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Author(s)	Chidumayo, Nozyechi N.; Yoshii, Kentaro; Saasa, Ngonda; Sakai, Misuki; Kariwa, Hiroaki
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Title: Development of a tick-borne encephalitis serodiagnostic ELISA using recombinant Fc-antigen fusion proteins

Running title: Envelope-Fc fusion proteins in TBEV diagnosis

Nozyechi N. Chidumayo^{a,b}, Kentaro Yoshii^a, Ngonda Saasa^c, Misuki Sakai^a, Hiroaki Kariwa^a

^aLaboratory of Public health, Graduate School of Veterinary Medicine, Hokkaido University, Japan.

^bClinical Studies Department, School of Veterinary Medicine, University of Zambia, Zambia.

^cDepartment of Disease Control, School of Veterinary Medicine, University of Zambia, Zambia

Corresponding author Kentaro Yoshii, Ph.D, D.V.M

Postal address: Laboratory of Public Health, Graduate School of Veterinary Medicine, Hokkaido

University, Kita-18 nishi-9, Sapporo, Hokkaido 060-0818 Japan

Tel/fax: +81-11-706-5213

E-mail: kyoshii@vetmed.hokuda.ac.jp

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Tick-borne encephalitis virus, Fc fusion protein, diagnosis, ELISA

Abstract

Current diagnostic tests for tick-borne encephalitis virus (TBEV) infections require high biosafety facilities for antigen preparation, and can cross-react with other flaviviruses. There is therefore a need to develop safe, inexpensive serodiagnostic tools with high specificity and sensitivity. In this study, a recombinant plasmid that expresses the membrane (prM) and envelope (E) proteins of TBEV fused to the Fc domain of rabbit IgG was constructed and expressed in mammalian cells. The E-Fc proteins were secreted as soluble homodimers, which retained reactivity with anti-TBEV and anti-rabbit IgG antibodies. The E-Fc proteins were then used to develop an enzyme-linked immunosorbent assay (ELISA) to detect TBEV antibodies in rodent and human sera. Compared with the neutralization test, the ELISA had over 90% sensitivity and specificity. In addition, the assay showed no cross reactivity with Japanese encephalitis antibodies. These findings suggest that the E-Fc ELISA may be a useful tool for TBEV serodiagnosis.

1 Introduction

Tick-borne encephalitis virus (TBEV) causes over 10,000 cases of encephalitis annually (Gunther & Haglund, 2005). TBEV belongs to the family *flaviviridae*, genus *flavivirus* and is divided into three subtypes (European, far-Eastern, and Siberian) of which the far-Eastern subtype is the most lethal. The virus is endemic in many parts of Europe and Asia (Banzhoff *et al.*, 2008, Gubler, 2007, Ludlam *et al.*, 2006). In addition to TBEV, some of these regions are also endemic to other flaviviruses such as Japanese encephalitis virus (JEV) and West Nile virus (WNV) (Banzhoff *et al.*, 2008, Gubler, 2007, Ludlam *et al.*, 2006). Diagnosis of flavivirus infection is challenging due to the similar clinical presentation and the cross-reactive nature of flaviviruses in serological tests (Chavez *et al.*, 2010). Laboratory diagnosis of flavivirus infections is based primarily on the identification of antibodies in neutralization tests (NT) and enzyme-linked immunosorbent assay (ELISA) (Ikawa-Yoshida *et al.*, 2011, Yoshii *et al.*, 2003). The neutralization test is highly specific, and is useful for differentiating flaviviruses that cross-react in other serological tests. However, neutralization tests are time-consuming, and high-biosafety-level facilities are required for the handling of the live virus. As a result of these limiting factors, the test cannot be routinely performed in diagnostic laboratories. ELISA is therefore the most commonly used assay to diagnose flavivirus infections, and commercial ELISA kits are available for TBEV (Yoshii *et al.*, 2003). The ELISA kits use inactivated whole viral antigens to detect TBEV specific antibodies, but cross-reactivity with other flaviviruses is a concern (Hobson-Peters, 2012, Ikawa-Yoshida *et al.*, 2011, Yoshii *et al.*, 2003). Commercial TBEV diagnostic kits have a sensitivity of 73-99% and specificity of 14-81% (Niedrig *et al.*, 2001). Cross-reactive antibodies from yellow fever, dengue virus, and Japanese encephalitis positive sera have all produced false positive results in these tests (Obara *et al.*, 2006, Niedrig *et al.*, 2001).

