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Original article

Development of a serodiagnostic IgM-ELISA for tick-borne encephalitis virus using subviral particles with *strep*-tag

Miki Nakayasu, Minato Hirano, Memi Muto, Shintaro Kobayashi, Hiroaki Kariwa, Kentaro Yoshii*

Laboratory of Public Health, Faculty of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan

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A B S T R A C T

Tick-borne encephalitis virus (TBEV) is a zoonotic agent causing severe encephalitis in humans. IgM antibody detection is useful for the serological diagnosis of TBEV infection, because IgM has high specificity for each flavivirus and indicates a recent infection. Commercial IgM-ELISA kits are somewhat expensive and difficulties in their sensitivity have been suggested due to their format and formalin-inactivated antigens. Therefore, the development of an inexpensive IgM-ELISA with high specificity and sensitivity is needed. In this study, a μ-capture ELISA was developed to detect TBEV-specific IgM antibodies using subviral particles (SPs) with *strep*-tag (*strep*-SP-IgM-ELISA). The results of our *strep*-SP-IgM-ELISA were highly correlated with diagnoses made by the neutralization test (sensitivity: 94.1%), and our *strep*-SP-IgM-ELISA could detect anti-TBEV IgM antibodies in patients who could not be diagnosed with the neutralization test. Besides, 51 of 52 positive samples by a commercial IgM-ELISA were also diagnosed as positive by our *strep*-SP-IgM-ELISA (98.1%), and our *strep*-SP-IgM-ELISA could detect anti-TBEV IgM antibodies in all samples that were inconclusive based on the commercial IgM-ELISA. Our *strep*-SP-IgM-ELISA will be useful for diagnoses in TBE-endemic areas.

1. Introduction

Tick-borne encephalitis virus (TBEV) belongs to the *Flavivirus* genus of the *Flaviviridae* family, and causes mild or moderate febrile illness and fatal encephalitis in humans. TBEV is divided into European, Siberian, and Far-Eastern subtypes. The Far-Eastern subtype is the most lethal. The virus is endemic in many parts of Europe and Asia, and more than 10,000 cases of the disease are reported annually (Banzhoff et al., 2008; Gubler, 2007; Ludlam et al., 2006; Erber et al., 2017).

Diagnostic methods for TBEV-infection in humans include direct detection of an infection by virus isolation or RT-PCR and the detection of anti-TBEV antibodies. Various tests are used for the serological diagnosis of tick-borne encephalitis (TBE). Neutralization test (NT) is considered the gold standard due to its high specificity and low cross-reactivity, and it is used as a definitive diagnosis especially in areas where two or more flaviviruses are endemic. However, the NT is time-consuming and requires a high-level biocontainment facility to handle live TBEV. The enzyme-linked immunosorbent assay (ELISA) is widely used for the serological diagnosis of TBE. ELISAs usually use formalin-inactivated virions as antigens, which can be handled safely. Anti-TBEV IgG ELISAs for detecting anti-TBEV IgG antibodies display relatively high sensitivity but often show cross-reactivity with other flaviviruses (Dobler et al., 1996; Holzmann et al.,1996; Niedrig et al., 2001). Furthermore, both the NT and IgG-ELISA require paired sera to differentiate a recent TBEV infection from a previous infection or vaccination against TBEV. IgM antibodies are the first antibodies to appear in response to initial antigen exposure, and the detection of IgM antibodies indicates recent infection. Diagnostic tests for anti-TBEV IgM are usually more specific than IgG tests in terms of cross-reactivity with other flaviviruses (Dobler, 2017). Anti-TBEV IgM-ELISAs are also commercially available, but they are somewhat expensive and some employ a two-layer ELISA format using formalin-inactivated virions coated directly on an ELISA plate, which may produce false-negative results due to IgG contamination (Dobler, 2017). Furthermore, inactivation of the virion by formalin was reported to alter its antigenicity (Heinz et al., 1995). Therefore, serological diagnostic methods that are safe, simple, and sensitive to TBEV must be developed.

