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Development of a serodiagnostic multi-species ELISA against Tick-borne encephalitis virus using subviral particles

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Abbreviations

AP: alkaline phosphatase

BHK-21: baby hamster kidney fibroblast

ELISA: enzyme-linked immunosorbent assay

FBS: fetal bovine serum

HEK293T: human embryonic kidney 293T

HRP: horseradish peroxidase

JEV: Japanese encephalitis virus

mAb: monoclonal antibodies

NT: neutralization test

OD: optical density

OPD: *o*-phenylenediamine dihydrochloride

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PEG: polyethylene glycol

SDS: sodium dodecyl sulfate

SPs: subviral particles

Strep-SP ELISA: ELISA using Strep-SPs

Strep-SPs: SPs with Strep-tag

TBEV: Tick-borne encephalitis virus

WNV: West Nile virus

Abstract

Tick-borne encephalitis virus (TBEV) is a zoonotic agent causing severe encephalitis in humans. A wide range of animal species could be infected with TBEV in endemic areas. A serological survey of wild animals is effective in identifying TBEV-endemic areas. Safe, simple, and reliable TBEV serodiagnostic tools are needed to test animals. In this study, ELISA was developed to detect anti-TBEV specific antibodies in multi-species of animals, using recombinant subviral particles (SPs) with an affinity tag and protein A/G. A Strep-tag was fused at the N terminus of the E protein of the plasmid coding TBEV prME. The E proteins with Strep-tag were secreted as SPs, of which Strep-tag was exposed on the surface. The tagged E proteins were associated with prM. The SPs with Strep-tag were applied as the antigen of ELISA, and TBEV-specific antibodies were detected by the protein A/G. Compared to neutralization test results, the ELISA showed 96.8% sensitivity and 97.7% specificity in rodents and 95.1% sensitivity and 96.0% specificity in humans, without cross-reactivity with antibodies to Japanese encephalitis virus. These results indicate that our ELISA would be useful to detect TBE-specific antibodies in a wide range of animal species.

Keywords; tick-borne encephalitis virus, flavivirus, subviral particles, ELISA

Introduction

Tick-borne encephalitis virus (TBEV) belongs to the Flaviviridae family and Flavivirus genus, and causes mild or moderate febrile illness, and subacute or 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 (Banzhoff et al., 2008; Gubler, 2007; Ludlam et al., 2006), and more than 10,000 cases of the disease are reported annually. Therefore, it is an important disease within the context of public health. TBEV is maintained in transmission cycles between *Ixodid* ticks and wild mammalian hosts, particularly rodents (Mansfield et al., 2009). A wide range of animal species are also infected with TBEV by the bite of infected ticks. To control clinical disease with vaccination of humans, it is important to specify the TBEV-endemic area and design an effective vaccination plan. An epizootiological survey of wild animals such as rodents, deer and goats is effective in detecting TBEV-endemic areas (Gerth et al., 1995; Klaus et al., 2012; Lindhe et al., 2009; Yoshii et al., 2011). Serological diagnosis is a useful method to perform an epidemiological survey.

A neutralization test (NT) has been used in areas where two or more flaviviruses are endemic, as it has high specificity for each virus. However, NT is time-consuming, and high-biosafety-level facilities are required to handle the live virus. An enzyme-linked immunosorbent assay (ELISA), based on inactivated tick-borne encephalitis (TBE) virions, is also widely used. ELISA is a useful diagnostic method because it could be handled safely. However, many commercial TBE-ELISA kits have shown cross-reactivity against antibodies to other flaviviruses (Dobler et al., 1996;

Holzmann et al., 1996; Niedrig et al., 2001a). Commercial ELISA kits are applicable mainly to infection in humans. A commercial ELISA kit for a wide range of animal species is currently available, but it is somehow expensive. Therefore, it is important to develop a safe and simple serological diagnostic method that could be applied to a wide range of animal species.

The expression of the entire prM and E genes are secreted as membrane-bound subviral particles (SPs) (Allison et al., 1995). The 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 diagnosis (Ferlenghi et al., 2001; Ikawa-Yoshida et al., 2011; Obara et al., 2006).

In this study, we constructed recombinant SPs in which the affinity tag, Strep-tag, was expressed on the surface. Furthermore, the SPs with Strep-tag (Strep-SPs) were applied to a new serodiagnostic ELISA that could detect anti-TBEV antibodies in a wide range of animal species by using protein A/G. The ELISA was evaluated by comparing the results with those from the NT.

Materials and Methods

Cell and virus. Baby hamster kidney fibroblast (BHK-21) cells were cultured on Eagle's minimum essential medium (Wako, Osaka, Japan) containing 8% fetal bovine serum (FBS) and were used for NTs. Human embryonic kidney 293T (HEK293T) cells were cultured at 37 °C in high-glucose Dulbecco's Modified Eagle's medium (Wako) supplemented with 10% FBS, L-glutamine, and penicillin/streptomycin.

The Oshima 5-10 strain of TBEV was used. It was isolated from a dog in

Hokkaido in 1995 and classified as the Far Eastern subtype (Takashima et al., 1997). The virus was propagated by intracerebral inoculation of suckling mice.