Recombinant protein technology could reduce the cost of diagnosis, and eliminate the need for the high-biosafety-level facilities required for neutralization tests and the production of whole-virus antigens. Furthermore, the use of viral proteins could improve the specificity of diagnostic tests (Chavez *et al.*, 2010, Hobson-Peters, 2012), reducing the number of false positive results in areas

endemic to more than one flavivirus. ELISA diagnostic tests using viral proteins (Chavez et al., 2010, Holbrook *et al.*, 2004, Ikawa-Yoshida *et al.*, 2011, Obara *et al.*, 2006, Yoshii *et al.*, 2003). Flaviviruses contain two surface proteins, envelope (E) and membrane protein (prM/M) (Yoshii *et al.*, 2003). In the immature virion, the prM and E proteins form heterodimers (Allison *et al.*, 1999). This interaction between prM and E is thought to protect the immature virion from premature activation and fusion with host cell membranes during transit through the trans-Golgi network (Allison *et al.*, 1999, Murray *et al.*, 2008, Rodenhuis-Zybert *et al.*, 2011, Zybert *et al.*, 2008). The E protein is the major surface glycoprotein has been shown to be useful in TBEV diagnostics and vaccine development (Chavez et al., 2010). The ectodomain (which is exposed on the viral surface) represents the N-terminal 80% of the E protein (Jaiswal *et al.*, 2004). The C-terminal 20% of the molecule consists of the transmembrane domains (which anchor the protein to the lipid envelope) (Allison *et al.*, 1999, Jaiswal *et al.*, 2004). X-ray crystallographic studies have revealed that approximately 90% of the ectodomain forms three structural domains designated I, II, and III (Allison *et al.*, 1999, Chavez *et al.*, 2010, Ikawa-Yoshida *et al.*, 2011). These domains are highly immunogenic, and are the major target for neutralizing antibodies (Chavez *et al.*, 2010). Previous studies demonstrated that recombinant proteins expressing prM and C-terminal truncated E protein (lacking the anchor region) are secreted as soluble E proteins (Allison *et al.*, 1999), making them suitable candidates for immunodiagnostic studies. (Brondyk, 2009, Palomares *et al.*, 2004, Shih *et al.*, 2002, Carter *et al.*, 2010, Lo *et al.*, 1998). This study describes the application of TBEV E-Fc proteins for TBEV diagnosis. The prM and E protein ectodomain were genetically linked to the Fc domain of rabbit IgG, and expressed in mammalian cells.

Materials and Methods

2.1 Cells

The 293T cells were cultured at 37°C in high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Nissui Pharmaceutical Co., Ltd) supplemented with 10% fetal calf serum, L-glutamine, and penicillin/streptomycin.

2.2 Serum

Ninety-seven serum samples from suspected TBE patients were tested. The serum samples were obtained from the Far Eastern Medical Center, Khabarovsk, Russia in 1998. In addition, 10 samples from Japanese encephalitis (JE)-infected Nepalese patients were obtained from Drs. Nakayama and Kurane (National Institute of Infectious Diseases, Tokyo, Japan) (Akiba *et al.*, 2001). Twenty serum samples demonstrated to have no flavivirus antibodies by the neutralization test were used as negative controls. Sixty-six samples collected from wild rodents that were captured in Kamiiso, Hokkaido between August 1996 and October 1997 were also tested for anti-TBEV antibodies. Thirty-two samples were positive for neutralizing antibodies against TBEV, and thirty-four were negative.

2.3 Preparation of recombinant antigens

A plasmid expressing the coding region of the prM and E protein ectodomain (amino acid residues 1-449) fused to the Fc region of rabbit IgG was constructed (Figure 1) using the pCAGprME plasmid containing the genes of TBEV Oshima 5-10 strain as the template (Yoshii *et al.*, 2003). The coding region of the prM and E protein ectodomain was amplified by PCR using the forward primer ggctctagagcctctgctaaccatgttc and reverse primer cggcatgcaaaaaggctgttaaaggcac. The PCR product was digested with *Sph*I and *Xba*I, ligated into a predigested pCAGGS-mCD150-RabFc plasmid (Kindly provided by Dr Konnai; Laboratory of Infectious Diseases, School of Veterinary Medicine, Hokkaido University, Japan), and the resulting plasmid was designated pCAGprME449-Fc. The plasmid was transfected into 293T cells using TransIT-LT1 reagent (Mirus Bio) following the manufacturer's

instructions. Cells were grown for 48 h at 37°C, washed in PBS, and supernatants were harvested and stored at -80°C

