-specific antibodies can be biased by the high level of cross-reactivity among related orthoflavivirus species, as it has been revealed in comparative studies on the commercially available diagnostic kits for TBE infection [33,34]. For instance, false WNV positive results may be generated with the sera of TBEV-infected subjects [35]. In addition, because the circulation of orthoflaviviruses can overlap temporally and spatially, the history of the patient regarding travel, previous infections and vaccinations should be considered when interpreting orthoflavivirus serological assays.

In previous studies, we have used recombinant NS1 (rNS1) proteins to detect anti-orthoflavivirus IgG in a multiplex NS1-based protein microarray [36–38]. The multiplex microarray approach allows a rapid, high-throughput simultaneous detection of antibodies against a number of viral proteins using a low specimen volume (e.g., 10 µL of serum). Here, we have sought to expand our NS1 IgG orthoflavivirus array for the specific and sensitive detection of TBEV IgG elicited by either natural infection or vaccination. To this end, we explored the added value of the reported high sensitivity and specificity of domain III of the E protein (EDIII) [39–41]. We could successfully show that the addition of flavivirus EDIII next to the routinely used antigen NS1 increases the sensitivity for TBEV IgG detection. The multiplex assay developed in this study would be a powerful single diagnostic tool to screen for orthoflavivirus infections in endemic regions.

2. Materials and Methods

2.1. Cloning and Expression

TBEV (GenBank acc. nr. AEP25267.2), WNV lineage 2 (AY532665.1), DENV serotype 1 (DENV₁) (T279761.2), DENV serotype 2 (DENV₂) (AII99332.1), DENV serotype 3 (DENV₃) (ALS05358.1), DENV serotype 4 (DENV₄) (ANC57613.1), ZIKV (MF438286.1), JEV (NP_059434.1) and USUV (AQM55263.1) NS1 synthetic genes (Baseclear, Leiden, The Netherlands) were codon-optimized for expression in human cells and sub-cloned

with a Gibson Assembly kit (NEB, Boston, MA) in the high-level mammalian expression vector pPPI4 presenting a hexahistidine (his) tag [42] (i.e., pPPI4-tPA(22 P/A)-SP-rNS1-6xHis and verified via Sanger sequencing. The NS1 genes were preceded by the signal secretion sequence tPA (tPA-SP) mutated at position 22 (22 P/A) p to improve the secretion of the recombinant proteins [43] (Figure 1). The codon-optimized DNA encoding the EDIIIs of TBEV, WNV, DENV₁₋₄, ZIKV, JEV and USUV flanked with a synthetic signal secretion sequence (Genscript Biotech, Netherlands) and the sequence coding for a rat IgG-Fc-tail were synthesized and sub-cloned in the pPPI4 expression vector by GenScript (i.e., pPPI4-synth-SP-rEDIII-Fc-6xHis) (Figure 1). All the recombinant proteins were produced in human embryonic kidney (HEK) 293F suspension cells maintained in FreeStyle™ 293 Expression Medium (Gibco, Thermo Fisher Scientific, Breda, The Netherlands), in a shaking incubator (125 r.p.m.) at 37 °C and 8% CO₂. The pPPI4 orthoflavivirus plasmids (125 µg) were transfected into HEK-293F cells using transfection-grade linear Polyethylenimine Hydrochloride “Max” (PEI MAX) (Polysciences, Bergstrasse, Germany) following the manufacturer’s recommendations. Cells were discarded 7 days post transfection via centrifugation at 4000 r.p.m. for 30 min at 4 °C. The secreted proteins were subsequently purified from the supernatant.

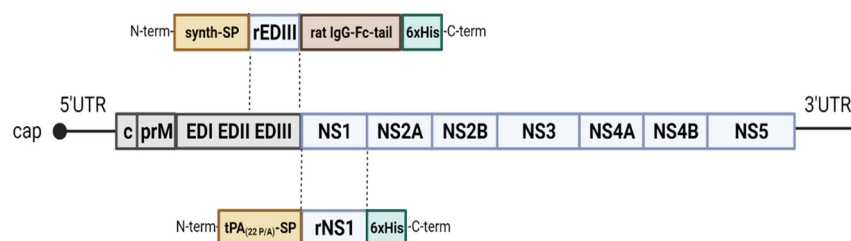


Figure 1. Orthoflavivirus genome organization. Schematic representation of expression vector pPPI4-tPA(22 P/A)-SP-rNS1-6xHis (lower) and vector pPPI4-synth-SP-rEDIII-Fc-6xHis (upper). The tick-borne encephalitis virus (TBEV) genome encodes 3 structural proteins, the capsid (C), the precursor membrane (prM) and the envelope (E) proteins, and seven non-structural proteins (NS). tPA(22 P/A)-SP corresponds to the tPA signal secretion sequence mutated at position 22 and presents at the N-terminus of the protein. rNS1, recombinant nonstructural protein 1, and 6xHis is the tag at the C-terminus used for protein purification. Synth-SP corresponds to the synthetic signal secretion sequence, at the N-terminus of the protein; rEDIII, recombinant envelop domain-III protein; and Fc, rat IgG-Fc-tail.

2.2. Purification of the Recombinant Proteins

The clarified supernatant was incubated with Ni-NTA resin (Bio-Rad, Veenendaal, The Netherlands) at 4 °C for 1 h, with gentle shaking. Beads were transferred to 25 mL cartridges and washed with 20 mL of washing buffer (50 mM Tris (pH 8), 300 mM NaCl and 30 mM imidazole). Recombinant proteins were eluted in 50 mM Tris (pH 8), 300 mM NaCl and 300 mM imidazole. The imidazole was removed via dialysis using a standard grade regenerated cellulose (Spectrum Chemical, Gardena, CA, USA) placed in 2 L of phosphate-buffered saline (PBS) overnight at 4 °C. Finally, proteins were concentrated on Amicon ultrafilters (EMD Millipore, Darmstadt, Germany), and the final concentration was determined using absorbance measurements at 280 nm using a Nanodrop. The purified recombinant proteins were analyzed via sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and the purity estimated was higher than 95% (Figure 2a,b). Proteins were stored at −80 °C prior to their utilization.