Serum. Seventy four serum samples from wild rodents (53 *Myodes rufocanus bedfordiae*, 18 *Apodemus speciosus* and one *A. argenteus*) captured in Hokuto, Hokkaido, between August 1996 and October 1997 were tested for anti-TBEV antibodies. The animal experiments were performed in accordance with the recommendations in the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology. The experimental protocols were approved by the Animal Care and Use Committee of the Hokkaido University (approval number: 13-0119).

One hundred and seven serum samples from TBE-suspected patients were also tested. The serum samples were obtained from the Far Eastern Medical Center, Khabarovsk, Russia in 1998. Ten samples from Japanese encephalitis virus (JEV) -infected Nepalese patients were obtained from Drs. Nakayama and Kurane (National Institute of Infectious Diseases, Tokyo, Japan). The study was approved by the ethics committee of Graduate School of Veterinary Medicine, Hokkaido University (Approved No. 26-1).

Plasmid construction. The pCAG-TBEV-M-E and pCAG-WNV-M-E plasmids, which are pCAGGS-based plasmids encoding the TBEV (Oshima 5-10 strain) or West Nile Virus (WNV; the NY-99 flamingo382-99 strain) signal sequence of prM, prM and E genes, was constructed as described previously (Yoshii et al., 2003).

To construct the pCAG-TBEV-M-StrepE and pCAG-WNV-M-StrepE plasmids, the coding sequence for the Strep-tag from the pCAG-OSF plasmid (kindly provided by Dr. Kamitani, Osaka University) was inserted before that for the E proteins. The pCAG-TBEV-M-E and pCAG-WNV-M-E plasmids were digested by *Age I* and *Pvu II*, and *Cla I* and *Bgl II*, respectively. The fragments for coding sequences for the C-terminal prM and signal sequence for E, Strep-tag, Flag-tag, and N-terminal E were amplified by polymerase chain reaction (PCR) and inserted into the digested plasmids using the In Fusion cloning kit (TAKARA, Shiga, Japan; Fig. 1).

Transfection. The plasmid was transfected into HEK293T cells using the 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 cells and culture supernatant were harvested and stored at -80˚C.

The supernatant containing SPs was concentrated by polyethylene glycol (PEG) precipitation or ultracentrifugation. In PEG precipitation, the supernatant was mixed with final concentrations of 10% PEG 8,000 and 1.9% NaCl, and incubated for 2 h at 4˚C. The SPs were precipitated by centrifugation at 11,000 x g for 20 min and were resuspended in phosphate-buffered saline (PBS). In ultracentrifugation, the supernatant was precipitated by centrifugation at 28,000 rpm for 1 h in CP80MX using P40ST-1239 rotor (Hitachi Koki, Tokyo, Japan), and were resuspended in PBS.

Detection of the E proteins by ELISA. The TBEV-E proteins in the cells and culture supernatant were treated with or without 1% Triton X-100 and detected by sandwich ELISA using the monoclonal antibodies (mAb) 1H4 (1:50) and 4H8 (1:1,000), recognizing the conformational epitopes of the E protein (Komoro et al., 2000). To detect the E proteins with the Strep-tag, the E proteins were captured by mAb 1H4 (1:50) and the Strep-tag was detected by Strep-Tactin conjugated with horseradish-peroxidase (HRP; 1:5,000; BIO RAD, Hercules, USA). The color reaction was developed by adding *o*-phenylenegiamine dihydrochloride (OPD; 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.

SDS-PAGE and Western Blotting. The samples were separated on 10% polyacrylamide-sodium dodecyl sulfate (SDS) gels or commercial 5-12% gradient polyacrylamide-SDS gels (ATTO, Tokyo, Japan) under non-reducing conditions. Proteins were transferred to polyvinylidene difluoride membranes, blocked, and incubated overnight with anti-TBEV E rabbit antibody (1:750 or 1:1,000)(Yoshii et al., 2004), anti-WNV E mouse antibody (1:1,000; Millipore, Darmstadt, Germany), or alkaline phosphatase (AP) -conjugated Strep-Tactin (1:1,000 or 1:5,000; BIO RAD). The rabbit antibodies were detected by AP or HRP-conjugated anti-rabbit IgG secondary antibodies (1:5,000; Jackson Immunoresearch, Pennsylvania, USA), and the mouse antibodies were detected by HRP-conjugated anti-mouse IgG secondary antibodies (1:10,000).

For the affinity precipitation, the E proteins in the cell lysate or supernatant were

precipitated using a Strep-Tactin Sepharose beads kit (IBA, Gottingen, Germany) for 4 h at 4˚C. They were used for subsequent SDS-polyacrylamide gel electrophoresis (PAGE) and western blotting analysis. The proteins on the membranes were incubated with the anti-prM (1:300) and anti-E antibodies (1:750), followed by the AP-conjugated secondary antibody (1:10,000).

Bands were visualized using the AP detection kit (Merck, Darmstadt, Germany) or chemiluminescent HRP substrate (Millipore).

Sucrose density-gradient centrifugation. The SPs were centrifuged by overnight equilibrium density centrifugation in a 10-50% sucrose gradient at 28,000 rpm at 4˚C for 16 h. Fractions of 0.5 ml were collected from the top of the tubes and analyzed by ELISA.

