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Development of enzyme-linked immunosorbent test for the detection of tick-borne encephalitis virus-specific antibody responses

Advanced studies' thesis

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Tick-borne encephalitis virus (TBEV) infection can lead to a severe disease called tick-borne encephalitis (TBE). Diagnosis is established by measuring TBEV specific IgM antibodies usually from blood serum using enzyme-linked immunosorbent assays (ELISA). Effective vaccines against TBEV are available in Europe. In rare cases a TBEV-infection can lead to a disease also in vaccinated individuals. This is called vaccination breakthrough (VBT) infection. The vaccines contain only structural TBEV proteins, and antibodies against nonstructural proteins (e.g. NS1) form only after TBEV infection. Therefore, vaccinees, infected individuals and VBTs can in principle be separated by their antibody response.

In this project, TBEV envelope (E) and non-structural (NS1) proteins were produced as glutathione S-transferase (GST) fusion proteins in insect cells (Sf-9). Protein production in Sf-9 cells was confirmed with western blot. Protein purification was performed using GST affinity column. The E protein was additionally produced in mammalian HEK293 cells with His-tag and purified with nickel-nitrilotriacetic (Ni-NTA) column. The purified proteins were utilized to set up the ELISA test using a panel of serum samples from TBEV infected and vaccinated individuals. Detection of E and NS1 specific antibodies was tested also using immunoblot strip assay.

E and NS1 proteins were expressed well in insect cells, however, the purification of NS1 was unsuccessful. The use of E proteins produced in insect cells (E-GST) and HEK293 cells (E-His) as antigens in ELISA was analyzed. The IgG ELISA parameters were successfully optimized with E-His protein, but IgM antibody levels were constantly low. In immunoblot strip assay, one infected serum sample contained antibodies that recognized the linearized E-GST protein.

In this study, a TBEV E-protein specific IgG ELISA test was developed. In the future, E-His ELISA test will be optimized for IgM antibody measurement. Because NS1 protein is important in separating the infected and vaccinated individuals, the challenges in NS1 protein production need to be resolved.

Keywords: tick-borne encephalitis virus, ELISA, serology, vaccine

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Introduction

Tick-borne encephalitis virus (TBEV) is a significant human pathogen that is transmitted primarily by ticks belonging to the *Ixodidae* family. TBEV is endemic in a wide region extending from Northern and Central Europe to Asia. The virus has three main subtypes: European (TBEV-Eu), Siberian (TBEV-Sib) and Far-Eastern (TBEV-FE).[1] *Ixodes ricinus* is the primary vector in Europe and *Ixodes persulcatus* in Russia, Far East Asia and certain regions of Eastern Europe. Approximately 0.1-5.0% of *Ixodes ricinus* ticks carry the virus. For *Ixodes persulcatus* ticks, the percentage of TBEV-positivity can be up to 40%.[2]

TBEV is a member of the genus *Flavivirus* that belongs to the family *Flaviviridae* [3]. Other flaviviruses include for example yellow fever, dengue and Zika viruses [4]. TBEV has a structure similar to other flaviviruses (Figure 1).

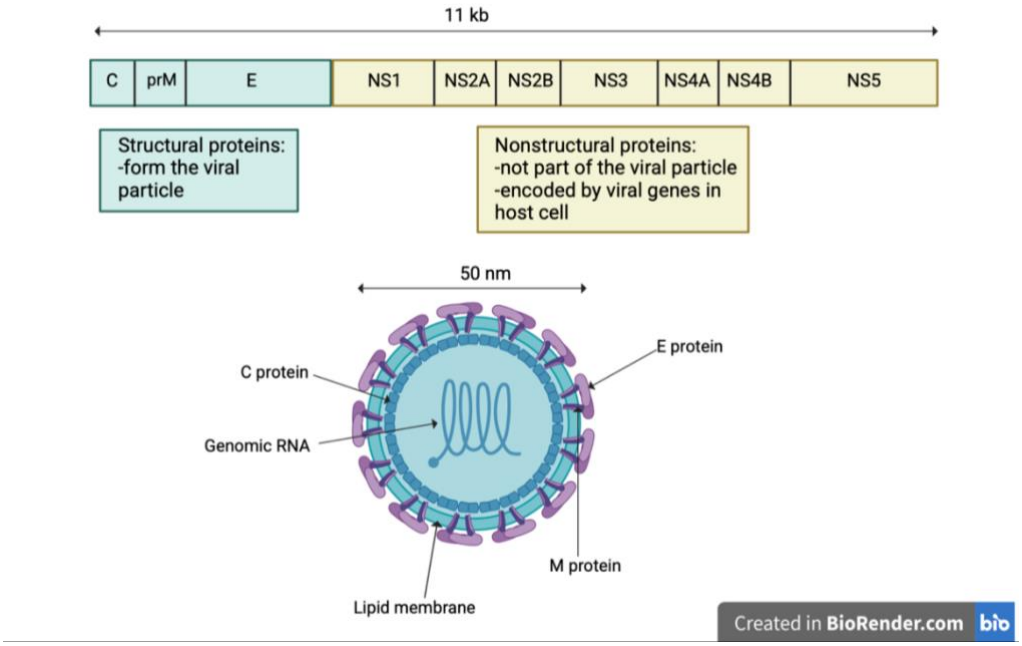

Figure 1. TBE virus structure. *TBEV has a positive-strand RNA genome that is approximately 11 kb long. The genome encodes a single polypeptide molecule of ~3400 amino acids. Host and viral proteases cleave the polypeptide at specific sites into three structural (C, E, M), and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5).* [1,3,5]*. The structural proteins construct the viral particle. Nonstructural proteins are not part of the viral particle, but are formed inside the host cell, when the virus replicates. TBEV virion is smooth and spherical and measures ~50 nm in diameter* [6,7]*. Image created based on article figures by Worku and Pustinjanac et al.* [1,8]

TBEV infections are usually asymptomatic, but infection can cause tick-borne encephalitis (TBE), a disease with central nervous system infection. CNS involvement can represent as meningitis, meningoencephalitis and meningoencephalomyelitis. [1,9] The subtype of TBEV affects the clinical characteristics and mortality of infection. The estimated mortality of TBEV-Eu is 1-2% and TBEV-Sib 6-8%. TBEV-FE has the highest mortality rate, 30%. [10] The incidence of TBE has risen in the past decades [11–13]. In Finland, reported TBE cases have steadily increased from the early 2000s (15-45 cases per year), and in 2024, up to 200 diagnosed cases were reported. [14–16]

No specific antiviral therapy exists for TBE but there are effective vaccines available. The vaccines available in Europe are Ticovac/FSME-IMMUN (Pfizer) based on the Neuroerfl strain of TBEV and Encepur (Bavarian Nordic) based on the Karlsruhe strain (K23). The vaccines contain inactivated TBE virus. [17,18] Despite the effective vaccines, vaccination breakthrough infections (VBT), i.e. infections in vaccinated individuals, have been detected. Breakthrough infections are reported especially in older age groups and in patients with comorbidities. [19–21]

TBE is diagnosed by measuring TBEV specific antibodies from blood serum and occasionally from cerebrospinal fluid using enzyme linked immunosorbent assays (ELISA). IgM antibodies are a sign of a recent infection. IgM antibody levels stay high for weeks to months after infection. IgG antibodies develop slower than IgM antibodies and stay elevated for the rest of the patient's life presumably mediating protective immunity. Individuals vaccinated against TBEV have high IgG antibody levels for over 5 years after vaccination, but booster vaccines are needed every 3-5 years to enhance immunity. [10,22,23]

The envelope (E) protein is the main target of neutralizing antibodies against the virus [5]. Antibodies against E protein form after infection and vaccination since the protein is present in virus [24]. As for antibodies against non-structural protein NS1, it has been suggested that NS1 specific antibody formation is induced especially after natural infection, since the vaccines contain no or very low amounts of NS1 protein. Therefore, NS1 protein could be used to differentiate infected from vaccinated individuals.[25]

The aim of the project was to set up an ELISA test for TBE serology that could differentiate infected individuals from vaccinees. As part of it, viral antigens (E and NS1 proteins) were produced as glutathione S-transferase (GST) fusion proteins in a baculovirus system in insect cells [26]. GST-purification system was used to purify the virus proteins [27]. TBEV E protein was additionally produced in mammalian cells. To adjust the performance of the test, antigen

concentration, serum dilution, and the concentration of the secondary antibody were optimized in ELISA.

Material and methods

Serum samples

To optimize ELISA conditions (antigen concentration, serum dilution, conjugate dilution), test samples were collected from TBEV-infected (3 anonymized patient samples from Clinical Microbiology, Tyks Laboratories) and TBEV-vaccinated individuals (2 anonymized samples collected for occupational safety assessment post vaccination). Samples from unvaccinated and uninfected individuals were used as negative controls (2 anonymized samples collected for occupational safety assessment pre-vaccination). The pre-vaccination samples are later referred to as negative controls. The same serum samples were also used in the immunoblot strip assay. Patient samples were initially obtained for diagnostic purposes, and the vaccinees' samples for occupational safety assessment, hence no ethical committee approval or clinical study permissions were required for the sample collection.