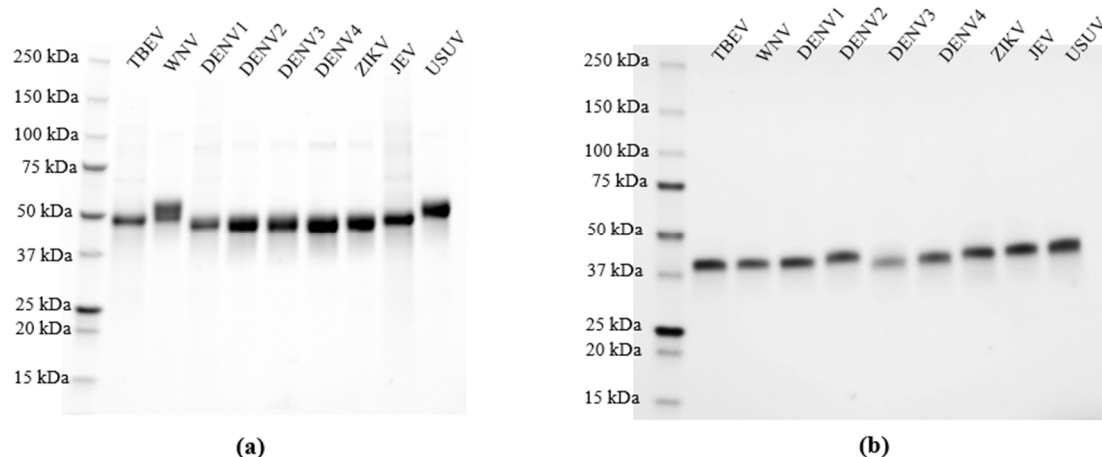


Figure 2. Production of orthoflavivirus recombinant NS1 for sensitive and specific microarray detection of orthoflavivirus IgG. (a) Recombinant NS1 and (b) recombinant EDIII of TBEV, West Nile virus (WNV) lineage 2, dengue virus (DENV) DENV₁, DENV₂, DENV₃, DENV₄, Zika virus (ZIKV), Japanese encephalitis virus (JEV) and Usutu virus (USUV) were purified and then analyzed via SDS-PAGE.

2.3. Serum Samples for Multiplex Protein Microarray Validation

For the validation of the protein microarray, sera from anonymized patients were used. Table 1 contains the information of each serum cohort. Patients were diagnosed according to internationally accepted criteria, combining clinical symptoms, epidemiological data and outcomes of routine serological methods (ELISA, IFA) [44]. DENV serum samples ($n = 11$) presented IgG against DENV without serotype specification. DENV sera were collected from returning travelers in the Netherlands between 2019 and 2020 and represent individual patients. ZIKV serum samples ($n = 15$) were collected in 2017 and 2020. Two sera were collected in Aruba and two in Bonaire. The remaining samples were collected in the Netherlands from returning travelers. All samples are individual serum samples. As DENV and ZIKV PRNT or VNT were not available, the routine diagnostic outcomes could be confirmed through PRNT or VNT only for the WNV and TBEV serum samples. Seven WNV serum samples representing individual patients were collected in 2020 in the Netherlands. TBEV sera ($n = 20$) were collected in 2008–2021 in the Netherlands. Fourteen samples were from individual patients, while for three patients, paired serum samples were available. For serum samples for which the date of onset of illness was known, the sera were taken >3 weeks post onset. In general, in the Netherlands, diagnostics for TBEV infection are requested late in the course of disease when neurological complaints have manifested and common etiology has been excluded. Serum samples from TBEV-vaccinated patients ($n = 13$) were collected in the Netherlands between 2007 and 2021 and represent individual patients. These patients indicated not to have received a previous YFV and/or JEV vaccination. All vaccinated serum samples were tested TBEV IgG positive and one sample presents TBEV IgM and IgG. While the vaccination statuses of the WNV, DENV and ZIKV patients were not available, the immunization statuses of 11 of 20 TBEV patients were known, of which one reported a previous yellow fever vaccination. Overall, for only ten patients, the date of onset of symptoms had been documented and the time between this date and the date of sample collection varied from five days to four months). The negative control serum cohort ($n = 35$) was collected in the Netherlands ($n = 30$) and in the Caribbean Netherlands ($n = 5$) between 2020 and 2023 and tested negative with routine orthoflavivirus diagnostics.

The sera from 556 nature management workers without TBEV vaccination were collected in the “Dutch forestry study”, as described elsewhere [45]. This study assessed the exposure to TBEV among professionals with a high risk for tick bites in the Netherlands.

Table 1. Overview of serum collection used for orthoflavivirus microarray validation.

Virus Species	Number of Samples	Sex	Group Age (in Years)	Positive Serology (ELISA/IFA) Specific IgG	Virus Neutralization Confirmed (VNT/PRNT)	Vaccination
DENV ₁₋₄	11	M (6/11) F (5/11)	19 to 70	11/11	N/D	N/A
ZIKV	15	M (3/15) F (12/15)	26 to 80	15/15 (CHIKV and DENV positive IgG IFA: 7/15)	N/D	N/A
WNV	7	M (3/7) F (4/7)	30 to 75	7/7	5/7	N/A
TBEV	20	M (9/20) F (8/20)	20 to 80	20/20	15/20	9/20 N/A 10/20 No vaccine 1/20 YF vaccine
TBEV Vaccinated group	13	M (4/13) F (9/13)	17 to 70	13/13	N/D	TBEV vaccine *
Negative group	35	N/A	N/A	Orthoflavivirus IgM/IgG Neg	N/D	N/A
Dutch forestry workers	556	N/A	22 to 88	TBEV IgG (10/556)	TBEV VNT (3/10)	No vaccine

DENV₁₋₄: dengue virus serotypes 1 to 4; WNV: West Nile virus; ZIKV: Zika virus; TBEV: tick-borne encephalitis virus; CHIKV: chikungunya virus. Vaccinated group: * TBEV vaccine (FSME-IMMUN[®], Pfizer). N/A, information not available. N/D, not determined, assay not available. VNT: virus neutralization test. PRNT: plaque reduction neutralization test. M: male; F: female; YF: yellow fever.

The current study was performed in accordance with the guidelines for the sharing of anonymous sera and patient data in observational scientific research in emergency situations as issued by the Commission on the Codes of Conduct of the Federation of Dutch Medical Scientific Societies. The sera of the Dutch forestry workers study were collected under approval # 16-767/D of the medical ethics committee of the University Medical Center in Utrecht, The Netherlands.

2.4. Multiplex Protein Microarray

Array slides were produced and treated as described previously [46,47]. TBEV rEDIII and the rNS1 of DENV₁, DENV₂, DENV₃, DENV₄, JEV, TBEV, USUV, WNV lineage 2 and ZIKV at concentrations of 1.5 mg/mL were mixed with 2× protein printing buffer (GVS, Sanford, FL, USA) and spotted in duplicate in three drops of 333 pL each on 24-pad nitrocellulose-coated slides (ONCYTE AVID, GraceBio Labs, Bend, OR, USA) by using a non-contact Marathon Arrayjet microarray spotter (Roslin, UK). After printing, slides were placed in a drying chamber overnight and stored at room temperature until further use. To avoid non-specific binding to the nitrocellulose surface, the printed slides were treated with Blotto-blocking buffer (ThermoFisher, Breda, The Netherlands) for 1 h at 37 °C in a moist chamber. In between steps, slides were washed 3 times with 1× PBS supplemented with 0.1% Tween. Sera were diluted in Blotto-blocking buffer containing 0.1% Surfact-Amps20 (ThermoFischer, Breda, The Netherlands), and tested in 3-fold dilutions ranging from 1:20 to 1:43,740. After the washing step, the slides were incubated with goat anti-human IgG, F(ab')₂ fragment specific, Alexa Fluor 647-conjugated (Jackson Immuno Research, West Grove, PA, USA), diluted 1:1000 in Blotto-blocking buffer with 0.1% Surfact-Amps20 and incubated for 1 h at 37 °C in a moist chamber. Finally, the slides were washed with sterile water and dried. Signals were quantified using a Power scanner (Tecan, Männedorf, Switzerland).

