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A method for rapid and high-yield production of the tick-borne encephalitis virus E and DIII recombinant proteins in *E. coli* with preservation of the antigenic properties

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ABSTRACT

Tick-borne encephalitis virus (TBEV) is a member of the Flavivirus genus and is the main pathogenic arbovirus circulating in Europe, Russia and China. The envelope (E) protein is exposed on the viral surface and is the main antigen that is employed in diagnostic tests based on the detection of protein-specific antibodies from serum samples of infected individuals. The high degree of similarity among the E proteins of flaviviruses can, in some cases, lead to cross-reactivity and false-positive results in serological tests. Increased specificity in the detection of positive sera for different Flavivirus infections is often obtained by using a portion of the E protein, namely, the DIII domain. Different strategies and expression systems have been described for E and DIII protein production. Here, we present the optimization of an easy and fast method for TBEV E and DIII antigen production and partial purification from *E. coli* inclusion bodies. The antigenic properties of the produced antigens are retained, as validated by ELISAs with anti-TBEV murine sera as well as sera from infected human patients. The potential applications of both proteins as diagnostic reagents were confirmed.

1. Introduction

Tick-borne encephalitis virus (TBEV) is a member of the Flavivirus genus and the Flaviviridae family. This genus includes more than 70 members characterized by a single-stranded RNA genome of positive polarity and an enveloped icosahedral capsid (Gould and Solomon, 2008). TBEV, West Nile virus (WNV), dengue virus (DENV), Zika virus (ZIKV), yellow fever virus (YFV) and Japanese encephalitis virus (JEV) are the most relevant human pathogenic flaviviruses, and the transmission of these viruses occurs via tick or mosquito bites (Heinz and Stiasny, 2012).

The main arthropod vectors involved in TBEV diffusion are ticks of the species *Ixodes ricinus* and *Ixodes persulcatus*, which are responsible for the endemic presence of the virus in Siberia, China, and central, eastern and northern Europe (Caracciolo et al., 2015; Pettersson et al., 2014; Süss, 2011). Ten to fifteen thousand cases of TBEV infection are reported annually (Bogovic and Strle, 2015), including infections caused by all three virus subtypes—European, Siberian and Far Eastern (Lindquist and Vapalahti, 2008). Generally, after an incubation period of 7–14 days, infection leads to mild symptoms such as fever, fatigue and headache, but in some cases, the virus reaches the central nervous system, causing severe encephalitis, spinal paralysis or death (Bogovic and Strle, 2015). To date, no drugs or specific therapeutic treatments have been approved for TBEV, although infection can be prevented via vaccination with purified, inactivated whole virus produced in Europe and Russia (Heinz and Stiasny, 2012; Kollaritsch et al., 2011). The main immunogenic component of the vaccine is the structural envelope (E) protein, which forms the surface of the virus.

The molecular structure of the E protein comprises three distinct domains (DI, DII and DIII), which together constitute the ectodomain (sE), which is connected by flexible hinges and anchored to the viral membrane by a stem region and two transmembrane domains (TMDs) (Füzik et al., 2018; Rey et al., 1995). As the E ectodomain is exposed on the viral surface, most of the antibodies elicited after vaccination are directed against this region of the protein (Pierson and Kielian, 2013; Zhang et al., 2017).

The E protein plays a key role not only in immunoprophylaxis but also as a tool used to confirm TBEV infections. In fact, most of the immunodiagnostic kits developed to date involve the detection of antibodies against the E antigen (Ackermann-Gäumann et al., 2018).

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However, the antigenic similarity between TBEV and other members of the same genus leads to the production of cross-reactive anti-E protein antibodies (Crill and Chang, 2004; Kimura-Kuroda and Yasui, 1986; Mansfield et al., 2011). The presence of such cross-reactive antibodies complicates the serodiagnosis of Flavivirus infections, which mainly relies on the detection of antibodies against the viral envelope by using inactivated virus or purified E antigen. An alternative target, to better discriminate infections caused by different members of the Flavivirus genus, is the DIII domain, which exhibits the highest sequence variability among the E proteins of flaviviruses. As reported in previous studies (Beasley et al., 2004; Chávez et al., 2010; Holbrook et al., 2004; Ludolfs et al., 2007), the use of the DIII domain alone can improve diagnostic specificity, reducing the detection of cross-reactive antibodies.