Infusion cloning and bacterial transformation

The genes encoding European strain NS1, Siberian strain NS1 and European strain E proteins were cloned into pBVboost plasmid (a plasmid for transferring an insert gene to baculovirus system) with GST at the N-terminal side [26]. The NS1 genes were full-length and the E gene was shortened (1-413 aa of 496 aa). European strain genes were in accordance with the sequence of TBEV virus strain Kumlinge A52 polyprotein (GeneBank accession number: GU183380.1), and the Siberian strain gene with the sequence of TBEV virus strain Latvia-1-96 polyprotein (GeneBank accession number: GU183382.1). European and Siberian strain NS1 genes were synthetized and cloned to the pBVboost vector by GeneUniversal, and the European E-gene was subcloned in house (see below).

Primers for the gene encoding E protein (forward: 5'-GGTTCCGCGT GGATCC AGCAGATGTACCCAC-3', reverse: 5'-ACGATGAATTCCCGG GGATCC CAGCCTCTCGATGCC-3,) were designed according to Infusion HD Cloning Kit instructions (Takara Bio). European TBEV prME in pUC57-Simple vector (GeneUniversal) was used as a template to amplify the shortened E gene using CloneAmp HiFi PCR Premix included in the

Infusion HD Cloning Kit. Target inserts were isolated by agarose gel electrophoresis and excised from gel. pBVboost -N-GST vector was linearized with FastDigest BamHI enzyme (Thermo Scientific) and dephosphorylation with FastAP thermosensitive alkaline phosphatase (Thermo Scientific) was done simultaneously to prevent recircularization of the plasmid. Target inserts and the linearized vector were purified with Nucleospin Gel and PCR Clean-up kit (Macherey-Nagel)

Infusion cloning procedure for spin-column purified PCR fragments and transformation procedure were done according to the Infusion HD Cloning Kit instructions. The transformation reaction was spread on a LB plate containing the appropriate antibiotic, for pBVboost N-GST plasmid gentamicin 10 µg/ml. The plates were incubated overnight at 37°C. The next day, bacteria colonies were picked from plates. Colonies were placed in tubes containing LB medium supplemented with 7 µg/ml gentamicin. Tubes were incubated with shaking at 220 rpm for 18-20 hours at 37°C. The following day, plasmid DNA was isolated from the bacteria with GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific)

Bacterial transformation was done similarly for European strain NS1 and Siberian strain NS1 in pBVboost-N-GST. Plasmid DNA was isolated with GeneJET Plasmid Miniprep Kit.

Recombinant baculovirus generation and protein production in insect cells

Recombinant baculoviruses were generated according to the protocol written by Airenne et al.[26] Briefly, the pBVboost plasmid containing the TBEV gene was transferred to baculoviral genome in DH10BacΔTn7 E. coli cells. The transfer was done in prechilled cuvettes (Gene Pulser Cuvette, E.coli, 0.2 cm) by electroporator (GenePulser Xcell, Bio-Rad). SOC medium was added for baculovirus preparation, and the cells were incubated with shaking at 250 rpm for 4 hours at 37°C. The samples were placed on LB agar plates containing 10% sucrose, 10 µg/ml tetracycline and 7 µg/ml gentamicin. Isolation of bacmid DNA was done according to the protocol with GeneJET Plasmid Miniprep Kit.

Spodoptera frugiperda (Sf-9) cells were seeded on 6-well plates (1,0 x 10^6 cells per well) in serum-free insect cell growth medium (Sf-900 II SFM, Gibco). Recombinant bacmid DNA was transfected to cells with Cellfectin transfection reagent (Invitrogen). Cells were incubated with transfection mixture for 4 to 5 hours. Transfection mixture was removed, and 2 ml of insect cell growth medium with 5% inactivated FBS and 10 µg/ml gentamicin, was added to cells. Cells were incubated for 3-4 days at 28°C. After the incubation, the supernatant from the transfected cells was

centrifuged at 500 g and the cleared virus-containing supernatant (p1 virus stock) was stored at +4°C.

T175 TC flasks (Sarstedt AG & Co.KG) of Sf-9 cells were infected with 500 µl p1 virus stock and cells were incubated for 4 days at +28°C. After the incubation, medium was collected and centrifuged at 1000 g. Supernatant (p2 virus stock) was stored at +4°C. For protein purification, T175 TC flasks of Sf-9 cells were infected with 2 or 4 ml of p2 virus stock.

Western blots to confirm protein production

Protein production in Sf-9 cells was confirmed with western blot. After primary viral stock had been collected, Sf-9 cells were washed with PBS and lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP40 (Sigma), 1 x cOmplete protease inhibitor (Roche)) was added to cells. 5X SDS sample buffer was added to the lysates, the mixtures were boiled for 5 min and the samples were loaded to sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels (Mini-PROTEAN TGX Precast gels, Bio-Rad Laboratories). The gels were run for 1 h at 180 V in 1x SDS running buffer (25 mM Tris, 192 mM glycine, 0,1% (w/v) SDS, pH 8.3).

Transfer to Amersham Protran nitrocellulose blotting membrane (Cytiva) was done with Trans-Blot SD Semidry transfer cell (Bio-Rad) at 15 V for 20 min. Membrane was incubated in blocking solution (5% milk in Tris-buffered saline with Tween 20 detergent (TBST, 0.1% Tween 20)) for 1 hour at RT. Membrane was washed with TBST and primary antibody, serum from rabbit immunized for GST (in house from THL), was diluted 1:10 000 to blocking solution and added to the membrane. Membrane was incubated with primary antibody overnight on spinner rack at +4°C. The next day, membrane was washed thrice in TBST and secondary antibody, goat-anti rabbit (IRDye 800 CW, LI-COR), 1:15 000 dilution in blocking solution was added to the membrane. Membrane was incubated for 1 hour in the dark on spinner rack. After TBST washes, membrane was imaged with Odyssey XF imager (LI-COR, Biosciences UK, Ltd).

Protein purification with GST affinity column

Sf-9 cells were collected from T175 flasks to the growth medium and transferred to falcon tubes that were centrifuged 7 minutes at 800 g. Some of the supernatant (p3 stock) was saved and rest discarded. The cell pellets were resuspended in PBS and centrifuged 7 minutes at 800 g. The pellets were resuspended into ice cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1xcOmplete protease inhibitor), 20 mL for 5xT175. Lysis reaction was incubated for 1 hour on ice. The lysate was centrifuged 30 minutes at 2800 g to remove the debris.

The GST affinity column was washed with 70% ethanol and 1xPBS to purify the column. Glutathione Sepharose 4B (GE Healthcare) was added to the column and washed with1x PBS. PBS was added to Glutathione Sepharose to equilibrate it. The immobilized glutathione, which is the substrate of GST, binds GST-tagged proteins in the column.

The supernatant with the dissolved GST-fusion protein was poured into the column. The supernatant was incubated in column for 1 hour by turning the column from time to time and then let to flow through the column. Column was washed with PBS to remove the components not bound to the column. Glutathione-bound GST-tagged protein was eluted from the column with 500 µl elution buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 20 mM glutathione (Profinia Glutathione Pack, BIO-RAD)). The glutathione in the elution buffer detaches GST-tagged protein from the immobilized glutathione and elutes it from the affinity column. Elution buffer was incubated on glutathione beads for 5-10 minutes before collection. The elution step was repeated four times to gain 4 x 500 µl eluates.

Samples were collected throughout the purification and ran on SDS-PAGE gel and the proteins on the gel were visualized with PageBlue Protein Staining solution (Thermo Scientific) to estimate protein concentration and purity. Protein purification eluates were pooled and buffer was exchanged into PBS using Vivaspin 2 (Sartorius) with 30 000 MWCO PES membrane. Protein concentration was measured with Pierce BCA Protein Assay Kit (Thermo scientific).

Protein production in HEK293 cells

The E protein was additionally produced with a C-terminal polyhistidine tail in human embryonic kidney (HEK293) cells as described earlier [28]. The gene encoding E protein was amplified using the European TBEV prME in pUC57-Simple vector (GeneUniversal). The sequence encoding prM protein was included in the TBEV-E-His construct, because prM facilitates the correct folding of E protein. E-His recombinant protein was purified with Protino Ni-NTA column (Macherey-Nagel) at +4°C. Ni-NTA complexes bind to His-tags with high affinity. Increasing imidazole concentrations were used to elute the protein. Imidazole binds to Ni-NTA and replaces its interactions with His-tag of recombinant protein.