Expression of the entire prM and E genes leads to the secretion of membrane-bound subviral particles (SPs) (Allison et al., 1995). These SPs consist of a viral envelope without a nucleocapsid or genomic RNA. Because SPs maintain antigenicity similar to that of authentic virions, they have been applied as substitutes for infectious virions in serological diagnoses (Ferlenghi et al., 2001; Ikawa-Yoshida et al., 2011; Obara et al., 2006). In a previous study, we developed SPs with a small peptide tag, *strep*-tag, which has high binding affinity for *strep*-tactin, and applied them to a new IgG-ELISA for a broad range of mammalian

* Corresponding author at: Laboratory of Public Health, Faculty of Veeterinary Medicine, Hokkaido University, kita-18 nishi-9, kita-ku, Sapporo, Hokkaido 060-0818, Japan.
E-mail address: kyoshii@vetmed.hokudai.ac.jp (K. Yoshii).

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species (Inagaki et al., 2015). This multi-species ELISA using SPs with *strep*-tag (*strep*-SPs) showed high specificity and sensitivity.

In this study, an ELISA for the detection of anti-TBE IgM was developed using *strep*-SPs of TBEV expressed in mammalian cells as antigens. The ELISA was evaluated using serum samples from TBE-suspected patients and the results were compared to those obtained using the NT and a commercial IgM-ELISA kit.

2. Materials and methods

2.1. Cells and virus

Baby hamster kidney fibroblast (BHK) cells were cultured in Eagle's minimum essential medium (Wako, Osaka, Japan) containing 8% fetal bovine serum (FBS). Human embryonic kidney 293 T (HEK293 T) cells were cultured at 37 °C in high-glucose Dulbecco's Modified Eagle's medium (Wako) supplemented with 10% FBS and penicillin/streptomycin. The Oshima 5–10 strain of TBEV was isolated from a dog in Hokkaido in 1995 and classified as the Far Eastern subtype (Takashima et al., 1997). The virus was propagated in BHK cells.

2.2. Serum

A total of 95 serum samples from 43 TBE-suspected patients were obtained from the Far Eastern Medical Center, Khabarovsk, Russia in 1998, including 86 paired serum samples from 34 patients and 9 single samples. The study was approved by the ethics committee of the Faculty of Veterinary Medicine, Hokkaido University (Approval No. 26-1).

2.3. Plasmid construction and transfection

The plasmid pCAG-TBEV-M-*strep*E, which is the pCAGGS plasmid encoding the TBEV (Oshima 5–10 strain) signal sequence for prM, the prM gene, the signal sequence for E, and the E gene N-terminally tagged with *strep*-tag sequences, was constructed previously (Inagaki et al., 2015).

The plasmid was transfected into HEK293 T cells using X-tremeGENE HP DNA Transfection Reagent (Roche, Basel, Switzerland), following the manufacturer's instructions. The cells were grown for 24 h at 37 °C, and the culture supernatant was harvested and stored at −80 °C. The supernatant containing SPs was concentrated by polyethylene glycol (PEG) precipitation. For PEG precipitation, the supernatant was mixed with final concentrations of 10% PEG 8000 and 1.9% NaCl, and incubated for 2 h at 4 °C. The SPs were precipitated by centrifugation at 16,000 × *g* for 20 min and resuspended in phosphate-buffered saline (PBS).

2.4. Serodiagnostic ELISA

Anti-human IgM goat IgG (1:300 dilution in carbonate buffer; Bethyl Laboratories, Inc., Montgomery, TX, USA) was added to 96-well microplates. After overnight incubation at 4 °C, the plates were washed five times with PBS with Tween 20 (PBST) before adding Block Ace (DS Pharma Biomedical, Osaka, Japan) and incubated at 37 °C for 1 h. After washing, the serum samples were added (1:100 dilution in PBST containing 1% bovine serum albumin), and the plates were incubated at 37 °C for 1 h before washing again. *Strep*-SPs were added (1:20 dilution in PBST containing 1% bovine serum albumin) and the plates were incubated at 37 °C for 1 h and washed again. The *strep*-SP antigen was detected using *strep*-tactin conjugated with horseradish peroxidase (Bio-Rad, Hercules, CA, USA). The color reaction was developed by adding *o*-phenylenegiamine dihydrochloride (Wako) in the presence of 0.07% H_2O_2 for 30 min at room temperature, and the optical density (OD) was measured at 450–630 nm. Negative control antigens were prepared from the supernatant of untransfected HEK293 T cells. The results were recorded as the positive/negative ratio (P/N ratio; OD value using *strep*-SPs to that using negative control antigen). All experiments were conducted in duplicate. We conducted every assay with a positive control in which a high level of anti-TBEV IgM was detected with a commercial IgM-ELISA.