Several commercial ELISA kits for TBEV diagnosis use the inactivated virus as detection antigen, requiring adequate equipment for cultivation of the virus under the appropriate biosafety conditions. Here, we describe a rapid and simple method for TBEV E and DIII antigen production in an *E. coli* expression system. After purification, the proteins retain their antigenic properties, as demonstrated by ELISA, testing sera from mice immunized against the virus as well as from infected human patients, suggesting the potential application of these proteins in diagnostic tests for TBEV infection. The availability of reliable reagents for detection of anti-TBEV antibodies is useful in the diagnostic field for improvement of the diagnosis of Flavivirus infections and assessment of the induction of an immune response after vaccination. Furthermore, flaviviruses surveys in seroepidemiological studies are becoming important due to climate change in recent decades and increasing international travel, leading to adaptation of Flavivirus vectors to new geographical areas and to consequent flaviviruses redistribution (Andersen et al., 2019; Heinz et al., 2015; Tokarevich et al., 2011).

2. Materials and methods

2.1. Cloning of the TBEV sE and DIII coding sequences

The sequence of sE of TBEV (Neudoerfl Western subtype, NCBI reference genome NC_001672.1, from aa 1 to aa 395) was codon-optimized for both *E. coli* (for protein production) *and Mus musculus* (for mice DNA immunization). Both sequences were obtained as a synthetic sequence in the pMAT vector (Invitrogen) between the BssHII and NheI restriction sites. The DIII domain sequence was obtained by PCR using the pMAT-sE plasmid as a template and primer pairs specific to the DIII region (from aa 300 to aa 395), with the BssHII and NheI restriction sites added for subsequent cloning. Both sequences were cloned into the prokaryotic expression vector pGEX 4T-1 (GE Healthcare) that had been previously modified to introduce the BssHII and NheI restriction sites and an in-frame FLAG tag at the C-terminus after the NheI site. Each ligation reaction was transformed into *E. coli* DH5αF' cells. Correct cloning of the recombinant genes was initially confirmed by PCR amplification using forward and reverse primers specific to the pGEX vector and fingerprinting of the DNA fragment. Positive colonies were subsequently confirmed by Sanger sequencing.

2.2. Optimization of protein expression

A positive colony carrying the sE sequence was inoculated in 2xTY medium with 100 µg/ml ampicillin and 0.1% glucose. Growth was conducted on a shaker platform at 30 °C, and protein expression was induced at OD_{600nm} = 0.6 with 0.2 mM or 1 mM IPTG. At different time points after induction, bacteria were centrifuged for 20 min at 3000 × *g* at 10 °C, and supernatants were stored at −80 °C. Pellets were weighed, resuspended in 10 ml/g of lysis buffer (20 mM Tris (pH 8), 500 mM NaCl, 0.1% Triton X-100) and stored at −80 °C. All the collected protein samples (supernatants and resuspended pellets) were analyzed by Western blot (WB) analysis for detection of Glutathione S-transferase (GST).

2.3. Partial purification of the TBEV sE and DIII recombinant proteins

After thawing the total lysates, 400 µg of lysozyme per ml of sample was added, and the samples were incubated on ice for 30 min with shaking. Then, 100 µg/ml DNase and 1 mM EDTA were added to the lysates, and the samples were incubated on ice for 45 min with shaking. The lysates were sonicated three times for 30 s and centrifuged for 20 min at 18,000 × *g* at 10 °C. The supernatants were discarded, and the pellets were frozen at −80 °C. After thawing, the pellets were resuspended with 1/10 of the starting culture volume of Triton-containing wash buffer (50 mM Tris (pH 8), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% Triton X-100), sonicated three times for 30 s and centrifuged at 11,000 × g for 15 min at 10 °C. The wash was repeated a second time, and pellets were resuspended with 1/50 of the starting culture volume of wash buffer without Triton. Four volumes of 6 M urea (final concentration 4.8 M) were added to one volume of sample in wash buffer, and the mixture was centrifuged at 15,000 × g for 10 min at 10 °C. The solubilized protein was dialyzed in PBS/urea buffer at 10 °C, gradually decreasing the urea concentration from 5 M to 15 mM over a 16-hour interval.

2.4. SDS-PAGE and Western blot analysis

The bacterial lysates after protein expression and the partially purified proteins before and after dialysis were mixed with a denaturing and reducing loading buffer, separated on a 12% polyacrylamide gel and subjected to Coomassie blue staining. Protein molecular weights were determined using standard protein molecular weight markers (Smobio). Protein samples were also transferred from the gel to a PVDF membrane for WB analysis. Nonspecific binding sites were blocked with 2% nonfat milk in PBS (MPBS). Anti-GST and anti-FLAG tag murine monoclonal antibodies (Sigma Aldrich) diluted 1:3,000 in 2% MPBS were used as primary antibodies and incubated with the membrane for 1 h at RT. Alkaline phosphatase-conjugated anti-mouse antibody (Thermo Fisher Scientific) diluted 1:5,000 in 2% MPBS was then incubated with the membrane for 1 h at RT. After three washes with 0.1% Tween 20 in PBS buffer (PBST), the blots were developed with BCIP/NBT (Thermo Fisher Scientific) in alkaline phosphatase buffer (100 mM Tris-HCl (pH 9.0), 150 mM NaCl, 1 mM $MgCl_2$).