Serodiagnostic ELISA. Ninety-six well EIA plates (Corning, New York, USA) were coated with Strep-Tactin (1:1,000) overnight at 4˚C and then blocked with Block Ace (DS Pharma Biomedical, Osaka, Japan). After washing with PBS containing 0.05% Tween 20, the antigen Strep-SPs were added (1:5 diluted of PEG precipitated supernatant), followed by serum samples (1:200). The TBEV-specific antibodies were detected by Protein A/G conjugated with HRP (1:2,000; Thermo Fisher Scientific, Massachusetts, USA), and reacted with OPD. Negative control antigens were prepared from the supernatant of untransfected HEK293T cells. The results were recorded as the P/N ratio (OD value using Strep-SPs to that using negative control antigen). The results of ELISA were compared with those of NT and were assessed by Pearson's chi-squared test in a 2 × 2 contingency table.

The commercial Immunozym FSME IgG kit (Progen Biotechnik, Heidelberg,

Germany) were used for comparison with the strep-SP ELISA.

Neutralization test. TBEV was incubated with serially diluted serum and inoculated to BHK cells. The cells were incubated with minimum essential medium containing 1.5 % carboxymethyl cellulose and 2 % FBS for 4 days. After 4 days of incubation, the cells were fixed with 10 % formalin and stained with 0.1 % crystal violet. Serum samples that produced a 50 % reduction in plaque formation of the TBEV on BHK cells in 12-well plates were determined by immunocytochemical staining. Serum samples ≥1:20 were judged to be positive for neutralizing antibodies against TBEV.

Results

Expression and characterization of Strep-SPs. The expression of the entire prM and E proteins, leading to the secretion of SPs, has been reported (Allison et al., 1995). To obtain the SPs on which the Strep-tag was surficially expressed, we constructed a plasmid expressing the prM and E proteins with the tag fused to the N-terminus of the E protein (the pCAG-TBEV-M-StrepE and pCAG-WNV-E-StrepE plasmids; Fig. 1). The Strep-tag is an eight-residue minimal peptide sequence (WSHPQFEK), with an intrinsic affinity toward streptavidin. Strep-Tactin was developed to improve the peptide-binding capacity of streptavidin. (Schmidt, 2007).

HEK293T cells were transfected with pCAG-TBEV-M-StrepE or pCAG-TBE-M-E, and the expression and secretion of the E proteins with the Strep-tag were detected by Western blotting. In both the cell lysate and supernatant, the E proteins were detected by anti-E specific antibodies, and the E proteins with the Strep-tag were detected by Strep-Tactin from the cells transfected with pCAG-TBEV-M-StrepE (Fig.

2A). The band for the E proteins with the Strep-tag from pCAG-TBEV-M-StrepE migrated slower than that of the E proteins without the tag from pCAG-TBE-M-E. The transfection of pCAG-WNV-M-StrepE resulted in the expression and secretion of the E protein of WNV with Strep-tag, as observed in the transfection with pCAG-TBEV-M-StrepE (Fig. 2B).

To examine whether the Strep-tag was expressed on the surface of the secreted SPs, the E proteins in the secreted SPs were directly captured by anti-E specific antibody and Strep-tag expressed on the surface of SPs was detected by Strep-Tactin by ELISA (Fig. 2C). While no reactivity was observed in the SPs from pCAG-TBE-M-E, the Strep-tag of the E proteins in the SPs from pCAG-TBEV-M-StrepE reacted with Strep-Tactin. These data indicated that the Strep-tag of the E protein in the supernatant was exposed on the surface of the SPs.

In intracellular immature virions, the prM and E proteins are associated to form prM-E heterodimers, which is an essential step in the proper maturation of the E protein (Allison et al., 1999). To confirm whether the addition of the Strep-tag affect the interaction between prM and E, the E protein with the Strep-tag in the cell lysate was precipitated by Strep-Tactin beads and separated by SDS-PAGE. The E and prM proteins were detected by Western blotting (Fig. 2d). The bands for E and prM were detected in the precipitated cell lysate. This result indicated that the interaction between the E and prM proteins was not affected by addition of the Strep-tag.

To examine the physical properties of the expressed E protein with the Strep-tag in the medium, the supernatants were subjected to sucrose density-gradient centrifugation analysis. The E protein in each gradient fraction was detected by ELISA. The signals of the E protein in the Strep-SPs were seen at a sucrose density of 1.13-1.16 g/cm^3, which