The commercial Immunozym FSME IgM kit (Progen Biotechnik, Heidelberg, Germany) was used for comparison with the *strep*-SP-IgM-ELISA. Tests were conducted according to the manufacturer's instructions.

2.5. Neutralization test

TBEV Oshima 5–10 strain (Takashima et al., 1997) was incubated with serially diluted serum and inoculated onto BHK cells at 37 °C for 1 h. After inoculation, the cells were incubated with minimum essential medium containing 1.5% carboxymethyl cellulose and 2% FBS for 4 days. Then the cells were fixed with 10% formalin for 30 min at room temperature and stained with 0.1% crystal violet for 10 min. Serum samples that produced a 50% reduction in plaque formation by TBEV on BHK cells in 12-well plates were diagnosed as positive. Serum samples ≥ 1:20 were judged to be positive for neutralizing antibodies against TBEV. The neutralizing antibody titer was determined as the reciprocal of the highest serum dilution that reduced viral plaque counts by 50% or more.

3. Results

In this ELISA, TBEV specific antibodies in serum samples were captured by anti-human IgM antibodies coated onto a plate. The *strep*-SPs reacted with the TBE-specific IgM antibodies and were detected with enzyme-conjugated *strep*-tactin. The optimal concentration for the *strep*-SP-IgM-ELISA was evaluated using serially diluted sera determined as positive or negative by a commercial ELISA. The *strep*-SP-IgM-ELISA method could distinguish positive sera with TBEV-specific IgM antibodies from a negative serum sample (Fig. 1). The P/N ratio of the negative serum was about 1.0, showing that the serum did not react with *strep*-SP antigens when compared to negative control antigens. In contrast, those of the positive samples were greater than 2.0. Because the maximum values of the P/N ratio were obtained at 100- and 200-fold dilutions, the optimal serum dilution was set to 100-fold.

Fig. 2 shows the distribution of P/N ratios for the serum samples in the *strep*-SP-IgM-ELISA. The samples were separated into two major groups: serum samples with P/N ratios < 1.3 were presumed negative for TBEV-specific IgM antibodies, while those > 1.9 were presumed positive. Because the two samples with P/N ratios between 1.4 and 1.7 were determined to be positive by NT and Smirnov-Grubbs test ($p < 0.01$), the cut-off value of the *strep*-SP-IgM-ELISA was set at P/N = 1.3.

Paired serum samples were collected from 43 patients suspected as

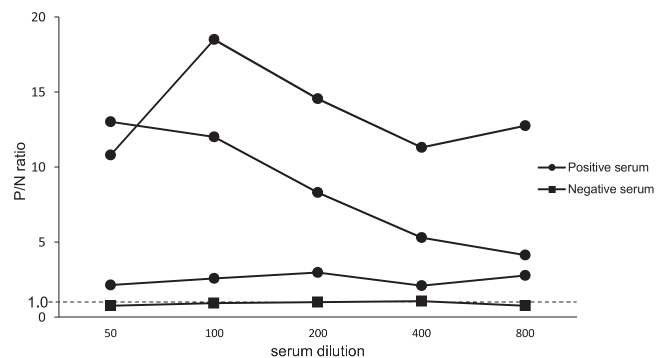

Fig. 1. Titration curve of TBEV antibody-positive human serum samples using the strep-SP-IgM-ELISA. One TBEV-negative serum sample was used as a control.

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