2.4 Characterization of the E-Fc protein

ELISA

The TBEV E-Fc protein was detected by sandwich ELISA using the monoclonal antibodies (mAb) IH4 and 4H8 to recognize the conformational epitopes of E-protein (Komoro *et al.*, 1999). To prepare the samples, the cells and supernatants were treated with 1% Triton X-100 in ELISA buffer (0.3% bovine serum albumin in phosphate buffered saline with 0.5% Tween 20). To detect membrane-bound E protein, the supernatants were harvested without treatment with Triton X-100. Samples were added to monoclonal antibody IH4-coated 96-well microtiter ELISA plates, previously blocked with Block Ace (Dai Nippon, Osaka, Japan), and E protein was detected by incubation with the biotinylated mAb IH4 or 4H8 and horseradish peroxidase (HRP)-conjugated streptavidin (Sigma). HRP activity was detected by the addition of o-phenylenediamine dihydrochloride (OPD; Sigma) in the presence of 0.03% (v/v) H₂O₂, and absorbance was measured at 450-620 nm.

SDS-PAGE and western blotting

The samples were separated on 7.5% polyacrylamide-SDS gels under reducing and non-reducing conditions. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes, blocked, and incubated for 1 h with anti-E antibodies (Yoshii *et al.*, 2004, Ikawa-Yoshida *et al.*, 2011). Membranes were then incubated with alkaline phosphatase-conjugated secondary antibody (1:6000) or HRP-conjugated anti-mouse IgG antibody (1:2000). Bands were visualized using an alkaline phosphatase kit (Merck, Darmstadt, Germany) or chemiluminescent detection reagent (GE Healthcare, United Kingdom) following the manufacturer's instructions. To detect the Fc domain, PVDF membranes were incubated with HRP-conjugated anti-rabbit IgG (1:2000), and signals were detected using chemiluminescent detection reagent (GE Healthcare, United Kingdom).

Immunoprecipitation

Cell lysates were immunoprecipitated using monoclonal antibody IH4-bound protein G-sepharose beads for 1 h at 4°C. Immunoprecipitated materials were solubilized, and then analyzed by SDS-PAGE and western blotting. The prM and E proteins were detected using anti-prM (1:800) and anti-E antibodies (1:10000), respectively, followed by alkaline phosphatase-conjugated secondary antibody (1:6000). Protein bands were visualized using an alkaline phosphatase detection kit (Merck Darmstadt Germany) following the manufacturer's instructions.

2.5 E-Fc serodiagnostic ELISA

The optimal dilutions for ELISA were determined using box dilutions. Supernatant (300 ng/ml E-Fc) was added to 96-well microtiter ELISA plates that had been sensitized with anti-rabbit IgG (1:600; Jackson ImmunoResearch) and blocked with Block Ace. Rodent or human sera (1:200) were added, followed by peroxidase-conjugated anti-mouse IgG (1:7000; Jackson ImmunoResearch) or anti-human IgG (1:20000; Jackson ImmunoResearch). Peroxidase activity was then detected using OPD, as described above. Data were reported as the P/N ratio [OD with the E-Fc antigen – OD with the negative antigen (supernatant from un-transfected 293T cells)]. The cut-off point was determined as the P/N value with the minimum difference between sensitivity and specificity.

3 Results

Characterization of E-Fc proteins

The pCAGprME449-Fc plasmid was constructed to express the coding regions of full-length prM and E ectodomain of TBEV Oshima 5-10 strain fused to the Fc region of rabbit IgG (Figure 1), and transfected into 293T cells. The E-Fc proteins were detected in cell lysates and supernatants (Figure 2a), indicating that the proteins were successfully expressed and secreted into the culture medium. Reactivity with anti-E (Figure 2b) and anti-rabbit IgG (Figure 2c) antibodies indicated that E-Fc proteins were properly processed. E-Fc protein monomers of the expected size (~75 kDa) were detected only in the cell lysates, whereas a band of ~150 kDa, corresponding to the molecular weight of E-Fc homodimers, was detected in both the cell lysates and supernatants.