253 was almost identical to that of SPs (1.12-1.14 g/cm^3), and it was disturbed by the
254 treatment of Triton-X 100 (Fig. 3). This result indicated that the E protein with the
255 Strep-tag was secreted as a membrane-bound structure similar to SPs.
256
257 ***Serodiagnostic Strep-SP ELISA.*** To examine whether ELISA using Strep-SPs
258 (Strep-SP ELISA) as the antigen could be used to detect the TBE specific antibodies in
259 serum samples from TBEV-infected individuals, several serially diluted wild rodent and
260 human sera were examined (Fig. 4). In this ELISA, Strep-SPs were captured by plate
261 coated with Strep-Tactin. The TBEV-specific antibodies in the serum reacted with the
262 Strep-SPs and detected by Protein A/G. In both rodent and human sera, all NT positive
263 sera had high P/N ratios, while NT negative sera had a P/N ratio of about 1.0 in
264 Strep-SP ELISA.
265 To determine whether Strep-SP ELISA was appropriate for serodiagnosis, rodent
266 and human serum samples were tested for TBEV antibodies, and the results were
267 compared with NT results. Thirty-one TBEV positive and 43 negative rodent serum
268 samples captured in TBEV-endemic areas were tested using Strep-SP ELISA, and the
269 specificity and sensitivity were determined by comparison with the NT results using the
270 corresponding cut-off value (Fig. 5A; Table 1). The difference between the sensitivity
271 and specificity was minimal when a cut off value of 1.34 was used. In this case, for
272 serodiagnosis of TBEV in rodents, Strep-SP ELISA had 96.8% sensitivity and 97.7%
273 specificity compared with the NT result ($P < 0.001$). We also tested 107 TBE suspected
274 human serum samples (Fig. 5B; Table 2). There were 82 TBEV-positive sera and 25
275 negative sera. The sensitivity of Strep-SPs ELISA was 95.1% and the specificity was
276 96.0% when the cut off value was 1.202 ($P < 0.001$). To determine if Strep-SP ELISA

277 could distinguish between anti-JEV and anti-TBEV antibodies, 10 serum samples
278 confirmed to have anti-JEV antibodies were tested. While commercial ELISA gave
279 inconclusive results in 3 samples, all samples were assessed to be negative in Strep-SP
280 ELISA, indicating that Strep-SP ELISA is not cross-reactive with the JEV antibody
281 (Table 3).
282

Discussion

To identify TBEV endemic areas, it is important to conduct a serological survey of wild animals (Yoshii et al., 2011). NT is the serodiagnostic method used to detect TBEV-specific antibodies regardless of animal species, but it requires time and a high-level of biocontainment for its application. Therefore, the application of SPs could be a useful approach to developing a serological diagnosis in a wide range of species without high-biosafety facilities. In this study, we prepared recombinant SPs of TBEV with the Strep-tag and applied them to serodiagnostic ELISA for TBEV-infected human and wild rodent sera.

The flavivirus E proteins have been shown to be important for immunodiagnostic and immunotherapeutic applications in several studies (Hermida et al., 2006; Holbrook et al., 2004; Wu et al., 2004). However, purification of the secreted proteins is cumbersome. Some fusion partners were used to easily purify tagged E-proteins (Beasley et al., 2004; Holbrook et al., 2004). The Strep-tag could be easily fused to a recombinant polypeptide in various fashions (Schmidt, 2007), and in this study, this tag was fused to the E protein for efficient capture of the Strep-SP antigen in ELISA without affecting antigenicity. In a recent study, it was reported that the antigenic structures of the E proteins were disturbed when the ELISA plate was coated directly with the viral particles as a solid-phase antigen (Stiasny et al., 2006). To avoid this antigenic disturbance by direct coating, the Strep-SPs were captured by Strep-Tactin coated on the plate in our ELISA.

In native virions, the E proteins are homodimers, extending in parallel on the surface of the viral envelope membrane (Rey et al., 1995). The E protein is composed of

ectodomains (I, II, and III), stem, and a trans-membrane anchor region. Domain III is known to possess the major antigenic epitopes (Chávez et al., 2010). In this study, the Strep-tag was fused at the N terminus of E, without disturbing the antigenic and conformational structures. Our results indicated that the Strep-tagged E proteins were secreted as SPs and the Strep-tag was exposed on the surface of the particles (Fig. 2).

The prM and E proteins must associate with each other shortly after synthesis for proper folding of E proteins (Lorenz et al., 2002). The conversion of immature virus particles to mature virions occurs in the secretory pathway and coincides with cleavage of prM into pr and M by the Golgi-resident furin or a furin-like enzyme (Stadler et al., 1997). The pr-region of prM is thought to stabilize the E protein and keep it from undergoing rearrangement to the fusogenic form in the reduced pH environment of the early secretory pathway (Guirakhoo et al., 1991). In a previous study, intracellular E proteins that were not fully mature were applied as antigens of serodiagnostic ELISA (Yoshii et al., 2003), but they showed relatively lower sensitivity and specificity, indicating that proper maturation is important to retain native antigenicity. In our study, addition of the Strep-tag in the E protein did not disturb the interaction between prM and E (Fig. 2D), and membrane-bound structures similar to authentic SPs were secreted in the culture fluid (Fig.3). These results suggested that the E protein with the Strep-tag was secreted through the proper maturation and secretory pathways by the support of prM.