2.5. Murine sera

Eight murine serum samples were used for ELISAs on recombinant TBEV proteins and control proteins. Two serum samples were obtained from BALB/c mice vaccinated with the FSME-IMMUN® human vaccine (Baxter). Five serum samples were derived from mice subjected to DNA immunization by gene gun technology (Bio-Rad, Hercules, CA, USA). Briefly, the optimized sE sequences of TBEV and WNV were cloned into the pcDNA3 vector (Invitrogen), and 1 µg of immunization DNA coated on 0.5 mg of 1 µm diameter gold nanoparticles was biolistically delivered into mice three times at fifteen-day intervals. Four mice were immunized with TBEV sE and one mouse with WNV sE. All tested sera were collected 15 days after the final immunization boost. A non-immunized mouse was used as a control.

2.6. Human sera

Nine human serum samples were used for ELISAs on recombinant TBEV proteins. Four serum samples were obtained from patients who tested positive for anti-TBEV antibodies, while five serum samples were obtained from negative controls. Antibody positivity was determined using the TBEV (FSME) IgG ELISA Kit (Immunolab GmbH).

2.7. Solid-phase ELISA

Partially purified TBEV proteins and BSA (negative control) were used to coat the ELISA plate (MaxiSorp, Nunc). Antigens were diluted in PBS at a final concentration of 5 µg/ml and incubated O/N at 4 °C. Coating solutions were discarded, and nonspecific binding sites were blocked with 120 µl of 2% MPBS for 45 min at RT. Four different dilutions (1:1,000; 1:2,000; 1:4,000; and 1:8,000) of each murine serum were used. Human sera were used at a uniform dilution of 1:1,000. Sera were incubated in antigen-coated plates for 1 h at RT. The detection was performed incubating 100 µl of anti-mouse IgG or anti-human IgG horseradish peroxidase-conjugated antibody (Jackson ImmunoResearch) diluted 1:5,000 in 2% MPBS for 1 h at RT. After three washes with PBST, the reaction was developed with 100 µl of TMB (3,3′,5,5′-tetramethylbenzidine, Sigma Aldrich) and blocked with 50 µl of 2 N sulfuric acid.

2.8. Statistical analysis

The data obtained with human sera were analyzed by GraphPad Prism 6 software. The statistical significance between the two groups of human samples (positive/negative for TBEV infection) was calculated by Student's *t*-test. For all analyses, differences were considered significant at p-values < 0.05.

3. Results

3.1. Cloning of TBEV sE and DIII sequences

The envelope of TBEV is the main viral antigen used in serodiagnostic tests, as most of the antibodies elicited after infection are directed against this structure. The viral envelope is formed by the E protein, which comprises three regions (DI, DII and DIII) constituting the so-called sE. Two TMDs are connected to the sE by a stem anchor (Fig. 1A). The DIII domain contains virus-specific epitopes, and previous studies have demonstrated that antibodies that bind to the DIII region are highly specific (Beasley et al., 2004; Chávez et al., 2010; Holbrook et al., 2004). To develop a rapid production protocol for suitable antigens for research and diagnostic applications, we decided to clone both the sE and DIII domain sequences as GST fusion proteins. The synthetic sE sequence and DIII domain were amplified with specific primers and cloned into a modified pGEX expression vector. The modified pGEX vector allows the production of the protein of interest as a fusion protein with GST at the N-terminus and a FLAG tag at the C-terminus (Fig. 1B). The pGEX plasmids carrying the sE and DIII sequences were transformed into DH5αF' cells, and single colonies were confirmed to be positive clones initially by PCR and fingerprinting and subsequently by Sanger sequencing.