2.5. Data Analysis

A data analysis was performed as previously described [46]. To read the csv files containing the raw data generated by ScanArray Express software version 4.0.0.0004 (PerkinElmer, Waltham, MA, USA), we used R studio v4.0.0, package “DRC” version 2.3-7 (R studio, Boston, MA, USA). A representative theoretical IgG titer (EC50) was calculated based on serial serum dilutions exactly as described previously [48]. For the first screen of samples from the “Dutch forestry study”, based on a single samples dilution (1/20), the analysis of median spot fluorescence foreground intensity determined using ScanArray Express (version 4.0) classified the samples in three groups: (i) samples presenting a saturation intensity signal (intensity > 60,000), (ii) samples presenting a sub-saturation intensity signal (intensity between 40,000 and 60,000) and (iii) samples presenting a low intensity signal (below 40,000). Sera were considered positive for TBEV-specific IgG when above background titers (≤ 10) for rNS1 and/or rEDIII were detected.

2.6. Plaque Reduction Neutralization Test (PRNT)

The PRNT was used as a confirmatory assay for orthoflavivirus infection. The European subtype of TBEV (TBEV-Eu, strain Salland) and WNV lineage 2 (B956 strain) were used in the PRNT. A549 cells (CCL-185™) were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL of penicillin–streptomycin. For the assay, cells were seeded at a density of 2.5×10^5 cells/well one day prior to infection. The assay was performed in duplicate using CELLSTAR 24-well cell culture multi-well plates (Greiner Bio-One, Frickenhausen, Germany) in a biosafety level 3 facility (BSL3). Serological specimens were diluted 1:8 in DMEM supplemented with 2% FBS and antibiotic. All diluted sera were heat-inactivated (56 °C, 30 min) before testing. In a 96-well plate, the sera were further diluted 2-fold from 1:8 to 1:2048 in DMEM supplemented with 2% FBS and antibiotic in a volume of 120 μ L. Next, virus suspension was mixed to each serum dilution and incubated at 37 °C for 1 h. The virus–serum mixtures were added onto pre-formed A549 cell monolayers and incubated for 1 h at 37 °C in a 5% CO₂ incubator. The cell monolayer was then overlaid with 3.2% carboxymethyl cellulose (CMC) medium (Acros Organics, Landsmeer, The Netherlands) in a cell culture medium (DMEM containing 2% FBS and antibiotic) and incubated at 37 °C in a 5% CO₂ incubator. After a 4-day incubation period, the cells were fixed by adding 10% formaldehyde in PBS and stained using 1% crystal (Acros Organics, Landsmeer, The Netherlands) violet in 20% ethanol. Plaques were counted, and the endpoint titers were expressed as reciprocal of the highest serum dilution showing a $\geq 50\%$ reduction in plaque counts compared to wells without serum.

2.7. Statistical Analysis

Data visualization and statistical analyses were performed in GraphPad Prism software (version 9.1.0). A non-parametric *t*-test was performed to assess the statistical differences of paired or unpaired samples. Significance was noted as ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns, not significant. The receiving operating characterization (ROC) curve was created by plotting the TBEV seropositive samples confirmed using the PRNT assay against the overall orthoflavivirus seronegative samples. To describe the performance of the TBEV rEDIII as a potent antigen to characterize TBEV serostatus, the area under the ROC curve was calculated as a single numerical measurement.

3. Results

3.1. Expression and Validation of Recombinant NS1 for Specific Orthoflavivirus IgG Detection Using Multiplex Protein Microarray

To improve the yield of NS1 expression in comparison to our previous system [36] and increase flexibility for novel constructs, we changed the pPPI4 eukaryotic expression vector to express our antigen panel of nine NS1 proteins from DENV₁₋₄, JEV, TBEV, USUV, WNV and ZIKV. This system yielded up to 10 mg of purified protein per liter of culture

with a purity of >95% (Figure 2a). The performance of the array was assessed using a panel of sera from patients with a probable or confirmed [44] ZIKV ($n = 15$), DENV ($n = 11$), WNV ($n = 7$) or TBEV ($n = 20$) infection, confirmed negative for these viruses ($n = 35$) based on routine diagnostic tests, and people who received TBEV vaccination ($n = 13$) (Table 1). Figure 3a–e show heatmaps of the calculated IgG titers of the serum cohorts for each antigen, with columns representing individual sera reactivity to all tested NS1 antigens. All negative control sera showed a negative or near negative IgG titer against the rNS1 (background level) (Figure 3a), indicating an absence of non-specific binding. Three of seven sera reported positive for WNV IgG through routine diagnostics, showing IgG responses exclusively against the WNV rNS1; one of seven reacted with both WNV rNS1 and USUV, JEV and DENV_{1–4} rNS1s, with the highest IgG titer for DENV₄ rNS1; and one of seven presented, in addition to WNV NS1 reactivity, a low, but above background, reactivity for USUV NS1. Two sera did not bind to any of the NS1 antigens at all (Figure 3b,c). All 15 pre-characterized ZIKV IgG positive sera showed an IgG titer against ZIKV rNS1. In addition, a high-level IgG reactivity to DENV_{1–4} rNS1 antigens was detected for eight of them, and for five of those, a low IgG titer against WNV, JEV and/or USUV NS1 was detected as well. Four of those presented a high IgG titer against DENV_{1–4} rNS1, and one presented a higher titer for DENV₁, DENV₂ and DENV₃ compared to ZIKV NS1 reactivity. Finally, four samples presented a low reactivity to only DENV₂ with two being close to background level (Figure 3d,e). All DENV control sera showed a titer for at least one of the DENV serotypes, rNS1, while six samples presented varying IgG titers for all serotypes. Two samples showed a titer for DENV₂ NS1 only, one sample for DENV₃ only, one sample for both DENV₁ and DENV₃ and one sample for both DENV₂ and DENV₃ (Figure 3f,g). In addition, two samples also presented an IgG titer just over the background level to ZIKV rNS1. Finally, four samples showed a reactivity for TBEV rNS1 ($n = 2$) or ZIKV NS1 ($n = 2$) at the background level (Figure 3f,g). Only 7 of the 20 pre-characterized sera in the TBEV-infection cohort showed IgG reactivity to TBEV rNS1, which included 6 sera corresponding to the three serum pairs in this cohort. The sera from the three serum pairs were taken at least 3 weeks since the onset of illness and 3–5 weeks apart, and showed an increase in rNS1 reactivity in time. Four of the seven reactive sera showed additional IgG reactivity to other rNS1 antigens. In these four cases, two presented a highest titer for TBEV rNS1 while two sera presented a higher titer against JEV, USUV and/or WNV rNS1 (Figure 3h,i). In total, 2 sera reacted with very low titers to non-TBEV rNS1 only, while 11 sera did not react at all.