In Strep-SP ELISA, Strep-SPs were used to detect TBEV-specific antibodies, and protein A/G was applied to detect the antibodies in a wide range of animal species. Strep-SP ELISA of both rodent and human sera showed over 95% sensitivity and specificity, indicating that it had enough diagnostic accuracy for screening purposes. It

was reported that infection and/or vaccination with other flaviviruses, including yellow fever virus, dengue virus, WNV, and JEV, could induce cross-reactive antibodies (Dobler et al., 1996; Holzmann et al., 1996; Niedrig et al., 2001a). Our Strep-SP ELISA showed no cross-reactivity with JEV antibodies while the commercial TBEV ELISA showed cross-reactivity in previous studies (Niedrig et al., 2001b; Obara et al., 2006). A recent report showed that the broadly flavivirus cross-reactive antibodies recognized antigenic sites that were not fully accessible on the surfaces of native TBE virions, but became significantly more exposed after disintegration of the envelope (Stiasny et al., 2006). It also suggested that the inactivation of virions in commercial ELISA might cause denaturation and/or disintegration on the virions, resulting in cross-reactivity against numerous flaviviruses (Heinz et al., 1995). However, in our Strep-SP ELISA, Strep-SPs did not require formalin inactivation, and it might lead to conservation of the native structure of particles in which the cross-reactive epitope were not fully accessible.

In our ELISA, Protein A/G was used to detect specific antibodies in rodent and human sera. Protein A/G is a product that has the Fc-binding domains of both Protein A and Protein G, and could be used to detect immunoglobulin in serological diagnosis (Bhide, 2004), due to its binding spectra toward polyclonal immunoglobulin from a wide range of animal species (Eliasson et al., 1988). In this study, our Strep-SP ELISA was examined in human and wild rodent antibodies. This ELISA could be further applied to numerous animal species that could be mammalian hosts of TBEV. Binding capacity of Protein A/G to mammalian immunoglobulin differs among species (Stobel et al., 2002), and our ELISA should be examined in more diverse species for efficacy of detection.

In summary, Strep-SP ELISA demonstrated high sensitivity and specificity, with no cross-reactivity with anti-JEV antibodies. Further examination by using sera from imported dengue case or yellow fever vaccination would expand the availability of the ELISA for diagnosis in human. The features of SP secretion are common in flaviviruses (Allison et al., 1995; Fonseca et al., 1994; Mason et al., 1991; Pincus et al., 1992). The strategy developed in this study could be applied to serodiagnostic methods for other flaviviruses, as shown in the results of the secretion of Strep-SPs of WNV. Strep-SP ELISA would be useful for epidemiological and epizootiological surveys in areas where several flaviviruses are endemic.

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502
503
504

505 **Figure legend**

506

507 **Fig. 1. Schematic of the plasmid expressing SPs with Strep-tag.**

508 The sequence for the Strep-tag was fused to the N' terminus of the E protein.

509

510 **Fig. 2. Expression and secretion of the E protein with Strep-tag.**

511 (A) HEK293T cells were transfected with pCAG-TBE-M-StrepE, pCAG-TBE-M-E, or
512 no plasmid (mock). Twenty-four hours after transfection, the E protein in the cell lysate
513 and supernatant was detected by Western blotting analysis using anti-E antibody or
514 Strep-Tactin.
515 (B) HEK293T cells were transfected with pCAG-WNV-M-StrepE, or no plasmid
516 (mock). Twenty-four hours after transfection, the E protein in the cell lysate and
517 PEG-precipitated supernatant was detected by Western bloting analysis using
518 anti-WNV-E-antibody or Strep-Tactin.
519 (C) The E protein secreted into the culture medium was captured by anti-E mAb without
520 detergent and the reactivity against Strep-Tactin was examined.
521 (D) Interaction between the prM and StrepE proteins. The E protein with Strep-tag in
522 the cell lysate was precipitated using Strep-Tactin and separated by SDS-PAGE. Bands
523 corresponding to each protein were detected using anti-prM and anti-E antibodies.

524

525 **Fig. 3. Equilibrium banding profiles of SPs.**

526 Supernatant of cells transfected with pCAG-TBE-ME (gray circle) or
527 pCAG-TBEV-M-StrepE with or without treatment with TritonX-100 (black or white
528 circles, respectively) was subjected to equilibrium density centrifugation and was

examined for distribution of the E protein by ELISA. The level of the E protein in the peak fraction was set at 1.0.

Fig. 4. Titration curve of TBEV antibody positive (A) wild rodents or (B) human sera for Strep-SP ELISA.

Two TBEV negative sera were used as controls. All sera were serially diluted and tested in duplicate.

Fig. 5. Relationship between cut-off value, sensitivity, and specificity for the Strep-SP ELISA.

Seventy-four wild rodents sample (A) and 112 human (B) samples were tested for TBEV antibodies.