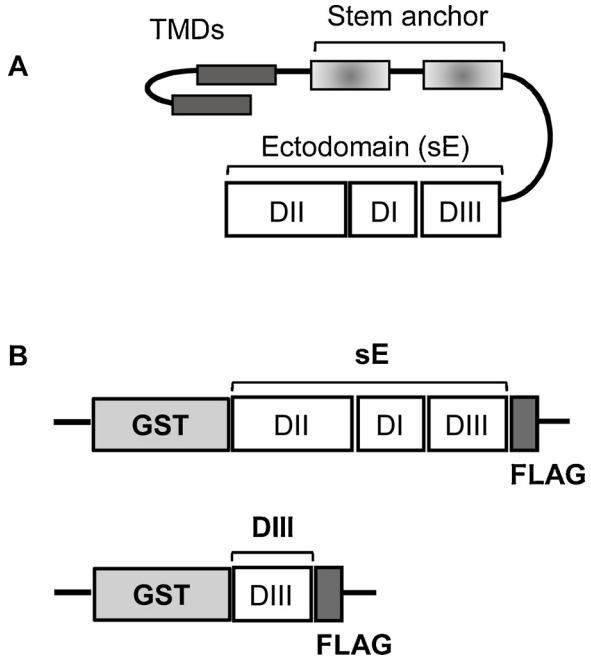


Fig. 1. TBEV envelope protein structure and cloning constructs.
A. Schematic structure of the envelope protein of TBEV, including the three domains (DI, DII, DIII) indicated as sE and two transmembrane domains (TMDs) linked to sE by the stem anchor region. **B.** Schematic representation of the two cloning constructs for TBEV protein production. Both constructs include the TBEV protein (sE or DIII) flanked by the GST protein (N-terminus) and FLAG tag (C-terminus).

3.2. Optimization of expression conditions

To optimize the expression conditions for the two proteins, different culture parameters were initially evaluated for the sE protein. The protein yield and solubility were initially evaluated after 6 h of growth in supernatant and bacterial pellet lysate by WB analysis to identify the fraction that contained the highest amount of antigen. As shown in Fig. 2A, although detected in both fractions, the protein was present mainly as an insoluble product in the pellet. Shorter induction times (1 h and 3 h) were also tested for improvement of yield and solubility. The result (Fig. 2B) suggested that there was no increase in the protein level in the supernatant, and the protein was also well expressed with a 1-hour induction in the total lysate. Finally, different IPTG concentrations (1 mM, compared to 0.2 mM) were evaluated with a 3-hour induction time. As shown in Fig. 2C, in both fractions, no substantial difference in expression was observed between the two IPTG concentrations used. In conclusion, the highest sE yields were observed in the pellet of the total lysate as inclusion bodies (IBs) after three hours of induction with a low concentration of IPTG.

3.3. Partial purification of the TBEV sE and DIII recombinant proteins

IBs were washed with a saline buffer containing minimal concentrations of denaturing agent (DTT) and detergent (Triton X-100). After washing, the protein aggregates were solubilized with urea. Two different final concentrations were tested: 3 M and 4.8 M. The best result in terms of solubilization was obtained with the higher concentration. To remove urea and avoid protein precipitation, the sample was dialyzed in PBS/urea buffer at 10 °C, gradually decreasing the urea concentration from 5 M to 15 mM over a 16-hour interval. As shown in Fig. 3, the total lysates of DIII (lane 1) and sE (lane 4) exhibited a main band at the expected molecular weights of 37 kDa and 70 kDa, respectively (including the 26 kDa GST-fused protein). The partially purified products (lanes 2 and 5) were much cleaner from the secondary products when compared with the total lysates thus confirming the efficacy of the washing steps. In addition, the proteins before (lanes 2 and 5) and after (lanes 3 and 6) dialysis were present at the same concentration, suggesting that this final step did not induce the formation of protein precipitates and confirming the solubility of the proteins.

To determine whether the partially purified proteins were full-length or degraded proteins, we tested the samples by WB analysis. As reported in Fig. 4, the bands detected upon loading the total lysates (lanes 1 and 4) exhibited the expected molecular weights with both the anti-GST and anti-FLAG antibodies; furthermore, no other bands were present, confirming the full length of the protein in the starting material. Protein quality was maintained in the samples after purification (lanes 2 and 5) as well as after dialysis (lanes 3 and 6), suggesting that the purification steps did not cause protein degradation.