In intracellular immature flaviviruses, prM and E associate to form prM-E heterodimers, which is an essential step in the proper maturation of the E protein (Allison et al., 1999). To determine if the addition of the Fc domain affected the interaction between prM and E, E protein was immunoprecipitated from cell lysates using a specific monoclonal antibody (IH4), and analyzed by western blotting (Figure 3). The E proteins were efficiently immunoprecipitated with mAb IH4, and the wild-type E protein (Ewt) and E-Fc protein-prM heterodimers could be co-immunoprecipitated. The thicker intensity of E and prM proteins in the E-Fc homodimer is due to more efficient immunoprecipitation of the homodimers as a result of direct binding of the Fc domain to the protein G-sepharose beads.

It was reported that recombinant proteins expressing the entire prM and E genes are secreted as membrane-bound subviral particles (virus-like particles lacking the nucleocapsid core), and that the E-proteins then form homodimers (Allison et al., 1999). To assess the physical form of the secreted E-Fc homodimers, sandwich ELISAs using the same mAb as the capture and detector antibody were performed in the presence or absence of detergent (Triton X-100). Lysis of the lipid envelope with Triton X-100 resulted in the dissociation of the Ewt homodimers, and loss of reactivity with the detection antibody due to competition for the epitope (Figure 4a). In contrast, the homodimerization

of E-Fc proteins was retained in the presence of Triton X-100, suggesting that homodimer formation was membrane-independent. Most fusion proteins are secreted as homodimers, presumably via a disulfide bond in the hinged domain of Fc (Lo et al., 1998). As expected, western blotting of the E-Fc protein under reducing conditions resulted in dissociation of the E-Fc homodimers, confirming that E-Fc homodimerization was due to disulfide bonds between E-Fc monomers (Figure 4b).

E-FC serodiagnostic ELISA

To determine whether the E-Fc ELISA is appropriate for serodiagnosis, serum samples were tested for TBEV antibodies, and the results were compared with neutralization tests. Sixty-six rodent samples were tested for the presence of anti-TBEV antibodies using the E-Fc ELISA, and the specificity and sensitivity were determined. At a cut off point of 1.0, the E-Fc ELISA had an equal sensitivity and specificity of 91.7% (Figure 5). Compared with the neutralization tests, the E-Fc ELISA accurately diagnosed 90.6% of the samples as TBEV positive, and 91.2% as TBEV negative (Table 1).

For the serodiagnosis of TBEV in humans, the E-Fc ELISA had 91.6% sensitivity and 91.7% specificity at a cut-off value of 1.1251 (Figure 6). Ninety-seven serum samples from suspected TBE patients were tested for anti-TBEV antibodies. The E-Fc ELISA correctly diagnosed 97.6% of the samples as positive TBEV positive (Table 2). Of the 12 samples negative in neutralization test, 10 samples were also negative using the E-Fc ELISA (Table 2). To determine if the ELISA could distinguish between anti-JEV and anti-TBEV antibodies, 10 serum samples confirmed to have anti-JEV antibodies were tested. All samples were assessed to be negative in the E-Fc ELISA, indicating that the antigen is not cross-reactive with JEV.

4 Discussion

Rapid and accurate flavivirus diagnostic tests are important for surveillance and proper medical management. The neutralization test is the most specific flaviviruses diagnostic test, but concerns over safety and the time necessary limit its application in routine diagnosis. TBEV ELISA kits that employ inactivated whole-virus as an antigen have been used extensively to detect anti-TBE antibodies. However, cross-reactivity with other flaviviruses can result in false positive results. High-biosafety-level facilities are also required for the preparation of the inactivated whole-virus antigens used in commercial ELISA kits. The generation of recombinant viral proteins is therefore an important approach to the development of flavivirus diagnostic tests that do not require high-biosafety facilities. The significance of flavivirus E proteins as antigens for immunodiagnostic and immunotherapeutic applications have been demonstrated in several studies (Hermida *et al.*, 2006, Wu *et al.*, 2004, Holbrook *et al.*, 2004). However, purification of the secreted proteins is cumbersome. Fusion partners are used purify tagged E-proteins easily by affinity chromatography providing an alternative for the production of viral proteins (Holbrook *et al.*, 2004, Beasley *et al.*, 2004). Constant fragment (Fc) of immunoglobulins are increasingly used as fusion partners in biomedical research to enhance protein expression, increase the stability of partner proteins, facilitate purification, and increase immunogenicity of partner proteins for vaccine development (Czajkowsky *et al.*, 2012, Flanagan *et al.*, 2007).