Fig. 1. Inagaki *et al*.,

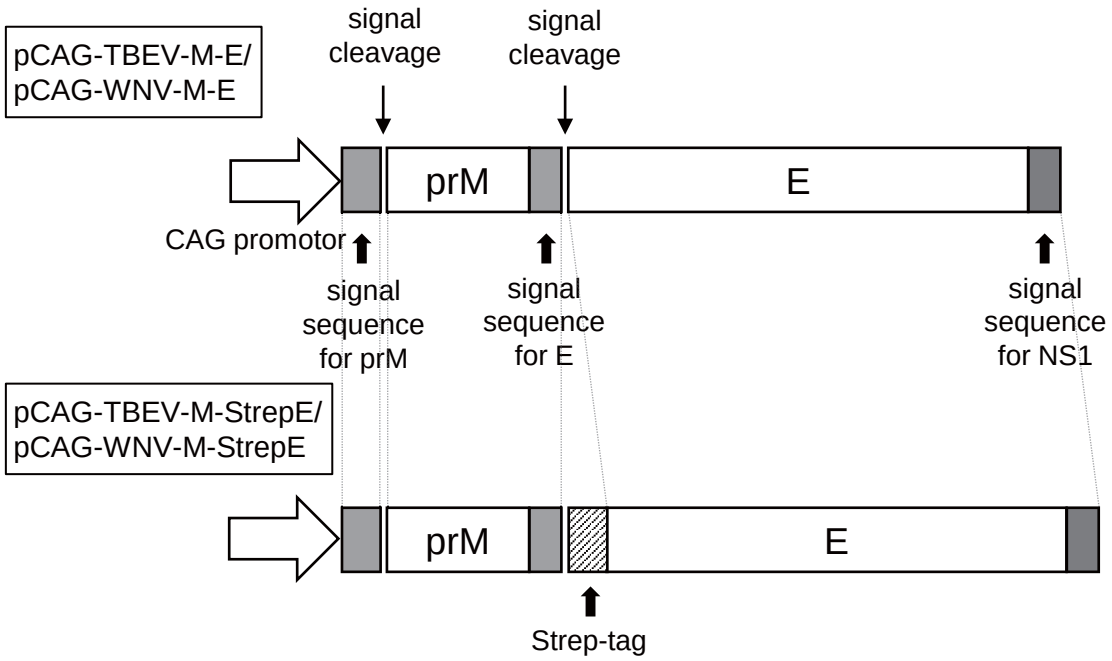


Fig. 2. Inagaki *et al*.,

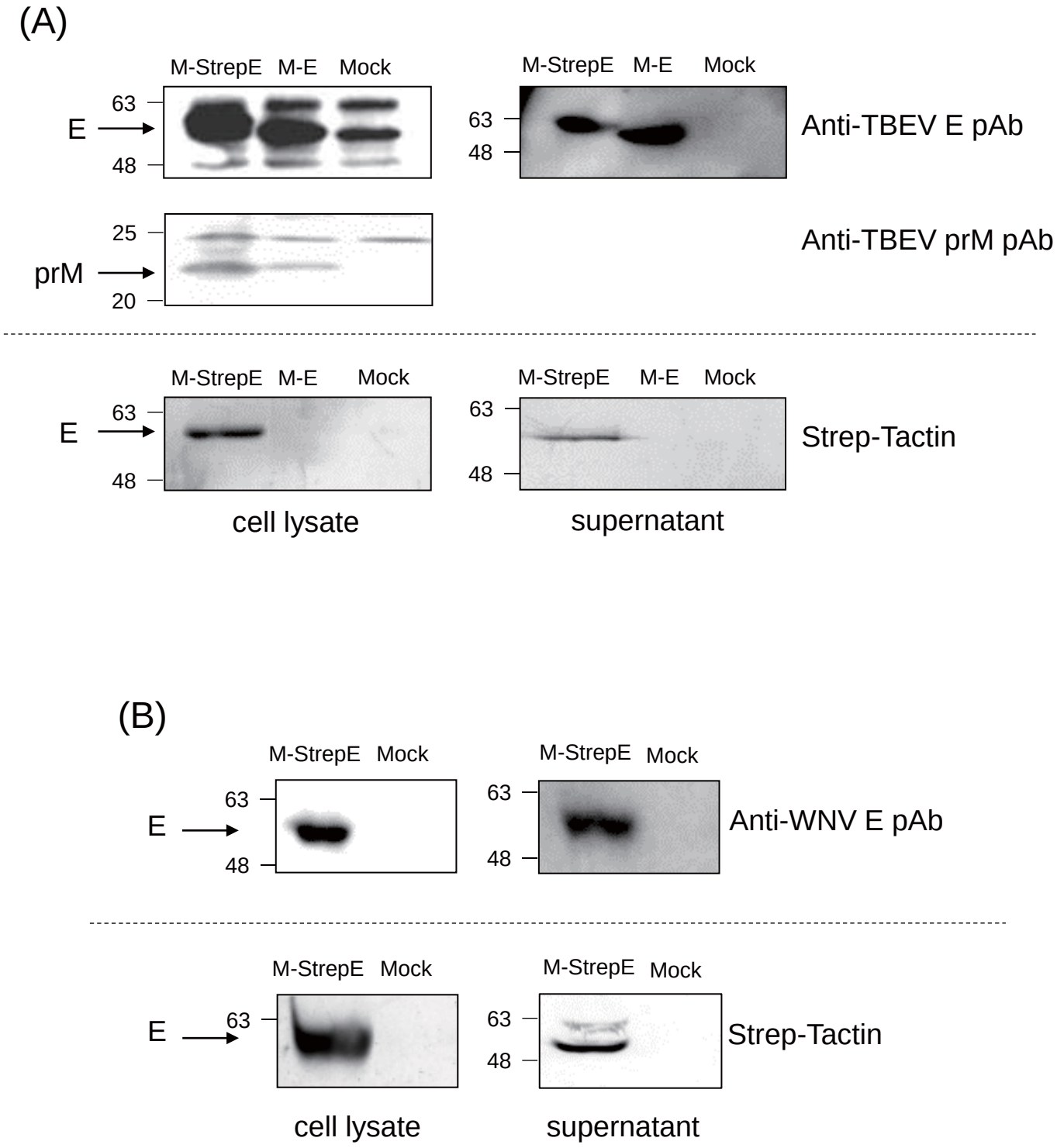