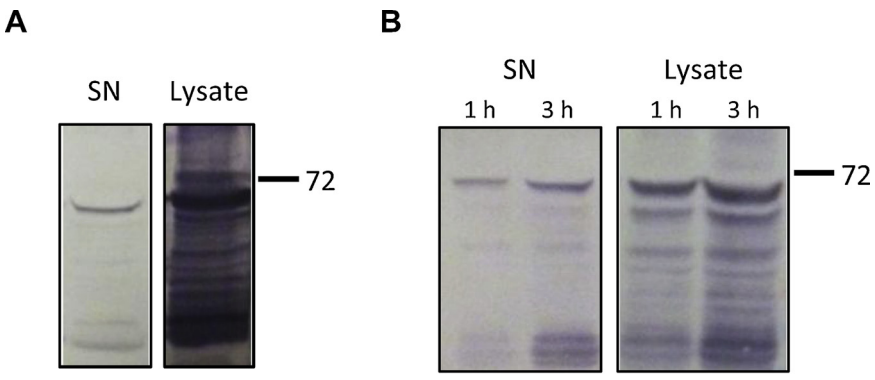
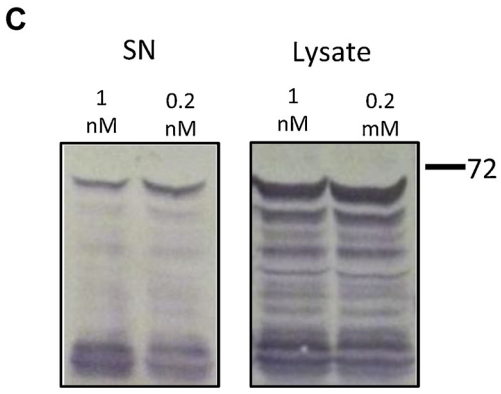


Fig. 2. Western blot analysis of different conditions for protein expression.
A. Detection of the sE antigen in the supernatant and total bacterial lysate after induction of protein expression. **B.** Detection of the sE antigen in supernatant and total bacterial lysate 1 and 3 h after induction of protein expression. **C.** Detection of the sE antigen in supernatant and total bacterial lysate 3 h after induction of protein expression with 0.2 mM and 1 mM IPTG. Bands were detected by incubation with first a mouse anti-GST antibody and then an anti-mouse alkaline phosphatase-conjugated antibody.



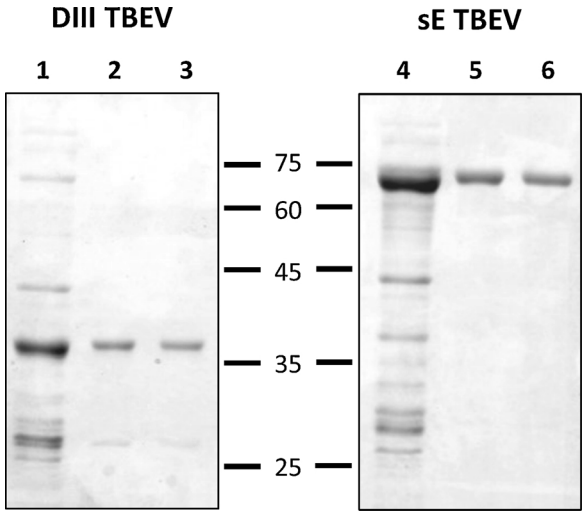


Fig. 3. SDS-PAGE of purified TBEV proteins.
SDS-PAGE of total bacterial lysates containing the sE and DIII proteins in denaturing and reducing conditions before purification (lanes 1 and 4) and after purification (lanes 2 and 5). The final products after dialysis were loaded in lanes 3 and 6. Standard molecular markers are indicated on the left and right sides.

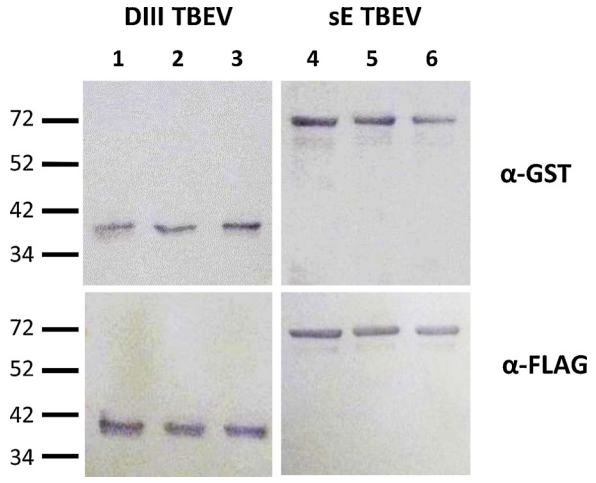


Fig. 4. Western blot analysis of purified TBEV proteins.
Western blot analysis of total bacterial lysates containing the sE and DIII proteins before purification (lanes 1 and 4), after purification (lanes 2 and 5) and after dialysis (lanes 3 and 6). Bands were detected by incubation with first a mouse anti-GST or anti-FLAG antibody and then an anti-mouse alkaline phosphatase-conjugated antibody. Standard molecular markers are indicated on the left side.