To further investigate the observed discrepancies in results with the WNV and TBEV cohorts between our microarray and routine diagnostic testing for WNV and TBEV rNS1, we further characterized specimens using plaque reduction neutralization (PRNT) assays, the gold standard used to confirm orthoflavivirus infection based on serology.

3.2. WNV and TBEV Plaque Reduction Neutralization Test

To understand the lack of reactivity for WNV rNS1 in the microarray of the two specimens from probable WNV patients, we characterized the complete WNV serum panel in a WNV-specific PRNT using WNV lineage 2. While the five WNV rNS1 reactive WNV patient samples were all able to neutralize WNV, the ones that did not show an IgG titer against WNV rNS1 in the array (Figure 3b and Table 1) did not neutralize WNV in the PRNT either, confirming the reactivity measured in the protein microarray.

To investigate the discrepant results among the 20 TBEV patient sera in the rNS1 microarray, a TBEV PRNT was performed (Table 1). Among the 20 probable TBEV infections, the TBEV-neutralizing antibody positivity was 75% (15/20) (titer $\geq 1:16$), including the 7 sera confirmed via the rNS1-based microarray. Eight sera were confirmed through the TBEV PRNT but not via rNS1 microarray, which is indicative of a lack of sensitivity of the protein microarray using NS1-based antigens.

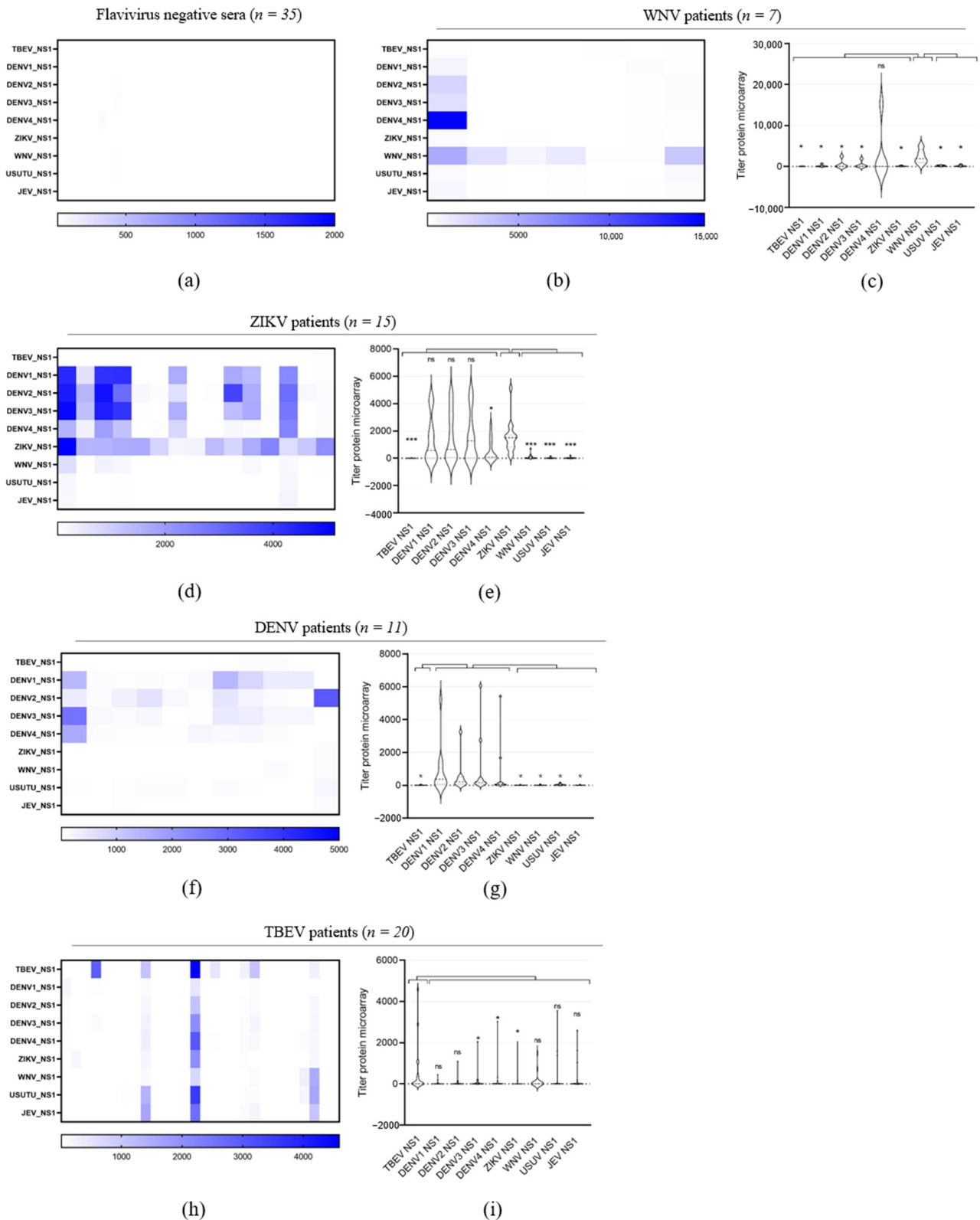


Figure 3. Validation of orthoflavivirus recombinant NS1 for sensitive and specific microarray detection of orthoflavivirus IgG. Heatmaps displaying microarray titers for the orthoflavivirus rNS1 of (a) orthoflavivirus negative reference sera, (b) WNV patients, (d) ZIKV patients, (f) DENV patients and (h) TBEV patients. (The color key of the titers is indicated at the bottom of each heatmap). The microarray antigens are listed on the left of each heatmap. Violin plots showing microarray titers of (c) WNV patients, (e) ZIKV patients, (g) DENV patients and (i) TBEV natural infection-positive sera

against the recombinant NS1 of TBEV, DENV1, DENV2, DENV3, DENV4, ZIKV, WNV, USUV and JEV. A non-parametric *t*-test was performed to assess the statistical differences of paired samples. Significance is noted as ***, $p < 0.001$; *, $p < 0.5$. (b) Receiving operating characterization (ROC) curve was created with GraphPad Prism software (version 9.1.0).