Enzyme immunoassay

96-well microtiter plates (Nunc Maxisorp, Thermo Fisher Scientific) were coated with TBEV NS1 and E antigens diluted in PBS (1, 2, 4, 6, and 8 µg/ml). Antigen plates were washed twice with washing buffer (0.05% Tween-20 in PBS) with Versa well washer (Thermo Fisher Scientific). 50 µl

of sample buffer (5% swine serum and 0.1% Tween-20 in PBS) was added per well. Serum samples were diluted in Megablock 96-well plates (Sarsted) to sample buffer. 50 µl of each sample was added per well. Final dilution of serum sample was 1:100 or 1:300. Dilution series of serum samples were also analyzed. Plates were incubated in +37°C for 2 hours. Plates were washed thrice with washing buffer. Horseradish peroxidase-(HRP) labeled polyclonal rabbit anti-human IgM and polyclonal rabbit anti-human IgG (Dako) were diluted into sample buffer. IgG was diluted 1:4000, 1:6000 or 1:8000 and IgM 1:2000 or 1:4000. 100 µl of conjugate dilution was added per well. Plates were incubated at +37°C for 1 hour. Plates were washed thrice with washing buffer. 100 µl of substrate solution (3, 3', 5, 5' -tetramethylbenzidine (TMB)) (Kementec Solutions) was added per well and incubated 20 minutes in dark at room temperature. 100 µl stop solution (0.1 M H_2SO_4) was added after incubation. The absorbance i.e. optical density (OD) was measured with Victor Nivo multimode plate reader (PerkinElmer) at 450 nm. Signal to background (SG/BG) values were counted by dividing values by the corresponding value of the neg2 sample.

Immunoblot strip assay

The European strain E and Siberian strain NS1 proteins that were produced in insect cells were used in the immunoblot strip assay. Before protein purification the cells infected with p2 viral stock were collected and pelleted. The pellets were resuspended in lysis buffer. After lysis, the mixture was centrifuged. The supernatant was used in protein purification. Remaining pellets of European strain E and Siberian strain NS1 (NS1 pellet was also treated with 6 M guanidine-HCl) were resuspended in PBS.

The resuspended pellets were mixed with 5X SDS sample buffer and boiled for 5 minutes. 300 µl of each sample was run on Criterion TGX precast gel (IPG + 1 well comb, Bio-Rad) at 180 V for 45 minutes in 1x SDS running buffer. Transfer to membranes was done with Trans-Blot SD Semidry transfer cell at 15 V for 30 min. The membranes were blocked in 5% milk in TBST for 1 hour in room temperature and washed with TBST. The membranes were cut to 4 mm strips and each strip was placed in separate container. TBST was added to them for storing. The strips were kept in +4°C until they were used.

NS1 and E strips were labeled with 1:100 dilution of the serum samples in blocking solution. The serum sample was incubated for 1 hour on mixer at +4°C. The secondary antibody was goat anti-human IgG (IRDye 800 CW, LI-COR), used in 1:15 000 dilution in blocking solution. The secondary antibody was incubated for 1 hour on mixer in room temperature protected from light. After TBST washes, strips were imaged with Odyssey XF imager (LI-COR, Biosciences UK, Ltd)

Detection of GST was used as control. The primary antibody was serum from rabbit immunized for GST (in house from THL) in 1:1000 dilution in blocking solution and secondary antibody was goat anti-rabbit (IRDye 800 CW, LI-COR), used in 1:15 000 dilution in blocking solution.

Results

TBEV protein production and purification

Sf-9 cells were transfected with recombinant bacmid DNA containing TBEV genes. The baculovirus infects cells resulting in a production of the protein of interest, i.e. E- and NS1 proteins of TBEV. Baculovirus formation and TBEV protein expression after Sf-9 cell transfection was confirmed with western blot. The molecular weight of TBEV-NS1-GST is 73-82 kDa and TBEV-E-GST 77-79 kDa. Both proteins were readily expressed in the Sf-9 cells (Figure 2). The gene encoding E protein was shortened by 83 amino acids, which explains the smaller molecular weight of E protein in the immunoblot. The GST tag that was used was also a bit smaller than the usual 26 kDa.

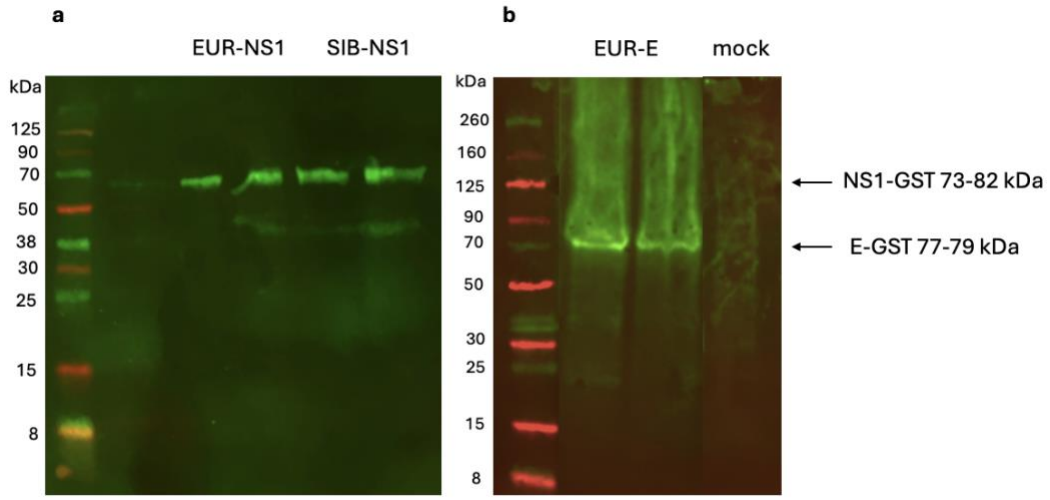

Figure 2. Immunoblot of European and Siberian TBEV NS1-GST proteins and European TBEV E-GST protein. *Insect cells were transfected with TBEV NS1-GST (a) and TBEV E-GST (b) recombinant bacmid DNA. Cells were lysed 4 days after transfection and lysates were run on SDS-PAGE gel in duplicates. Primary antibody was serum from rabbits immunized for GST (in house from THL, 1:1000) and secondary antibody goat-anti-rabbit IgG (IRDye 800 CW, LI-COR, 1:15 000). Arrows indicate the proteins of interest.*

Sf-9 cells were infected with p2 baculovirus stocks for mass production and protein purification. The infection was incubated for 5-6 days after which cells were collected to lysis buffer.

After the GST affinity column purification, the samples of pre- and post-purification were analyzed on SDS-PAGE. As shown in Figure 3a, NS1 remained in the pellet after purification (red arrow). To dissolve the pellet-bound NS1 protein, the NS1 lysate was treated with 8 M UREA, 6 M guanidine-HCl or 5% Ethylene glycol, and the pre- and post-purification samples were analyzed with SDS-PAGE. However, regardless of these strong dissolving treatments NS1 still remained in the pellet (Figure 4). On contrary to NS1 protein, E protein was successfully eluted from the affinity column, however its amount remained small in both purifications (Figure 3b, 3c, red arrows). To maximize the protein amount, E protein eluates from the two purifications were pooled. Protein concentration was measured with Pierce BCA Protein Assay Kit and it was approximately 375 µg/ml. The purified E-GST protein was subsequently used in ELISA.