Fig. 2. Inagaki *et al*.,

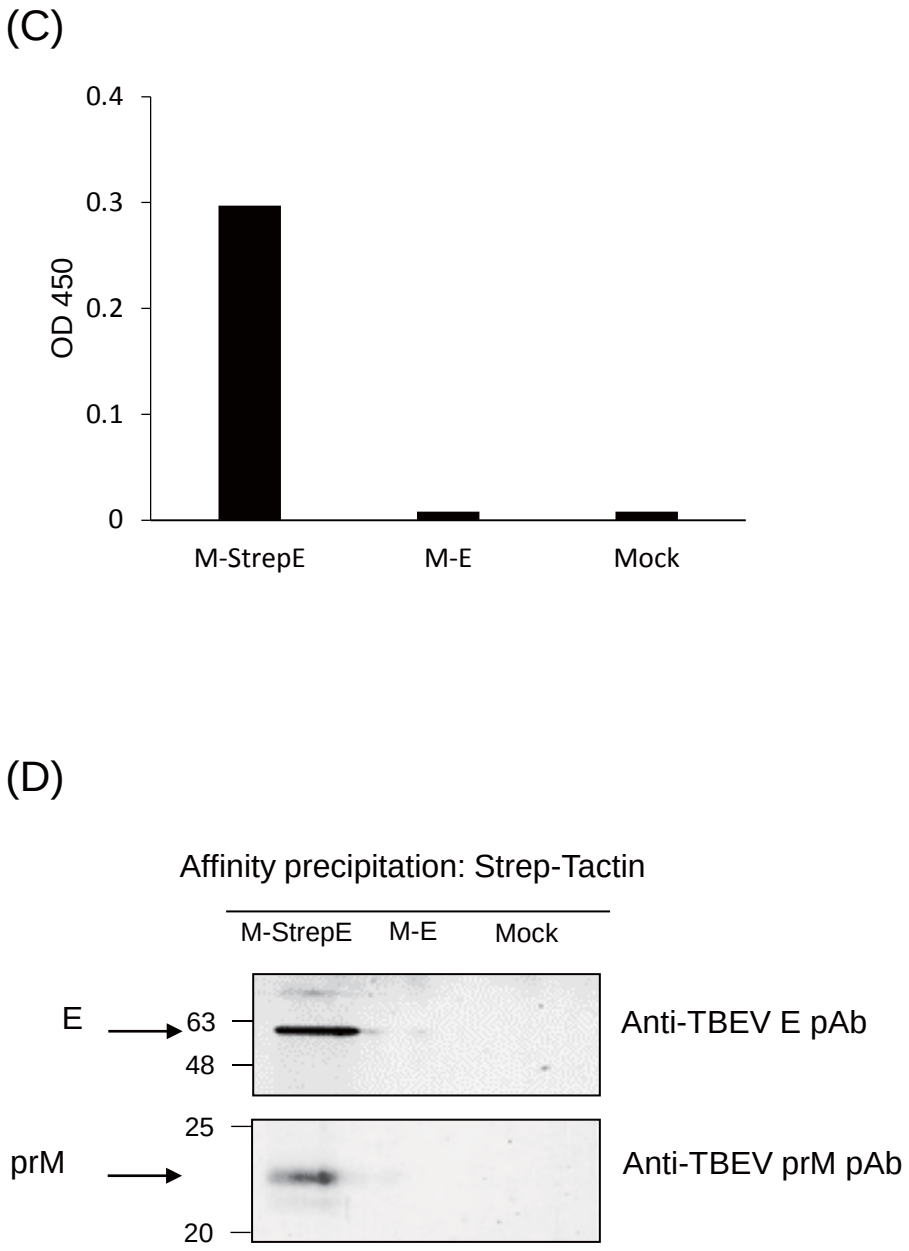


Fig. 3. Inagaki *et al*.,

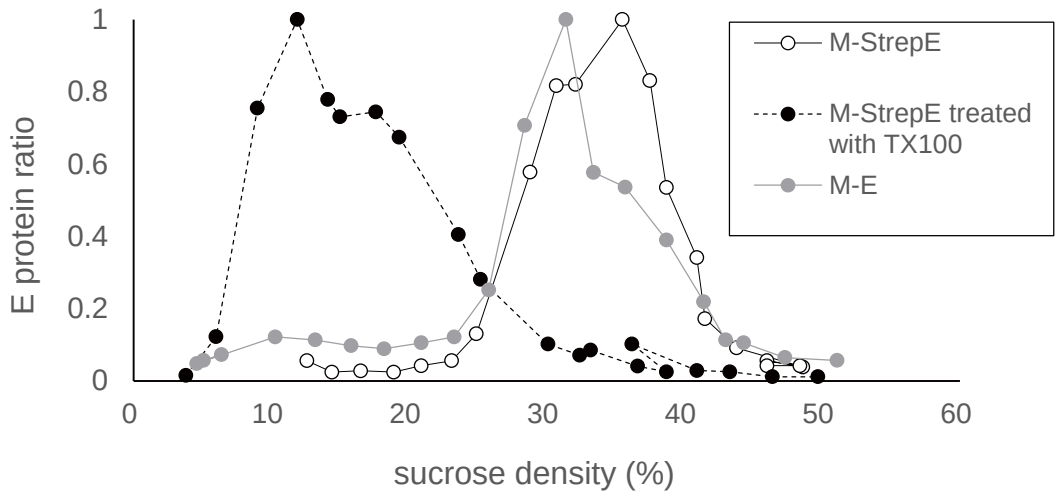
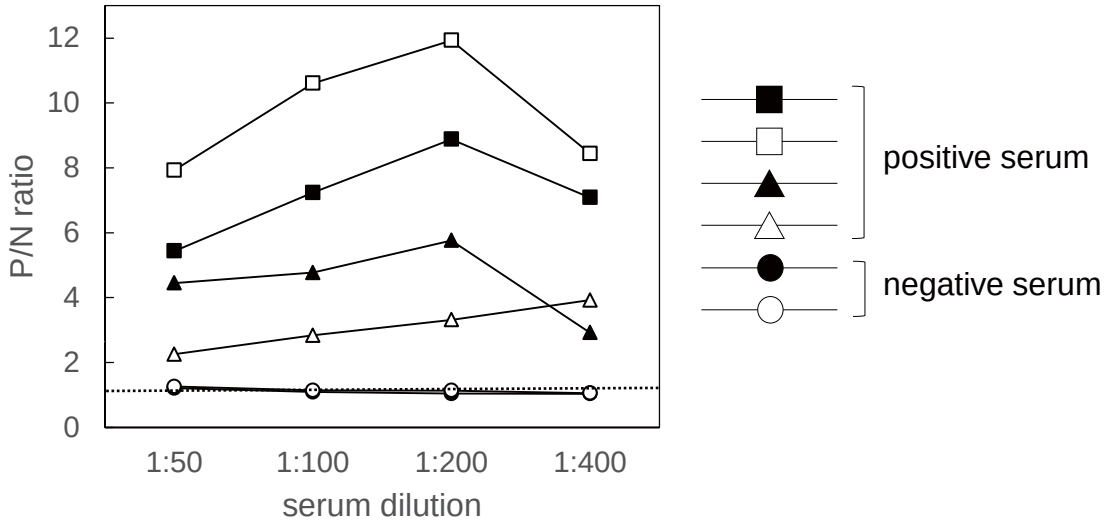