3.3. Evaluation of TBEV EDIII as Antigen for TBEV IgG Detection

To improve the sensitivity for TBEV IgG detection while maintaining or even improving the high level of specificity, we evaluated the parallel use of the orthoflavivirus antigen EDIII in the microarray. TBEV, DENV1–4, ZIKV, WNV, USUV and JEV rEDIII were expressed in mammalian cells as secreted proteins (Figure 1b). As available TBEV vaccines do not lead to the production of NS1 in vaccinees but do elicit E protein-directed antibodies, the sera of people vaccinated against TBEV can be used as a positive control for TBEV rEDIII. As expected, these serum samples ($n = 13$) presented IgG reactivity for TBEV rEDIII, whereas no or only background IgG reactivity against TBEV rNS1 was detected (Figure 4). The microarray with TBEV rEDIII demonstrated a specific reaction against TBEV infection and vaccination sera versus WNV, DENV and ZIKV sera and the negative cohort (Figure 4b). While only 7 of the 20 sera of probable TBEV patients showed reactivity against TBEV rNS1 (Figure 3h,i), 15 of these presented IgG reactivity to TBEV rEDIII antigen. This was completely consistent with the PRNT results (Table 1). The samples that were not able to neutralize TBEV did not show any reactivity against rEDIII either. The comparison of PRNT and microarray results shows that low microarray titers nearly always correspond to a low or a lack of neutralization potency. Only IgG titers against rEDIII > 100 on microarray correspond to measurable neutralization titers, as sera that presented negative on the PRNT showed titers < 100 . Lastly, we tested the reactivity of the TBEV vaccinated sera to other orthoflavivirus rEDIII proteins (Figure 4c,d). Only a low level of IgG reactivity was detected against WNV, DENV1–4, ZIKV, USUV and JEV rEDIII, but a much higher titer was found for TBEV rEDIII (Figure 4c,d). So, despite some cross-reactivity, the observed titers clearly indicated TBEV exposure.

Next, we evaluated the reactivity of the TBEV natural infection sera to WNV, DENV1–4, ZIKV, USUV and JEV rEDIII proteins (Figure 5). The samples presented only low levels of IgG reactivity to WNV, DENV1–4, ZIKV, USUV and JEV rEDIII compared to those of IgG reactivity to TBEV rEDIII. Within those samples, some serum samples presented high IgG reactivity to WNV, USUV or JEV rEDIII (Figure 5a,b) as well as IgG reactivity to rNS1 antigen for those orthoflaviviruses (Figure 3h,i), which is most probably indicative for a previous orthoflavivirus infection.

To evaluate the performance of the microarray test with rEDIII as an antigen to detect TBEV infection, a receiving operating characterization (ROC) analysis [49] was performed. The ROC analysis was created by plotting the 15 TBEV seropositive samples confirmed through the PRNT assay against the overall orthoflavivirus seronegative samples. The ROC curve analysis indicates that the protein microarray is accurate in determining the TBEV serostatus relative to the PRNT50 assay results with an area under the curve (AUC) of 0.970 (Figure 5c).