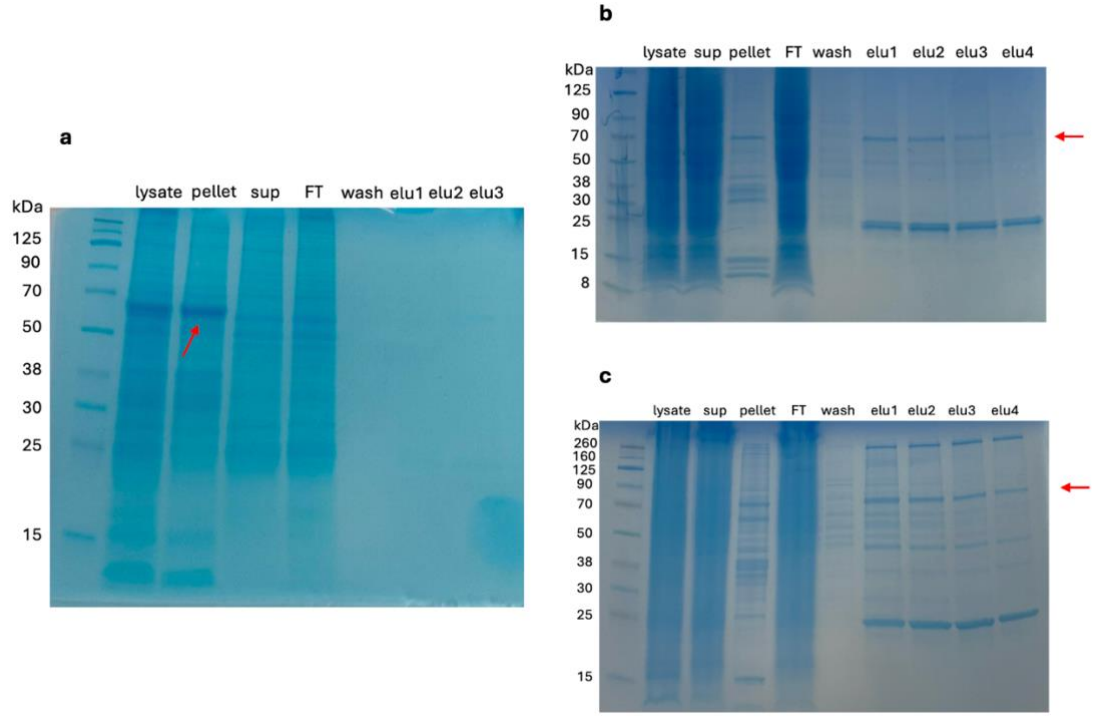

Figure 3. Affinity column purified Siberian TBEV NS1-GST and European TBEV E-GST. *Sf-9 cells were infected with p2 baculovirus stocks, incubated for 5-6 days, collected to lysis buffer and produced recombinant GST-tagged proteins were bound to 1 ml of glutathione beads. a. Five T175 flasks of Sf-9 cells were infected with 2 mL p2 TBEV NS1-GST baculovirus stock per T175. The infection was incubated for 6 days. The molecular weight of NS1-GST is 73-82 kDa. b. Two T175 flasks of Sf-9 cells were infected with 2 mL p2 TBEV-E-GST baculovirus stock per T175. The infection was incubated for 5 days. The molecular weight of E-GST is 77-79 kDa, c. Five T175 flasks of Sf-9 cells were infected with 4 mL p2 TBEV-E-GST baculovirus stock per T175. The infection was incubated for 5 days. sup = supernatant, FT = flowthrough, elu = eluate*

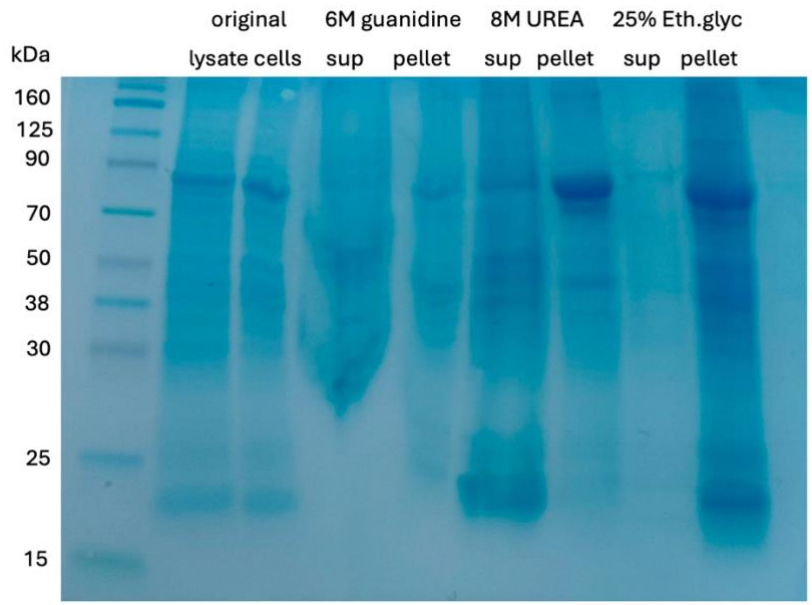

Figure 4. Dissolving TBEV SIB-NS1-GST from pellet. *The pellet of lysis of TBEV-SIB-NS1-GST production was split to three aliquots. The aliquots were treated with 1 ml of 8M UREA, 6M guanidine-HCl or 25% Ethylene glycol for 1h at RT. Supernatant and pellet were separated for each lysis product with 10 min 15000 g centrifugation. sup=supernatant, eth.glyc= ethylene glycol.*

Immunoblot strip assay

The purpose of the immunoblot strip assay was to examine whether TBEV vaccination or infection-induced antibodies in human serum samples recognized the proteins produced in insect cells, especially NS1 that could not be purified. NS1 and E strips were labeled with the serum samples mentioned earlier. One of the serum samples (inf2) recognized the linearized E protein (Figure 5), however, none of the sera recognized linearized NS1 protein. Buffer was used as a negative control for the staining, and anti-GST antibody was used as a positive control to ensure that the correct sized protein was detectable on the strip. The samples were analyzed twice to confirm the results.

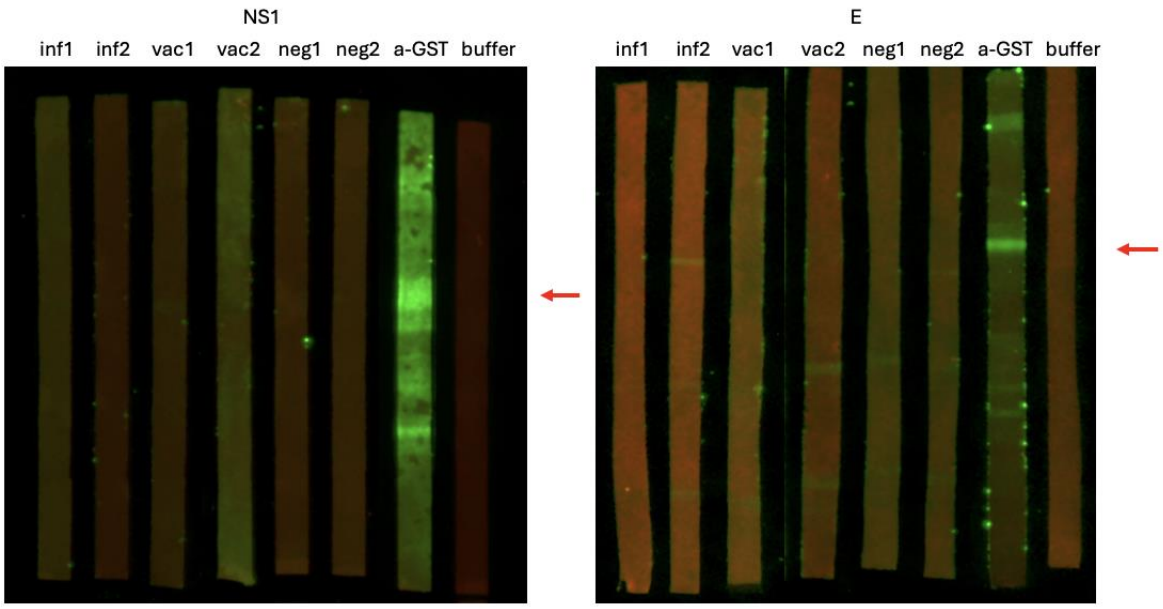

Figure 5. Detection of antibodies binding to linearized TBEV NS1 and E. *Immunoblot strip assay was used to detect IgG antibodies capable to bind linearized TBEV NS1-GST and E-GST in the 1:100 diluted serum samples. Anti-GST antibody served as the positive control. The size of the positive band is indicated with an arrow.*

Detection of anti-envelope antibodies in serum samples with ELISA

ELISA method is more convenient than the strip assay for the detection of antibodies from serum samples. To test if the purified E-proteins could be used as antigens for the detection of E-specific antibodies in serum samples, an ELISA assay was set up. First, to assess the optimal antigen concentration the TBEV-E antigen in three concentrations (1, 2 and 4 µg/ml) was coated onto wells. Human serum IgG and IgM antibodies bound to E-proteins were detected with anti-human IgG HRP conjugate (1:8000) or anti-human IgM HRP conjugate (1:4000). These conditions were tested with the E protein produced in insect cells (TBEV E-GST) and in HEK293 cells (TBEV E-his) (Figure 6). For TBEV E-GST ELISA serum samples were diluted 1:300 and for TBEV E-His ELISA 1:100 and 1:300.