In this study, recombinant TBEV E-Fc fusion proteins were generated from a plasmid vector, and successfully expressed in 293T cells. The plasmid expressed the entire prM and E protein ectodomain to facilitate prM-E interactions, which are necessary for the proper folding and maturation of the E protein. Importantly, fusion of the E protein to the Fc domain did not inhibit prM-E heterodimer formation (Figure 3), suggesting that the two proteins folded independently and that the E domain retained some of the functionality of the Ewt. The E-Fc proteins were secreted as soluble proteins that formed detergent-stable homodimers, presumably via disulfide bonds between Fc domains. These findings are consistent with the oligomeric characteristics of soluble fusion proteins in other studies (Lo *et al.*, 1998, Loureiro *et al.*, 2011, Zaharatos *et al.*, 2011). Importantly, the E-Fc protein had no

cross-reactivity with anti-human and anti-mouse IgG antibodies (data not shown), suggesting that the antigen was a good candidate for multi-species serodiagnostic studies.

The recombinant antigens were used for the development of an ELISA to detect TBEV-specific antibodies. The E-Fc protein was used as an antigen without any further concentration or purification. The E-Fc ELISA demonstrated over 90% sensitivity and specificity, and was able to detect anti-TBEV antibodies in both human and rodent sera. Although cross-reactive antibodies from other flaviviruses commonly produce false positive results in ELISAs, Japanese encephalitis positive sera tested negative in our E-Fc ELISA. The E-Fc ELISA could therefore be used to distinguish between JEV and TBEV infections. Previous studies using E-wt derived from TBEV Oshima 5-10 as antigens for TBEV diagnostic ELISAs reported over 90% sensitivity and specificity (Ikawa-Yoshida et al., 2011, Obara et al., 2006). Our findings correlate well with E-wt ELISA results, indicating that the Fc protein has comparable antigenicity.

In summary, the E-Fc ELISA demonstrated high sensitivity and specificity, with no cross-reactivity with anti-JE antibodies. The E-Fc ELISA could therefore be applied for TBEV serodiagnosis in regions endemic for both TBEV and JEV.

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Figure Legends

Fig. 1. Construction and expression of TBEV E-Fc fusion proteins. (a) Schematic representation of TBEV E protein and E-Fc fusion proteins. The anchor region of the E protein was replaced with the Fc region of rabbit IgG.

Fig. 2. Expression and reactivity of TBEV E-Fc protein. (a) Expression of E-Fc proteins. 293T cells were transfected with pCAGprME (expressing wild type E protein/Ewt), pCAGprME449-Fc (expressing E-Fc protein), or no plasmid (mock). Forty-eight hours after transfection, E proteins were detected in the supernatant and cell lysates by sandwich ELISA using anti-E mAb 1H4 and 4H8. (b) SDS-PAGE analysis of E protein expression and reactivity using anti-E antibody (c) SDS-PAGE analysis of E protein expression and reactivity using anti-rabbit IgG antibody.

Fig. 3. Heterodimer formation of prM and E proteins. Cell lysates were immunoprecipitated using anti-E mAb 1H4, and separated by SDS-PAGE. Bands corresponding to proteins were detected using anti-prM and anti-E antibodies.

Fig. 4. Analysis of physical characteristics of the homodimer form of E-Fc proteins. (a) Comparison of the reactivity of E-Fc proteins with membrane-bound E protein homodimers (without Triton X-100) and E protein monomers (with Triton X-100). Supernatants from transfected 293T cells were treated with 1% Triton X-100. The amount of E-protein homodimers was determined before and after solubilization by ELISA using mAb 1H4 as the capture and detection antibody. (b) Western blotting of E-Fc proteins under reducing conditions (with β -mercaptoethanol) and non-reducing conditions (without β -mercaptoethanol). Proteins were detected using anti-E antibodies.

Fig. 5. Relationship between cut-off value, sensitivity, and specificity in the E-Fc ELISA. Sixty-six rodent samples were tested for TBEV antibodies. The cut-off value (broken line) was set as the point at which the difference between the sensitivity and the specificity was minimal.

Fig. 6. Relationship between cut-off value, sensitivity, and specificity for the E-Fc ELISA. One hundred and seventeen human samples were tested for TBEV antibodies. The cut-off value (broken

406 line) was set as the point at which the difference between the sensitivity and the specificity was
407 minimal.

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