3.4. Antigenic properties of the partially purified sE and DIII recombinant proteins

The antigenicity of the produced proteins was first checked by ELISA using murine sera that had undergone two different immunization procedures against TBEV. The first immunization method was based on gene gun technology, involving the use of gold particles coated with a DNA expression vector encoding the antigen of interest. In our procedure, the DNA-coated particles are biolistically delivered into the animal epidermis, leading to antigen production by epidermal transfected cells and immune system stimulation. Four mice were immunized with DNA encoding the sE of TBEV, while the sE sequence of WNV, a member of the same genus as TBEV, was used to immunize a control mouse to check for possible nonspecific signals. As shown in Fig. 5A, both the sE and DIII antigens could be used to detect the presence of antibodies in the sera of mice immunized with the TBEV

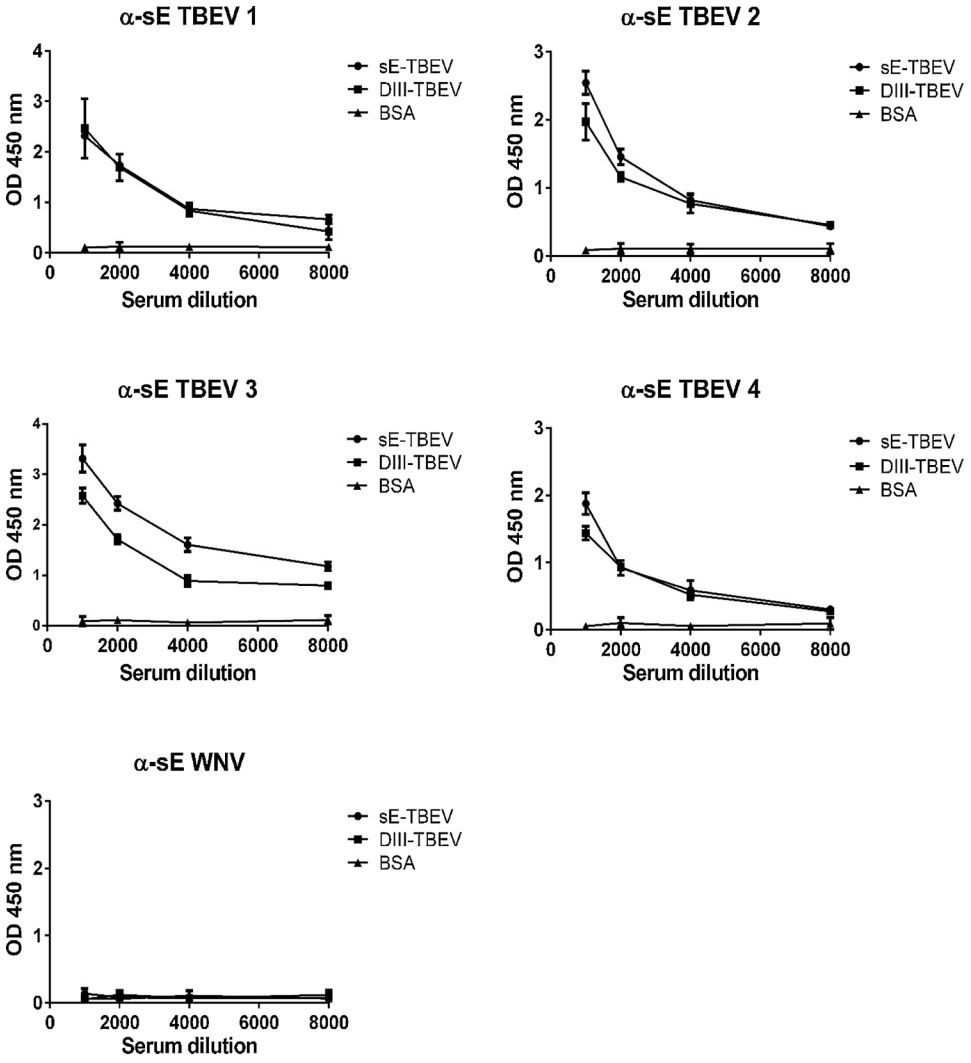


Fig. 5. ELISA of purified TBEV proteins with murine sera obtained after DNA immunization.
ELISA was performed with serum samples from four TBEV sE protein-immunized mice and one serum sample from a WNV sE protein-immunized mouse. All sera were tested on BSA (negative control) and the TBEV sE and DIII recombinant proteins in four different dilutions (indicated on the X axis). The optical density at 450 nm is reported on the Y axis. The standard deviation values were calculated for technical duplicates for each dilution.