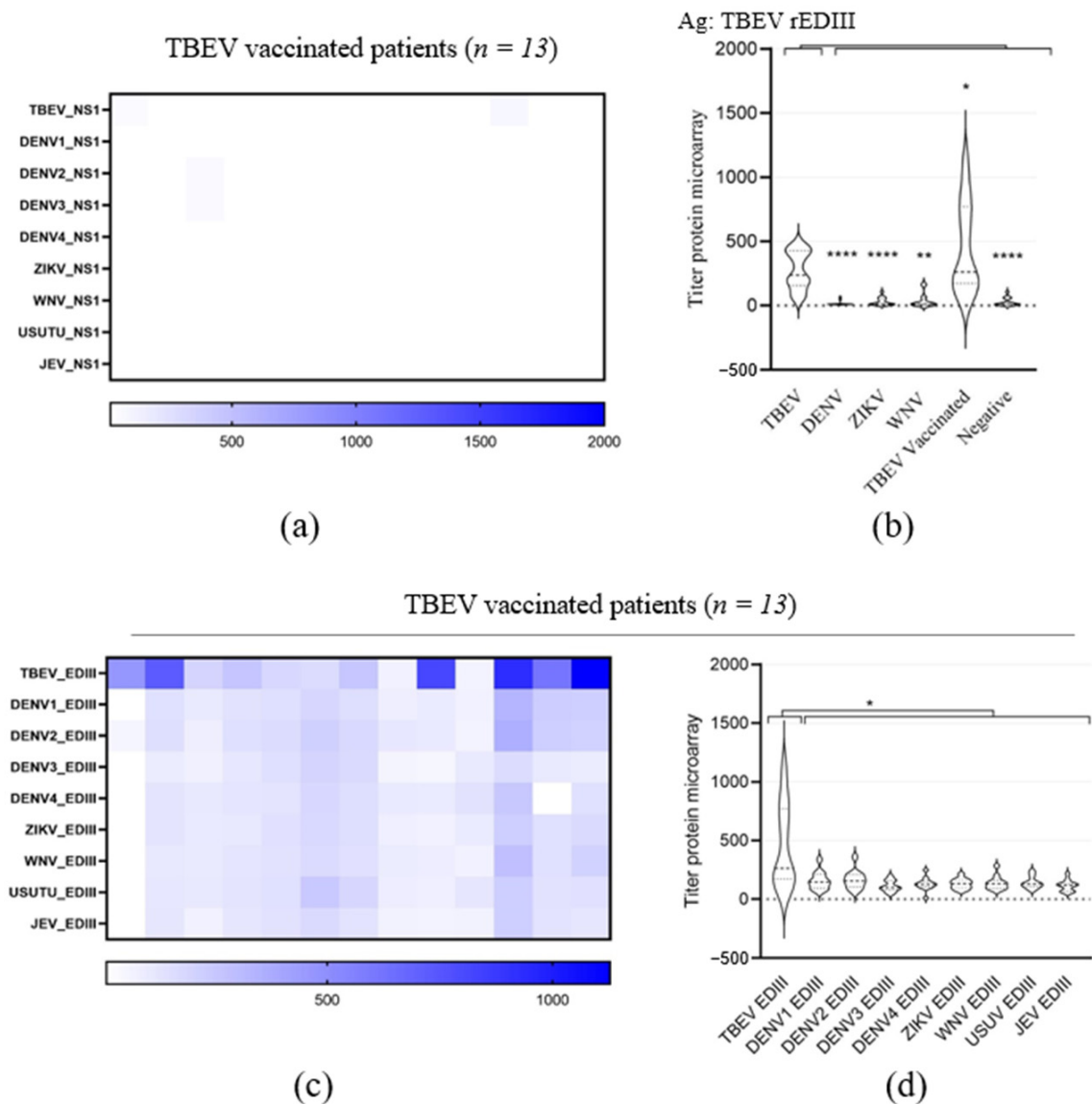


Figure 4. (a) Heatmap displaying microarray titers for the orthoflavivirus rNS1 of orthoflavivirus TBEV-vaccinated patients. (The color key of the titers is indicated at the bottom of the heatmap). The microarray antigens are listed on the left of the heatmap. (b) Violin plot showing microarray titers of the pre-characterized TBEV, dengue virus (DENV), Zika virus (ZIKV), West Nile virus (WNV) sera and the negative orthoflavivirus sera against TBEV rEDIII. TBEV vaccinees were used as the positive control. (c) Heatmap displaying microarray titers for the orthoflavivirus rEDIII of orthoflavivirus TBEV-vaccinated patients. (The color key of the titers indicated at the bottom of the heatmap.) The microarray antigens are listed on the y-axis. (d) Violin plot showing microarray titers of TBEV-vaccinated serum samples against the recombinant EDIII of TBEV, DENV1, DENV2, DENV3, DENV4, ZIKV, WNV, USUV and JEV. A non-parametric T-test was performed to assess the statistical differences of paired samples. Significance is noted as ****, $p < 0.0001$; **, $p < 0.01$; *, $p < 0.05$. The order of patient samples is the same for each heatmap.

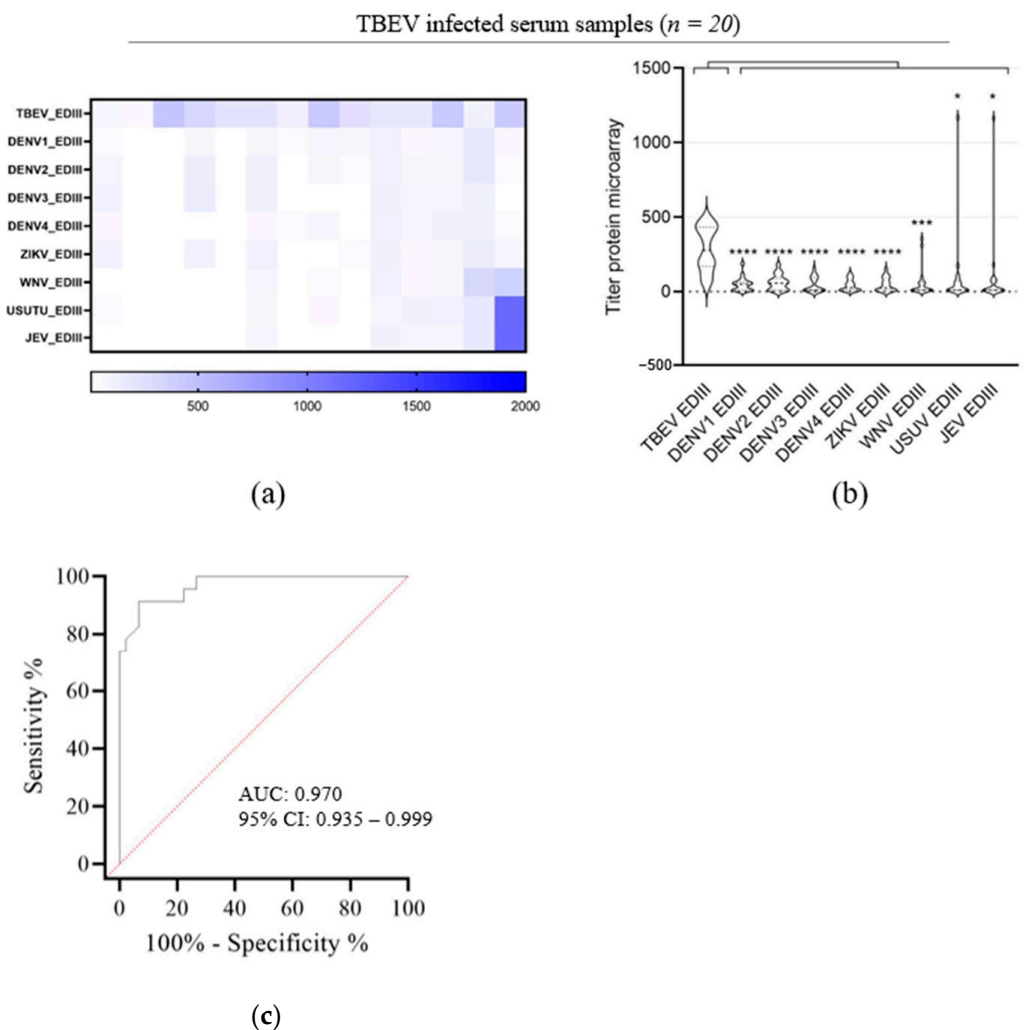


Figure 5. TBEV-infected serum samples against recombinant EDIII. (a) Heatmap displaying microarray titers for the orthoflavivirus rEDIII of orthoflavivirus TBEV natural infection-positive sera. (The color key of the titers is indicated at the bottom of each heatmap). The microarray antigens are listed on the y-axis. (b) Violin plots showing the microarray titers of TBEV natural infection-positive sera against the recombinant EDIII of TBEV, DENV1, DENV2, DENV3, DENV4, ZIKV, WNV, USUV and JEV. A non-parametric T-test was performed to assess the statistical differences of paired samples. Significance is noted as ****, $p < 0.0001$; ***, $p < 0.001$; *, $p < 0.5$. (c) Receiving operating characterization (ROC) curve was created with GraphPad Prism software (version 9.1.0). The order of patient samples is the same as in Figure 3.

We also evaluated the reactivities of the DENV, ZIKV and WNV serum samples against the range of orthoflaviviruses rEDIII antigens (Figure 6). ZIKV serum samples showed an IgG reactivity to all orthoflavivirus rEDIII but with higher titers for DENV1–4 and ZIKV rEDIII (Figure 6a,b). DENV serum samples presented an IgG titer against DENV1 rEDIII only (Figure 6c,d). For sera from positive WNV patients, high-level IgG reactivity against WNV rEDIII and the most closely related JEV and USUV rEDIIIs was detected with the highest titer against WNV rEDIII. In addition, those sera presented IgG titers against DENV1–4, ZIKV and TBEV rEDIII close to the background (Figure 6e,f).