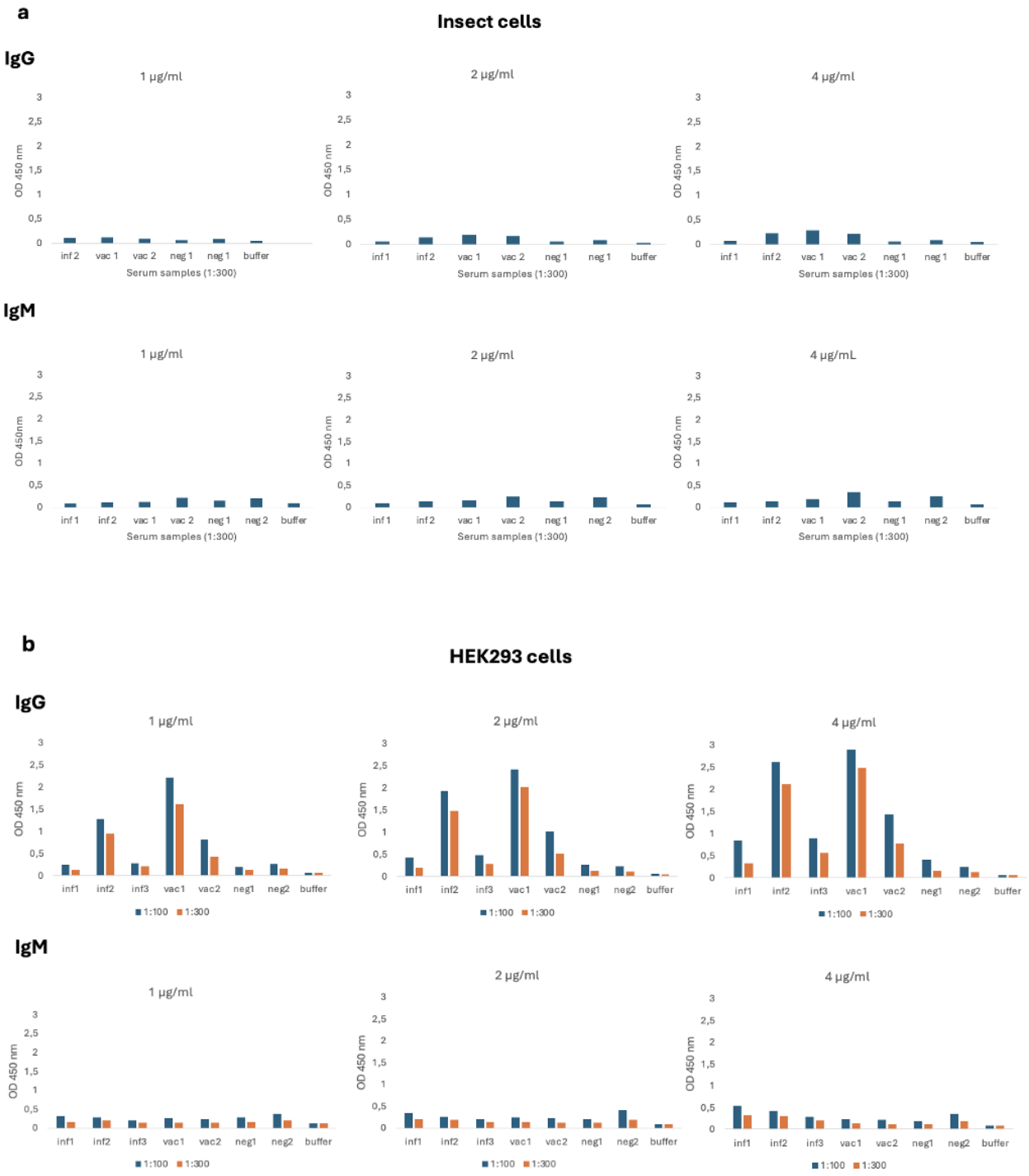

Figure 6. ELISA with TBEV-E-GST produced in insect cells (a) and TBEV-E-His produced in HEK293 cells (b). *TBEV E protein was coated in 1 µg/ml, 2 µg/ml and 4 µg/ml concentrations. Anti-human IgG HRP conjugate was used in 1:8000 dilution and anti-human IgM HRP conjugate in 1:4000 dilution. a. Serum samples were diluted 1:300. b. Serum samples were diluted 1:100 and 1:300. Serum samples from three TBEV-infected patients (inf1, inf2, inf3) and two vaccinees (vac1, vac2) were used in the assay. Two negative serum samples from uninfected and unvaccinated individuals (neg1, neg2) and the dilution buffer were used as negative controls.*

The OD values in ELISA were very low with TBEV-E-GST for both IgG and IgM; all values were under 0.5 (Figure 6a). When the GST background was deducted, the values were even lower (Figure 7). However, the OD values for IgG were higher when TBEV E-His was used as an antigen (Figure 6b). IgM values were very low, but IgG values reached appropriate levels with the settings used. OD values above 1 were reached already with 1 µg/ml antigen concentration, and the OD values rose as the antigen concentration was increased. The best OD values for IgG were reached with 4 µg/ml concentration of E-antigen. On contrary to GST, the His-tag background was not deducted from these results, since it has previously been shown to yield low OD values [28].

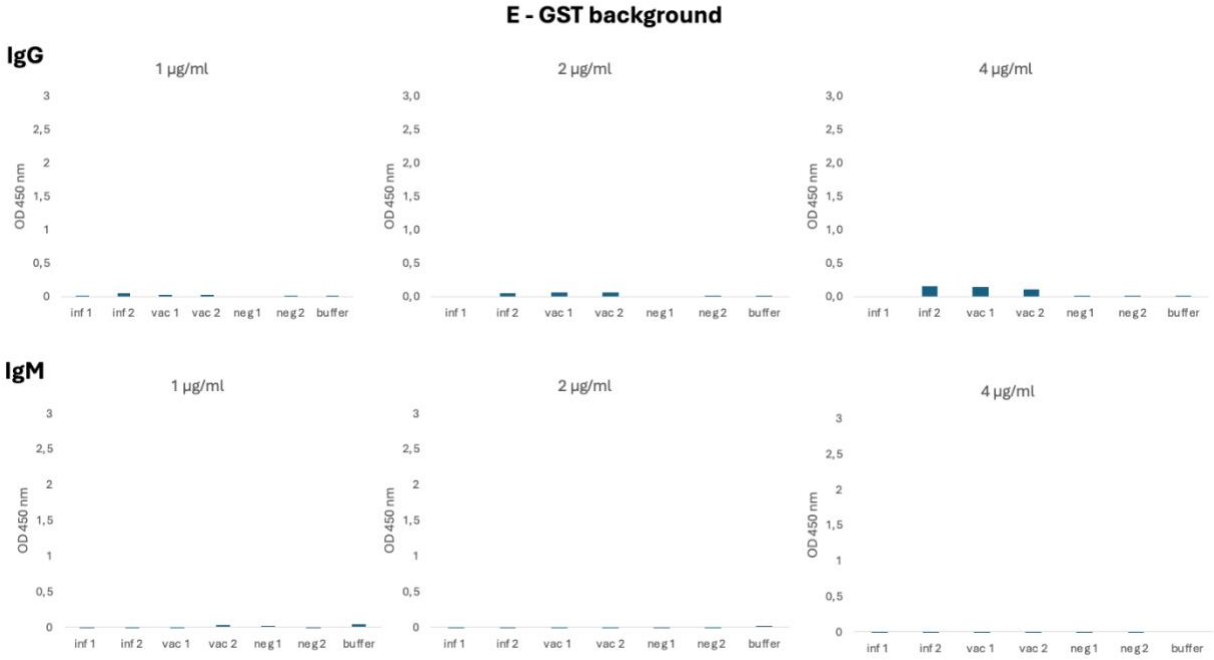

Figure 7. ***TBEV-E-GST ELISA results with GST background deducted,*** *TBEV E protein and GST were coated in 1 µg/ml, 2 µg/ml and 4 µg/ml concentrations. Anti-human IgG HRP conjugate was used in 1:8000 dilution and anti-human IgM HRP conjugate in 1:4000 dilution. Serums were diluted 1:300. GST OD values were deducted from E-GST OD values to remove the background. Serum samples from two TBEV-infected patients (inf1, inf2) and two vaccinees (vac1, vac2) were used in the assay. Two negative serum samples from uninfected and unvaccinated individuals (neg1, neg2) and the dilution buffer were used as negative controls.*

Serum gives always background in ELISA, and the serum dilution has to be optimized for each antigen. Calculation of signal to background (SG/BG) ratios is one method to estimate the used settings. For TBEV E-His 1:300 dilution of serum samples was better than 1:100, because with the higher dilution the background values, i.e. the values of negative samples, also begun to rise (Figure 8). The highest SG/BG ratios were obtained with 4 µg/ml antigen coating concentration and 1:300 serum dilution.

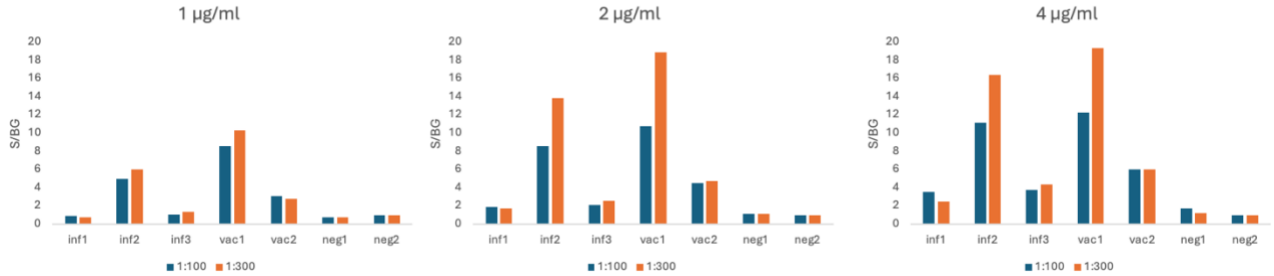

Figure 8. SG/BG ratios for TBEV-E-His produced in HEK293 cells. *All values were divided by the corresponding values of neg2 sample, representing background. Serum samples from three TBEV-infected patients (inf1, inf2, inf3) and two vaccinees (vac1, vac2) were used in the assay. Two negative serum samples from uninfected and unvaccinated individuals (neg1, neg2) and the dilution buffer were used as negative controls.*

To examine if further optimization of the TBEV E-GST antigen coating concentrations would result in higher OD values, TBEV E-GST was used in 4, 6 and 8 µg/ml concentrations. Serum samples were diluted 1:100 and 1:300. For the optimal detection of human serum IgG and IgM antibodies bound to E-proteins anti-human IgG HRP conjugate dilutions 1:4000 and 1:6000 and anti-human IgM HRP conjugate dilutions 1:2000 and 1:4000 were tested (Figure 9).