antigen. Positivity was detected over a wide range of serum dilutions, with samples exhibiting positivity at up to a 1:8,000 dilution. All sera were negative with BSA as a control protein, even at low dilutions. When the serum of the mouse immunized with WNV genes was used, no reaction was detected at any of the dilutions tested, confirming the specificity of the antibody response. The robustness of the results was confirmed by a second ELISA that was performed using two serum samples derived from mice vaccinated using the TBEV vaccine. In this case, as shown in Fig. 6, both vaccinated mice exhibited high positive signals for both recombinant proteins and at all the tested dilutions but were negative for the control protein. The serum of a nonvaccinated mouse, used as a negative control, did not react with any protein.

The antigenicity of the TBEV proteins produced was finally tested using human serum samples from four confirmed infected patients and five negative controls. As shown in Fig. 7, the positive sera reacted with both the produced antigens, presenting a binding signal that was significantly different compared with the negative controls. The difference between positive/negative samples was clearly higher for the DIII antigen than for the sE antigen (Fig. 7), as confirmed by statistical analysis.

4. Discussion

Human infections caused by TBEV are generally diagnosed by ELISAs based on the detection of antibodies against the virus (Holzmann, 2003). The same tests are also used to verify stimulation of the immune response after vaccination. The main antigen that elicits protective antibodies during both natural infection and vaccination is the E protein of the viral surface. Therefore, most of the available diagnostic tests rely on this protein and its ability to detect the antibodies present in serum samples. Different systems and purification methods have been proposed for E antigen production, including eukaryotic and insect cell transfection (Allison et al., 1995; Golotin et al., 2018; Marx et al., 2001). The use of prokaryotic cells for protein purification has three main advantages: high yields, reduced costs and short production times.

Here, we have described a rapid and simple method for TBEV E antigen production in bacteria. Similar to most Flavivirus members, TBEV harbors a glycosylation site on the E protein, which is therefore lost in the prokaryotic expression system. However, glycosylation is mainly associated with the infectivity of the virus, and loss of the glycosylation should not significantly affect the capability of the antigen to detect the immune response in naturally infected or vaccinated subjects

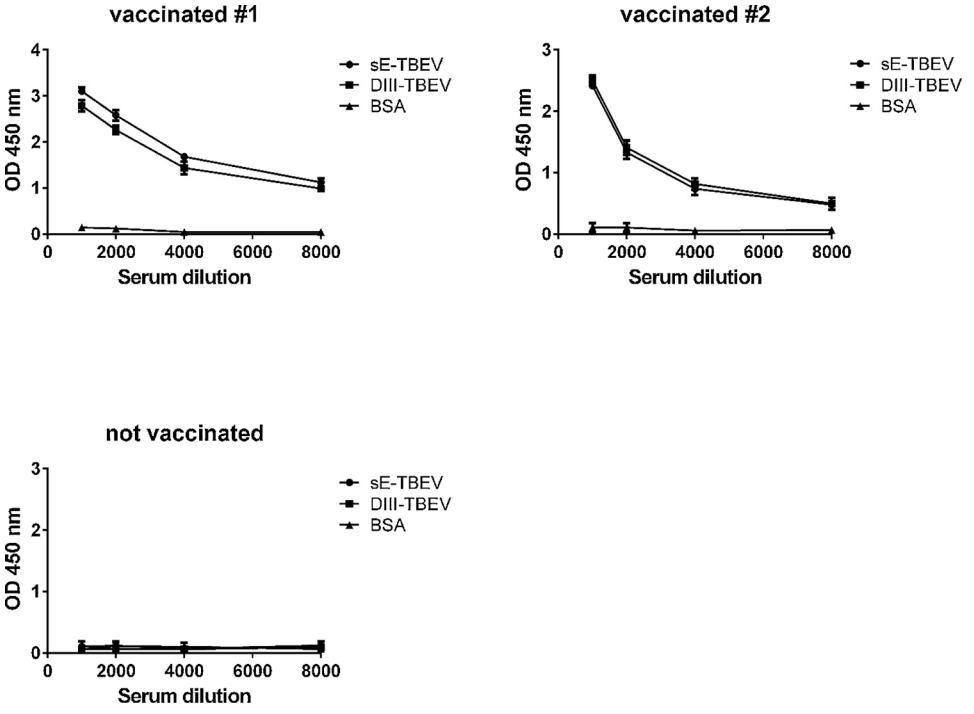


Fig. 6. ELISA of purified TBEV proteins with murine sera obtained after vaccine immunization.
ELISA with serum samples from two TBEV-vaccinated mice and one serum sample from a nonimmunized mouse. All sera were tested on BSA (negative control) and the TBEV sE and DIII recombinant proteins in four different dilutions (indicated on the X axis). The optical density at 450 nm is reported on the Y axis. The standard deviation values were calculated for technical duplicates for each dilution.