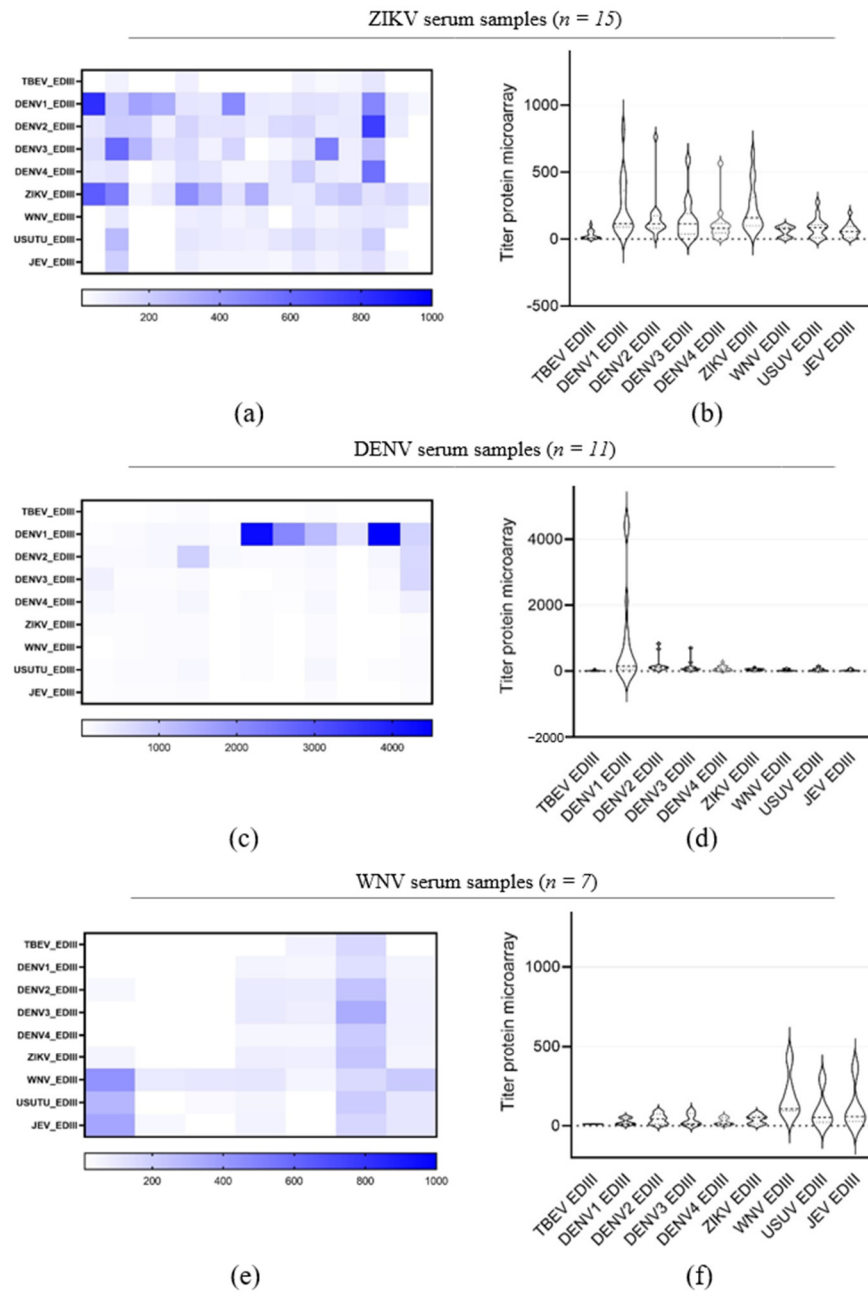


Figure 6. Evaluation of recombinant orthoflavivirus EDIII as an antigen. Heatmaps and violin plots showing the microarray titers of ZIKV-positive sera (a,b), DENV-positive sera (c,d) and WNV-positive sera (e,f) against the recombinant rEDIII of TBEV, DENV1, DENV2, DENV3, DENV4, ZIKV, WNV, USUV and JEV. The order of patient samples is the same as in Figure 3.

3.4. Performance Assessment of rNS1/rEDIII Microarray for TBEV IgG Detection in High-Risk Cohort of Forestry Workers

Finally, we assessed the performance of the array in a public health application, using the sera collected from 556 participants in the “Dutch forestry workers” study. Participants who reported to have received a TBEV vaccination were excluded [45]. To pre-select TBEV-antigen reactive sera for full titration, all 556 sera were first screened for TBEV antigen reactivity by using a single serum dilution (1/20) against the 18 orthoflavivirus antigens (9 rNS1 and 9 rEDIII). The serum samples ($n = 5$) presenting a specific positive signal for TBEV antigens (rNS1 and/or rEDIII) with little to no cross-reactivity to other orthoflavivirus, were titered, as previously described. None of the five serum samples showed an IgG titer against TBEV rNS1 (Table 2). Three samples presented a high IgG reactivity to TBEV rEDIII

with a titer > 100, and two serum samples had an IgG titer against TBEV rEDIII < 100 but above the background level (Table 2). We then compared these results with the previous results [45]. The five samples that tested TBEV positive (rNS1 and/or rEDIII reactivity) in the microarray were indeed all included in the 10 serum samples that tested positive in the TBEV ELISA while only three of them had tested positive in a TBEV PRNT (Table 2) [45]. The TBEV-neutralizing sera corresponded to the three sera with a titer > 100 against rEDIII on the microarray, thereby confirming our previous assertion that only IgG titers against rEDIII > 100 on the microarray can result in measurable neutralization titers.

Table 2. All TBEV serology results of sera ($n = 5$) that tested TBEV IgG positive for rNS1 and/or rEDIII in the orthoflavivirus microarray.

Sample	Microarray Value		TBEV ELISA Value *	VNT Titers *	Interpretation
	NS1 TBEV	EDIII TBEV			
1	≤10	218	6.1	120	Confirmed TBEV infection
2	≤10	126	3.0	30	Confirmed TBEV infection
3	≤10	101	1.3	30	Confirmed TBEV infection
4	≤10	36	2.6	Neg	Possible TBEV infection
5	≤10	40	2.4	Neg	Possible TBEV infection

* From [45].

4. Discussion

The diagnosis of TBEV infection often relies on serological methods [50,51]. However, orthoflavivirus diagnosis based on serology is highly complex due to the spatiotemporal overlap of the circulation of closely related orthoflaviviruses that elicit cross-reacting antibody responses and have overlapping disease manifestations [51,52]. Comparative virus neutralization assays are the gold standard for a serology-based confirmation of orthoflavivirus infection, but are not routinely performed by diagnostic labs due to complexity and the need for high-containment laboratories. Our multiplex arbovirus approach allows for a rapid, high-throughput simultaneous detection of antibodies against a number of orthoflavivirus proteins using a low specimen volume (e.g., 10 µL). We have sought to expand our NS1 IgG orthoflavivirus array for the specific and sensitive detection of TBEV IgG elicited by either natural infection or vaccination. To this end, we explored the added value of the reported high sensitivity and specificity of the domain III of the E protein (EDIII) [25–31,53]. We showed that the addition of orthoflavivirus EDIII next to the routinely used antigen NS1 increased the microarray sensitivity for TBEV IgG detection and enabled us to set a microarray titer cut-off (titer 100) that is predictive for positivity in the TBEV PRNT. While only 7 of 15 TBEV PRNT-positive sera showed reactivity against TBEV rNS1, all 15 sera were reactive with TBEV rEDIII on the microarray. Sera that were TBEV IgG positive in routine diagnostic tests but negative for TBEV rNS1 and rEDIII in the microarray could not neutralize TBEV. We cannot 100% exclude that some of the sera in the TBEV-infection cohort were taken very early in the course of the disease when the NS1 IgG responses have not yet appeared [54,55]. As TBEV-neutralizing antibody responses are mainly targeting the E protein, this might result in a positive neutralization test, a negative rNS1 array and a positive array EDIII outcome. However, TBEV infections are typically being diagnosed very late in the course of infection in the Netherlands, when more common etiology has been excluded and when NS1 IgG responses can be expected to be abundant.