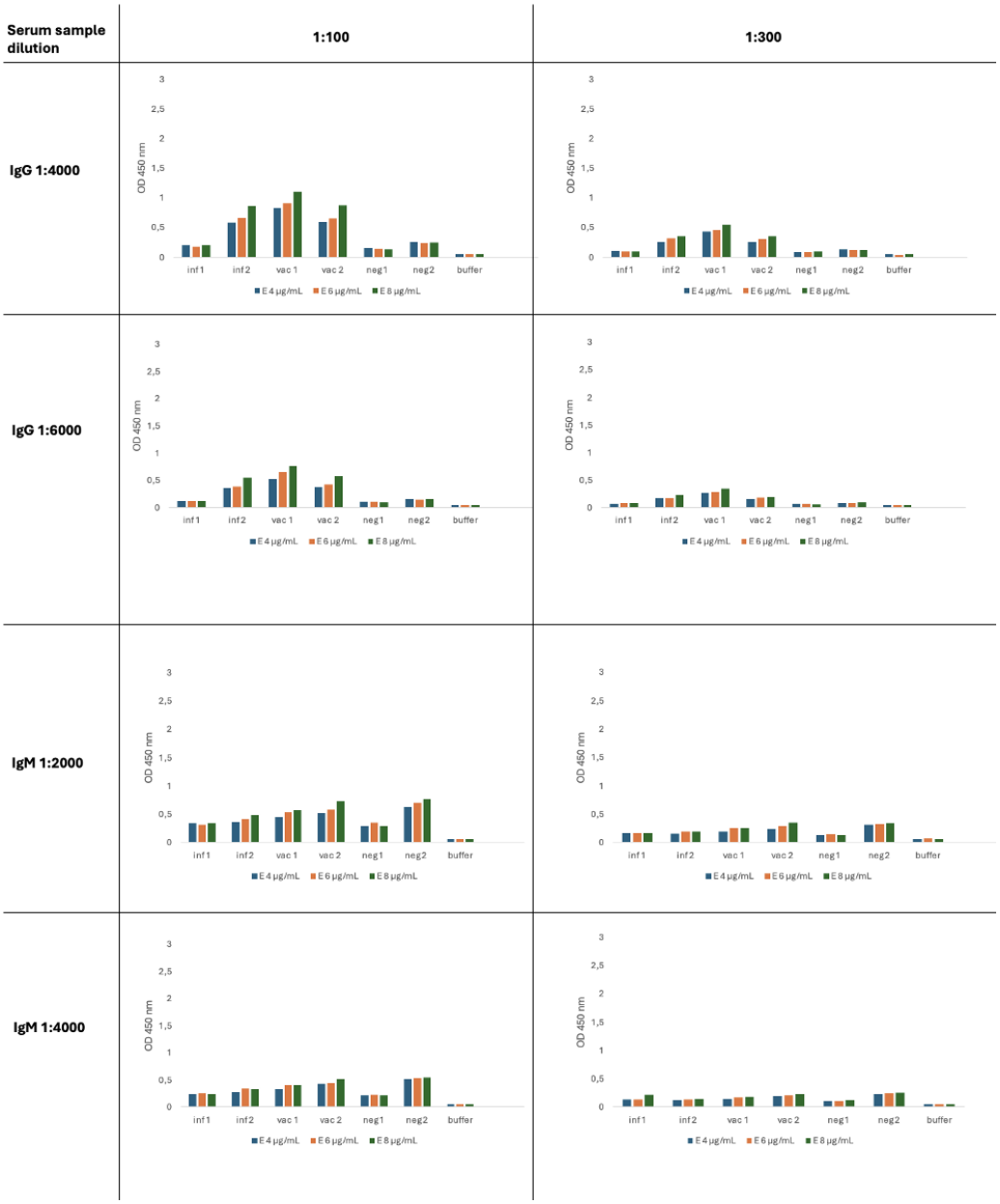

Figure 9. Optimization of TBEV-E-GST ELISA with different antigen concentrations and conjugate dilutions. *TBEV E protein was coated in 4 µg/ml, 6 µg/ml and 8 µg/ml concentrations. Serum dilutions 1:100 and 1:300 were used. Anti-human IgG HRP conjugate was diluted 1:4000 or 1:6000 and anti-human IgM HRP conjugate 1:2000 or 1:4000. Serum samples from two TBEV-infected patients (inf1, inf2) and two vaccinees (vac1, vac2) were used in the assay. Two negative serum samples from uninfected and unvaccinated individual (neg1, neg2) and the dilution buffer were used as negative controls.*

Compared to Figure 6a and TBEV-E-GST antigen concentrations up to 2 µg/ml, the OD values rose slightly with higher, up to 6 µg/ml antigen concentrations. As with the mammalian cell produced TBEV-E-His, the insect cell produced of TBEV-E-GST protein worked as TBEV-specific antigen in ELISA; highest responses were seen in TBEV infected and vaccinated individuals and increasing the antigen concentration elevated the OD values. However, the OD values remained

constantly low compared to TBEV-E-His and only with very high TBEV-E-GST antigen concentration (8 µg/ml) OD values above 1 were reached. GST background was not subtracted from these OD values.

In addition to antigen concentration and serum dilution, the HRP-conjugate dilution has effect on the performance of ELISA. Based on the TBEV-E-His SG/BG values for IgG, 1:300 serum dilution and 4 µg/ml antigen concentration were selected to the next optimization step to determine the best conjugate dilution. In addition, a longer incubation time for the substrate of HRP, TMB, was tried to see if it would yield higher OD values. Anti-human IgG HRP conjugate was tested at 1:4000, 1:6000 and 1:8000 dilutions and 20 min and 30 min incubation times for TMB were compared (Figure 10).

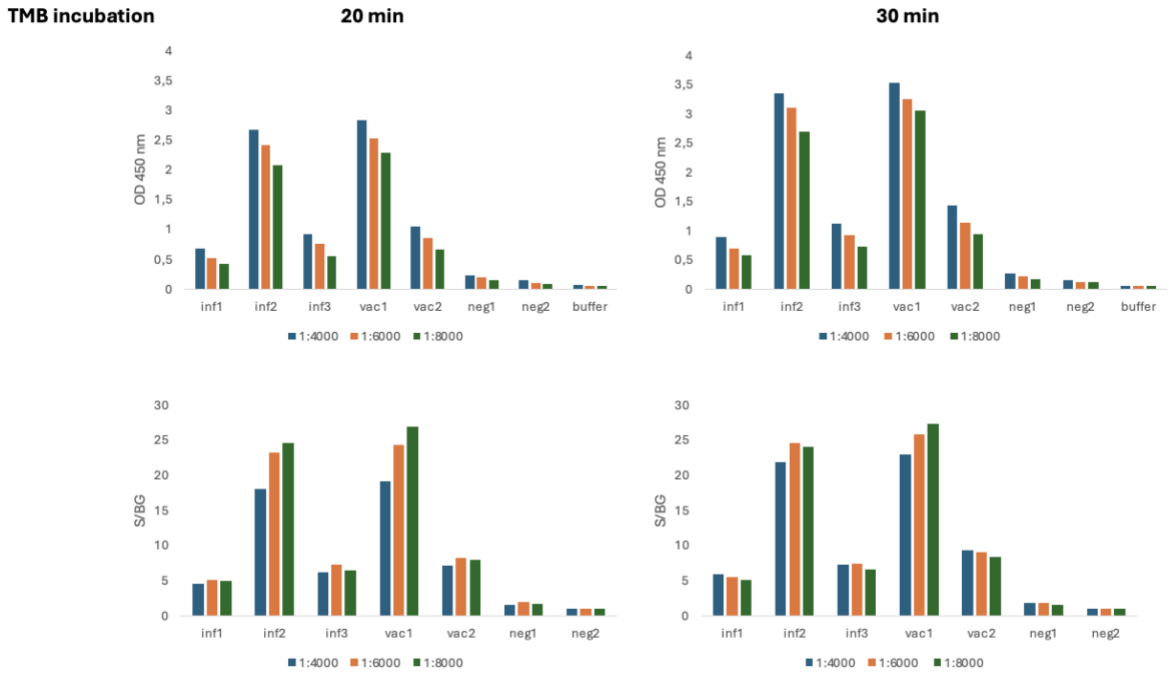

Figure 10. ELISA with TBEV-E-His, different conjugate dilutions and substrate incubation time. *TBEV E antigen concentration was 4 µg/ml. Anti-human IgG-HRP conjugate dilutions were 1:4000, 1:6000 and 1:8000. Substrate (TMB) incubation time was either 20 or 30 minutes. To calculate SG/BG values (bottom figures), all values were divided by the corresponding values of neg2 sample. Serum samples from three TBEV-infected patients (inf1, inf2, inf3) and two vaccinees (vac1, vac2) were used in the assay. Two negative serum samples from uninfected and unvaccinated individuals (neg1, neg2) and the dilution buffer were used as negative controls.*

Based on the SG/BG values the best conjugate dilution for anti IgG with TBEV-E-His was 1:8000. The substrate incubation times had no effect and the SG/BG values were similar with 20

min and 30 min TMB incubation times. Higher conjugate dilutions or longer TMB incubation time did not bring further advantage, since the background values started to rise also (Figure 10).