(Fontes-garfias et al., 2017; Goto et al., 2005; Yoshii et al., 2013).

Considering that a recurrent problem in Flavivirus diagnosis is the cross-reactivity caused by similarities among members of this genus (Allwinn et al., 2002; Litzba et al., 2014), we produced both the E protein and the DIII domain, exhibiting the highest degree of sequence diversity among flaviviruses. The two recombinant antigens were partially purified from protein aggregates formed within bacteria and dialyzed. Our method, based on optimization of bacterial growth and induction conditions, involves only sequential washes for solubilization of protein aggregates without the need for a chromatographic step. After dialysis, the final proteins obtained were highly pure, with no relevant contaminants or degradation products detected by SDS-PAGE and WB assays. Compared to previous works (Saejung et al., 2006; Tan et al., 2010; Tripathi et al., 2012), in which the protein amounts obtained ranged from 2 to 140 mg/L, our protocol resulted in final yields of up to 50 and 100 mg/l for the DIII and E antigens, respectively.

A fundamental property tested after protein production is the preservation of antigenicity, notwithstanding loss of conformation and glycosylation of the E protein. We demonstrated that murine sera derived from DNA immunization with the TBEV E protein, or from TBEV-vaccinated animals, are highly reactive on both E and DIII recombinant antigens. Furthermore, no cross-reactivity was observed using the serum of a control mouse immunized against the related WNV co-circulating with TBEV in some areas of Southern Europe. The two viruses belong to the same genus but different serocomplexes; therefore, signal specificity appears to be related to phylogenetic distance (Papa et al., 2011).

Finally, we analyzed the sera of TBEV-infected human patients. Additionally, in this case, the produced antigens were able to detect the immune response of the positive samples, whereas the negative controls did not react with the proteins. The increased difference in binding observed for the DIII antigen between positive and negative samples is consistent with previous data (Beasley et al., 2004; Chávez et al., 2010; Holbrook et al., 2004), demonstrating the capability of this E protein domain to better discriminate the presence of virus-specific antibodies than the sE domain. These data highlight the suitability of the adopted method for partial purification of the TBEV antigen in a fast and inexpensive manner with maintenance of the functional properties of the antigen. Considering the high degree of structural similarity among flaviviruses, we speculate that the same protocol can be applied to produce the same antigens from other important members of the Flavivirus genus. The availability of such proteins is useful not only for classic serological tests for Flavivirus infections but also for the development of new diagnostic technologies (Balasuriya et al., 2006; Tagliabue et al., 2017; Wong et al., 2004) for easy and fast detection of the virus. One of the main objectives of point-of-care systems is the low cost of reagents, which is consistent with the method for TBEV antigen production described herein.

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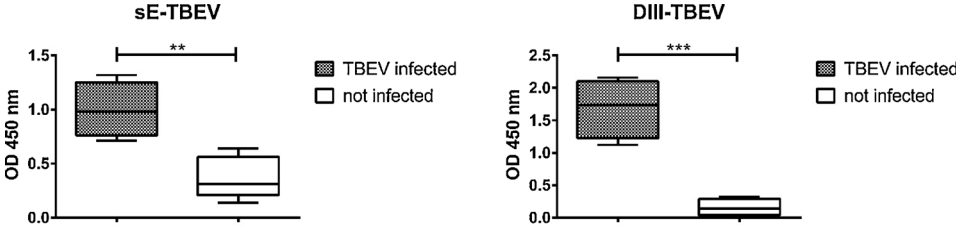

Fig. 7. ELISA of purified TBEV proteins with human sera obtained from positive and negative TBEV-infected patients.
ELISAs with human serum samples from four confirmed positive patients and five negative controls. All sera were tested on sE and DIII recombinant proteins at a dilution of 1:1,000. The optical density at 450 nm after subtraction of the absorbance of the negative control (BSA) is reported on the Y axis. The statistical significance of the difference between the two serum groups was calculated by *Student's t*-test ($p < 0.05$). p-values: 0.004 (**) and 0.0002 (***). The standard deviation values were calculated for the means of technical duplicates for each tested serum.

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