Holbrook et al. described results for both the sensitive and specific detection of TBEV IgG using rEDIII. They observed that rabbit antisera raised against TBEV rEDIII protein reacted specifically with TBEV EDIII but not with rEDIII from other orthoflaviviruses such as DENV, WNV and JEV, similar to our observations with TBEV infection and vaccination sera [31] (Figures 4c and 5). A study by the same authors with WNV rEDIII showed

some cross-reactivity of rabbit anti-WNV rEDIII serum with rEDIII from viruses in the same JEV-serocomplex [32]. In line, our data show a similar low cross-reactivity of WNV serum samples with the rEDIII of the two other JEV serocomplex viruses, USUV and JEV (Figure 6e). JEV is a travel-related infection that is rarely imported to Europe, while USUV has low endemic circulation in birds in the Netherlands and human infections are rare [56–59]. It is therefore unlikely that the observed heterologous reactivity is due to past infections. When compared to the rEDIII, our results show a higher specificity of rNS1 for the detection of WNV infection. This is in line with previous observations by Natalie et al. [36]. The detected reactivity with DENV rNS1 can most probably be explained by a travel-related previous infection with DENV. After malaria, dengue is the second-most diagnosed illness in travelers [60].

Previous studies have shown that ZIKV rEDIII is a useful antigen to discriminate between ZIKV and DENV infections [39,40,61]. We, however, observed clear reactivity (but less than that for the homologous antigen–antiserum combination) of ZIKV sera with the rEDIII of the four DENV serotypes but not vice versa (Figure 6a,b). The absence of cross-reactivity between DENV sera and ZIKV rEDIII was also observed by Premkumar et al. for late convalescent sera. However, they did observe some cross-reactivity with DENV sera taken in early convalescence [41]. It is, however, difficult to discern between cross-reactivity and past infections in the observed reactivity of ZIKV sera with DENV rEDIII. Within the orthoflavivirus genus, DENV and ZIKV are phylogenetically closely related but ZIKV and DENV are also co-circulating in large parts of the world [62]. Dengue is a regularly diagnosed illness in travelers [60], and the reactivity of the sera in our ZIKV cohort with DENV rEDIII could very well reflect a previous infection in the returning traveler with an acute ZIKV infection. The presence of DENV IgG in these specific samples of our ZIKV serum cohort was also observed in the rNS1-based microarray (Figure 3d).

Previous epitope analyses of both DENV E protein and NS1 indicated that NS1 could be more useful for specific serology as NS1 contains more virus-specific epitopes [63–67]. Indeed, while the DENV serum cohort presented varying anti-rNS1 IgG titers against the four DENV serotypes, the sera reacted only with DENV1 rEDIII. To correctly evaluate the use of rNS1 versus rEDIII in the serotyping of DENV infection, a serum cohort of serotyped DENV infections will be needed.

Serological discrimination between TBEV infection and vaccination is important to diagnose and study vaccine breakthrough cases of infection [68]. Multiple studies demonstrated that the detection of NS1-specific antibodies could be a tool to distinguish TBEV-specific antibody responses elicited by infection from those elicited by vaccination based on inactivated viruses [69–72]. Nevertheless, anti-NS1 IgG responses were measured in some vaccinees with a presumed absence of history of TBEV infection [70,71] possibly due to the presence of trace amounts of NS1 protein in preparations of the two TBEV vaccines marketed in Europe [73]. We observed no rNS1 antigen responses in our cohort of vaccinees but were surprised by the absence of rNS1 reactivity in the majority (13 of 15) of our PRNT-confirmed infection sera (Figures 3h and 4a). Although this lack of sensitivity could be overcome by the addition of rEDIII to the microarray analysis (Figure 5), we cannot completely exclude that 5 sera of the 13 infection sera that did not react with rNS1 were not related to TBEV vaccination. Due to privacy regulations, we could not relate back to detailed anamnestic data for those sera. Although uncertainties with respect to the infection versus vaccination status of these five sera exist, the well-defined cohort of confirmed non-vaccinated workers in professions with high risk for tick exposure confirmed the observed higher sensitivity of rEDIII versus rNS1 in the detection of TBEV infections [45].

Our results with the confirmed non-vaccinated high-risk tick exposure study cohort and the combined rNS1/rEDIII microarray are consistent with earlier analyses on this cohort [45]. The sera of three workers that were positive for TBEV infection in the routine ELISA and in the PRNT in the study showed anti-rEDIII IgG reactivity but no anti-rNS1 reactivity on our microarray. Besides the observations in some studies that the currently employed TBEV vaccines might elicit low-level anti-NS1 immune responses and thereby

hamper a serology-based distinction between vaccine- and infection-related induced immunity, the data here demonstrate that the absence of NS1-induced immune responses in combination with the presence of EDIII-induced responses does not identify vaccination cases with 100% certainty.

To be more conclusive in discriminating vaccine-induced and infection-induced immune responses, a better understanding of the kinetics of those immune responses in vaccination and infection cohorts with different clinical courses is needed for a more precise interpretation of the data. In conclusion, we showed that for orthoflavivirus IgG serology, with a focus on TBEV, a combined analysis of rEDIII- and rNS1-induced immunity has the potential to provide sensitive and specific data.

Author Contributions: Conceptualization, C.V., J.R., D.E. and C.R.; methodology, C.V., S.S., G.-J.G., and M.N.H.; validation, C.V., J.R., D.E. and C.R.; formal analysis, C.V. and C.R.; investigation, C.V., D.E. and C.R.; data curation, C.V., J.R. and C.R.; writing—original draft, C.V. and C.R.; supervision, D.E. and C.R.; project administration, J.R., D.E. and C.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The protocol for the cross-sectional serological survey among nature management organizations in the Netherlands as described in [45] was approved by the medical ethics committee of the University Medical Center in Utrecht, the Netherlands, permit number 16-767/D.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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