The objective in the ELISA optimization in addition to low background is that positive and negative samples can be clearly separated. Thus, to assess if the ELISA could be further improved with another serum dilution and to confirm the specificity of the results, serum samples were analyzed and diluted out in a dilution series (1:30, 1:100, 1:300, 1:1000, 1:3000, 1:10 000, 1:30 000, 1:100 000) with both TBEV-E-GST and TBEV-E-His ELISAs. Based on the results obtained above, TBEV-E-GST concentration was selected to be 8 µg/ml and TBEV-E-His concentration 4 µg/ml. With TBEV-E-GST the anti-human IgG HRP conjugate dilution was 1:4000 and with TBEV-E-His 1:8000 (Figure 11).

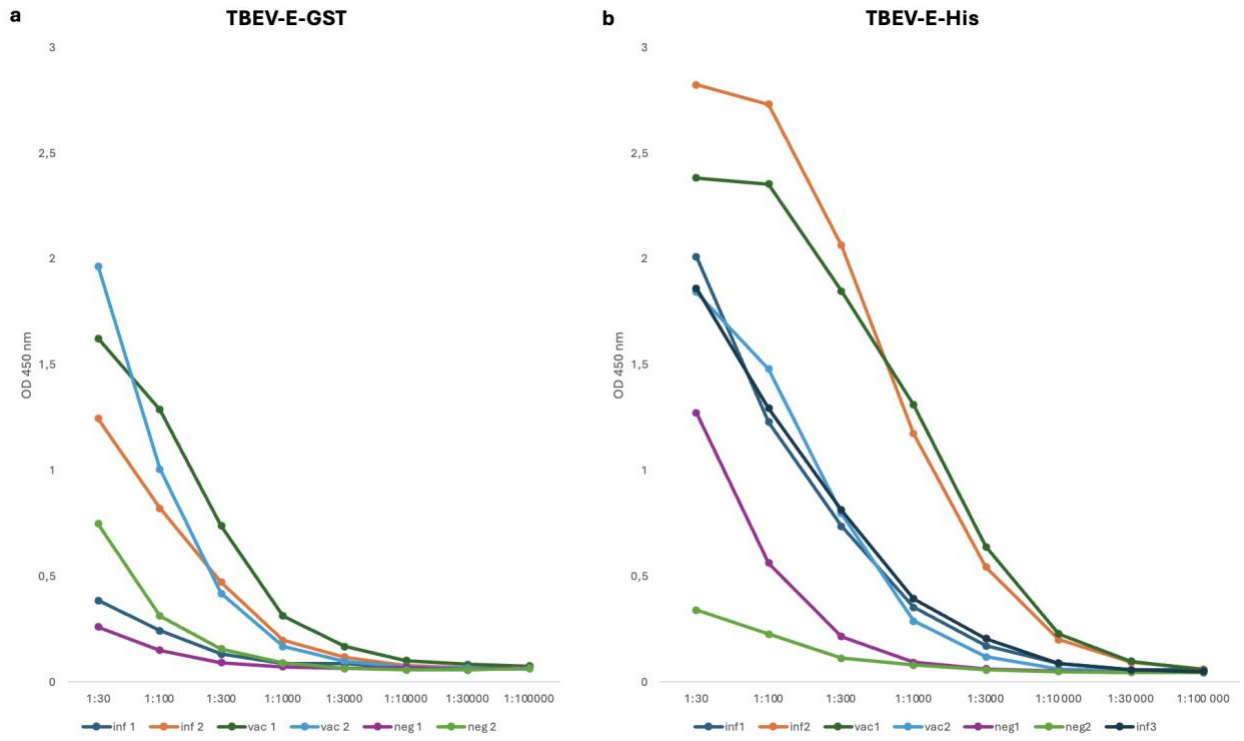

Figure 11. Dilution series with serum samples. *Serum samples were diluted in a dilution series (1:30, 1:100, 1:300, 1:1000, 1:3000, 1:10 000, 1:30 000, 1:100 000). a. TBEV-E-GST was coated at 8 µg/ml concentration and anti-human IgG HRP conjugate was used in 1:4000 dilution. b. TBEV-E-His was coated at 4 µg/ml concentration and anti-human IgG HRP conjugate was used in 1:8000 dilution. Serum samples from two TBEV-infected patients (inf1, inf2) and two vaccinees (vac1, vac2) were used in the assay. Two negative serum samples from uninfected and unvaccinated individuals (neg1, neg2) and the dilution buffer were used as negative controls.*

In the serum dilution series, with TBEV-E-His and 1:300 serum sample dilution, the individuals with high antibody levels, moderate antibody levels and almost negative antibody levels were

distinctly separated (Figure 11b). With TBEV-E-GST the antibody positive and negative samples were also separated with 1:100 and 1:300 serum sample dilutions, but not as clearly as with TBEV-E-His. Additionally, inf1 sample could not be separated from the negative samples with TBEV-E-GST (Figure 11a).

Discussion

The aim of the project was to set up an ELISA test method to detect and differentiate TBEV-specific antibody responses in vaccinees and infected individuals. To achieve this, TBEV NS1 and E proteins had to be produced and ELISA conditions (antigen concentration, serum dilution, conjugate dilution) needed to be optimized. In the time frame of this thesis, we were unable to produce NS1 applicable for ELISA. We were able to produce and purify E antigen in insect cells, and E protein worked as antigen in ELISA for the detection of anti-E IgG antibodies in human sera. However, a very high antigen concentration (8 µg/ml) was needed to reach OD values above 1 indicating that the purity of the protein was not sufficient. The envelope protein was additionally produced in mammalian cells (HEK293). This protein worked well in ELISA to detect anti-E IgG antibodies, and with optimized ELISA parameters we were able to observe clear differences between E-antibody positive and negative sera.

We had challenges in protein production in insect cells and subsequent GST-based purification. In our purifications, NS1 remained in pellet and purified E protein amounts remained low compared to the amounts achieved in earlier studies. For instance SARS-CoV-2 and Ebola virus proteins have been produced in insect cells and purified with GST-based purification system previously with success [28,29]. One explanation for the low E-GST protein production could be that the sequence that encodes prM protein was not included in the sequence cloned into the pBVboost vector. prM facilitates appropriate folding of E protein and ensures that E protein reaches its correct conformation. [30] Production of TBEV-E-His in mammalian cells was conducted with a construct including prM, resulting in sufficient amounts of E-protein suitable for ELISA. However, this successful production could also be due to cell line used (insect vs. mammalian).

Neutralizing antibodies in TBEV infections and vaccinations develop predominantly against E protein epitopes. Antibodies against E protein form after vaccination and infection, but the titers are usually higher after infection [24,31]. During the TBEV infection, NS1 protein is secreted as soluble antigen inducing substantial antibody formation in infected individuals. NS1 antibody formation is induced also in breakthrough infections in TBEV vaccinated. The TBEV vaccines that are in use in

Europe contain no or low amounts of NS1. Therefore, vaccination does not induce NS1 antibody formation similar to infection, and NS1 could be used in differentiating vaccinated and breakthrough infected individuals. [25,32,33]

Cross-reactivity of antibodies against other flaviviruses is a substantial issue in serodiagnostics and needs to be taken into account when the test is being set up. Several NS1-based antibody assays have been developed. In an Austrian study, serum samples from infected, vaccinated and seronegative individuals were analyzed with TBEV NS1 and virion IgG ELISA. One sample group had received Japanese encephalitis (JE) and yellow fever (YF) vaccinations in addition to TBE vaccination. None of the samples from the vaccinated groups showed response in NS1 IgG ELISA. [33] In another study, the NS1-directed antibody responses of infected, vaccinated and seronegative sample groups were compared. Additionally, groups with dengue / West Nile (DENV/ WNV) virus infection, YF vaccination and TBE/JE vaccination were included. TBEV infections could be clearly separated from other sample groups indicating that NS1 antibodies did not significantly cross-react with other flaviviruses. [25]

The aim of this project was also to differentiate acute TBEV infection from VBT. Individuals with acute TBEV infection have high IgM antibody levels for weeks to months after infection. Patients with breakthrough infection develop very high IgG antibody levels early. IgM antibody development is delayed, and IgM antibodies are low or absent at the time when they usually appear [17,22] Therefore, acute TBEV infection can be distinguished from breakthrough infection by IgM response.

In the ELISA with TBEV-E-His, IgM responses were constantly low, also in the infected individuals. IgM antibody measurement is more time sensitive than IgG measurement. We had only three serum samples from infected individuals. It is possible that their IgM antibody levels had already dropped. Therefore, it can't be stated whether our method is applicable for IgM antibody measurement or not.

As detected in the immunoblot strips, one of the infected individuals had antibodies against the linear epitope of E protein. In ELISA, E-protein specific IgG antibody responses were seen in all infected and vaccinated individuals. In an Austrian study, immunoblot strip assay was used with TBEV virus positive antisera from vaccinated individuals. The linear antigen epitopes were recognized by the TBEV-specific antibodies in the human sera. [34] We used the pellet after the cells had been lysed and centrifuged, since all NS1 protein remained in the pellet and for E protein some

amount also remained in the pellet. It is possible that our proteins were not optimal for antibody binding. The immunoblot strip method was not specially optimized, so it might not have been sensitive enough to detect the antibodies.

TBEV serology is important especially in diagnosis of TBEV infection, however, it has also potential to differentiate vaccinees, infected individuals and VBT's. In the time frame of this thesis, we were able to successfully set up an E-specific IgG ELISA. Further analyzes are needed to see whether the test is applicable for IgM antibody measurement as well. We were able to produce NS1 protein, but the purification was unsuccessful. NS1 is a key antigen in TBEV serology. In order to set up the NS1-specific ELISA, we need to be able to produce purified NS1 protein.

References

1. Pustijanac, E. *et al.* Tick-Borne Encephalitis Virus: A Comprehensive Review of Transmission, Pathogenesis, Epidemiology, Clinical Manifestations, Diagnosis, and Prevention. *Microorganisms* **11**, 1634 (2023).
2. Bogovic, P. Tick-borne encephalitis: A review of epidemiology, clinical characteristics, and management. *World J. Clin. Cases* **3**, 430 (2015).
3. Pulkkinen, L. I. A., Butcher, S. J. & Anastasina, M. Tick-Borne Encephalitis Virus: A Structural View. *Viruses* **10**, 350 (2018).
4. Van Leur, S. W., Heunis, T., Munnur, D. & Sanyal, S. Pathogenesis and virulence of flavivirus infections. *Virulence* **12**, 2814–2838 (2021).
5. Velay, A. *et al.* Tick-borne encephalitis virus: molecular determinants of neuropathogenesis of an emerging pathogen. *Crit. Rev. Microbiol.* **45**, 472–493 (2019).
6. Füzik, T. *et al.* Structure of tick-borne encephalitis virus and its neutralization by a monoclonal antibody. *Nat. Commun.* **9**, 436 (2018).
7. Pulkkinen, L. I. A. *et al.* Molecular Organisation of Tick-Borne Encephalitis Virus. *Viruses* **14**, 792 (2022).

8. Worku, D. A. Tick-Borne Encephalitis (TBE): From Tick to Pathology. *J. Clin. Med.* **12**, 6859 (2023).

9. Kaiser, R. Tick-Borne Encephalitis. *Infect. Dis. Clin. North Am.* **22**, 561–575 (2008).

10. Chiffi, G., Grandgirard, D., Leib, S. L., Chrdle, A. & Růžek, D. Tick-borne encephalitis: A comprehensive review of the epidemiology, virology, and clinical picture. *Rev. Med. Virol.* **33**, e2470 (2023).

11. Suss, J. Tick-borne encephalitis in Europe and beyond--the epidemiological situation as of 2007. *Euro Surveill. Bull. Eur. Sur Mal. Transm. Eur. Commun. Dis. Bull.* **13**, 18916 (2008).

12. Tick-borne encephalitis Annual Epidemiological Report for 2020. (2020).

13. Tick-borne encephalitis Annual Epidemiological Report 2022. (2022).

14. Puutiaisaivotulehduksen esiintyvyys Suomessa - THL. *Terveyden ja hyvinvoinnin laitos* https://thl.fi/aiheet/infektiotaudit-ja-rokotukset/taudit-ja-torjunta/taudit-ja-taudinaiheuttajat-a-o/puutiaisaivotulehdus/puutiaisaivotulehduksen-esiintyvyys-suomessa (2025).

15. Tonteri, E. *et al.* Surveillance of endemic foci of tick-borne encephalitis in Finland 1995-2013: evidence of emergence of new foci. *Euro Surveill. Bull. Eur. Sur Mal. Transm. Eur. Commun. Dis. Bull.* **20**, (2015).

16. Dub, T. *et al.* Game Animal Density, Climate, and Tick-Borne Encephalitis in Finland, 2007-2017. *Emerg. Infect. Dis.* **26**, 2899–2906 (2020).

17. Ruzek, D. *et al.* Tick-borne encephalitis in Europe and Russia: Review of pathogenesis, clinical features, therapy, and vaccines. *Antiviral Res.* **164**, 23–51 (2019).

18. TBE-rokote eli ‘punkkirokote’ - THL. *Terveyden ja hyvinvoinnin laitos* https://thl.fi/aiheet/infektiotaudit-ja-rokotukset/rokotteet-a-o/tbe-rokote-eli-punkkirokote (2024).

19. Hansson, K. E. *et al.* Tick-borne Encephalitis Vaccine Failures: A 10-year Retrospective Study Supporting the Rationale for Adding an Extra Priming Dose in Individuals Starting at Age 50 Years. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.* **70**, 245–251 (2020).

20. Nygren, T. M. *et al.* Tick-borne encephalitis vaccine effectiveness and barriers to vaccination in Germany. *Sci. Rep.* **12**, 11706 (2022).

21. Loew-Baselli, A. *et al.* Seropersistence of tick-borne encephalitis antibodies, safety and booster response to FSME-IMMUN® 0.5 ml in adults aged 18-67 years. *Hum. Vaccin.* **5**, 551–556 (2009).

22. Puutiaisaivokuumeen ja borrelioosin laboratoriolöydökset. *Duodecim Terveyskirjasto* https://www.terveyskirjasto.fi/kpp00006 (2021).

23. Beran, J., Xie, F. & Zent, O. Five year follow-up after a first booster vaccination against tick-borne encephalitis following different primary vaccination schedules demonstrates long-term antibody persistence and safety. *Vaccine* **32**, 4275–4280 (2014).

24. Kubinski, M. *et al.* Tick-Borne Encephalitis Virus: A Quest for Better Vaccines against a Virus on the Rise. *Vaccines* **8**, 451 (2020).

25. Girl, P. *et al.* Tick-Borne Encephalitis Virus Nonstructural Protein 1 IgG Enzyme-Linked Immunosorbent Assay for Differentiating Infection versus Vaccination Antibody Responses. *J. Clin. Microbiol.* **58**, e01783-19 (2020).

26. Airenne, K. J., Laitinen, O. H., Mähönen, A. J. & Ylä-Herttuala, S. Preparation of Recombinant Baculoviruses with the BVboost System. *Cold Spring Harb. Protoc.* **2009**, pdb.prot5181 (2009).

27. Kielkopf, C. L., Bauer, W. & Urbatsch, I. L. Purification of Fusion Proteins by Affinity Chromatography on Glutathione Resin. *Cold Spring Harb. Protoc.* **2020**, 102202 (2020).

28. Jalkanen, P. *et al.* A Combination of N and S Antigens With IgA and IgG Measurement Strengthens the Accuracy of SARS-CoV-2 Serodiagnostics. *J. Infect. Dis.* **224**, 218–228 (2021).

29. Melén, K. *et al.* Production, purification and immunogenicity of recombinant Ebola virus proteins − A comparison of Freund's adjuvant and adjuvant system 03. *J. Virol. Methods* **242**, 35–45 (2017).

30. Lorenz, I. C., Allison, S. L., Heinz, F. X. & Helenius, A. Folding and dimerization of tick-borne encephalitis virus envelope proteins prM and E in the endoplasmic reticulum. *J. Virol.* **76**, 5480–5491 (2002).

31. Remoli, M. E. *et al.* Anti-tick-borne encephalitis (TBE) virus neutralizing antibodies dynamics in natural infections versus vaccination. *Pathog. Dis.* **73**, 1–3 (2015).

32. Albinsson, B. *et al.* Distinction between serological responses following tick-borne encephalitis virus (TBEV) infection vs vaccination, Sweden 2017. *Euro Surveill. Bull. Eur. Sur Mal. Transm. Eur. Commun. Dis. Bull.* **23**, 17–00838 (2018).

33. Stiasny, K., Leitner, A., Holzmann, H. & Heinz, F. X. Dynamics and Extent of Non-Structural Protein 1-Antibody Responses in Tick-Borne Encephalitis Vaccination Breakthroughs and Unvaccinated Patients. *Viruses* **13**, 1007 (2021).

34. Marx, F., Gritsun, T. S., Grubeck-Loebenstein, B. & Gould, E. A. Diagnostic immunoassays for tick-borne encephalitis virus based on recombinant baculovirus protein expression. *J. Virol. Methods* **91**, 75–84 (2001).