Fig. 4. Inagaki *et al*.,

(A) wild rodent sera



(B) human sera

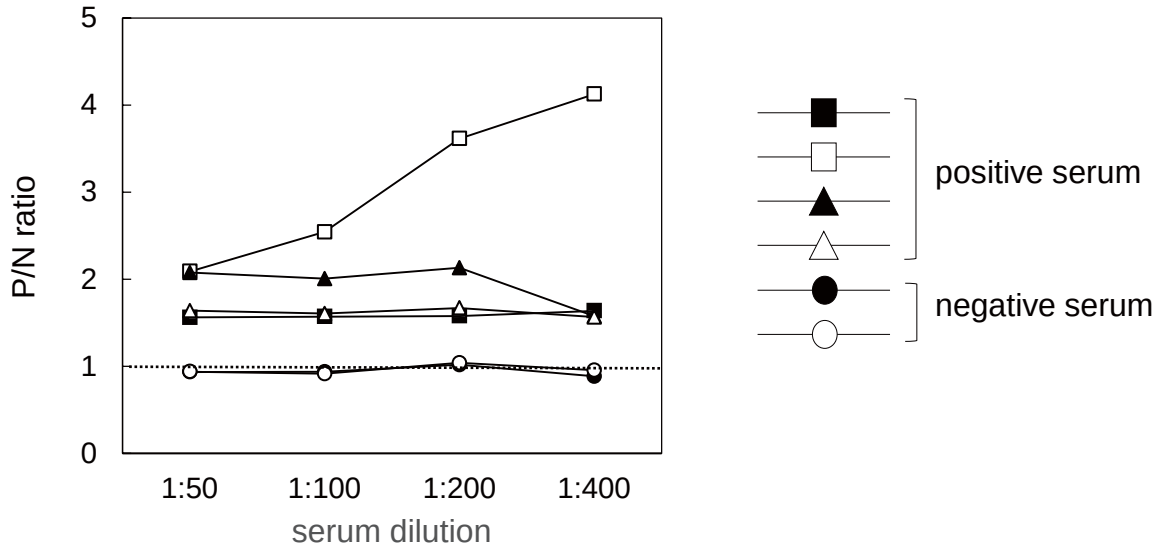


Fig. 5. Inagaki *et al.*,

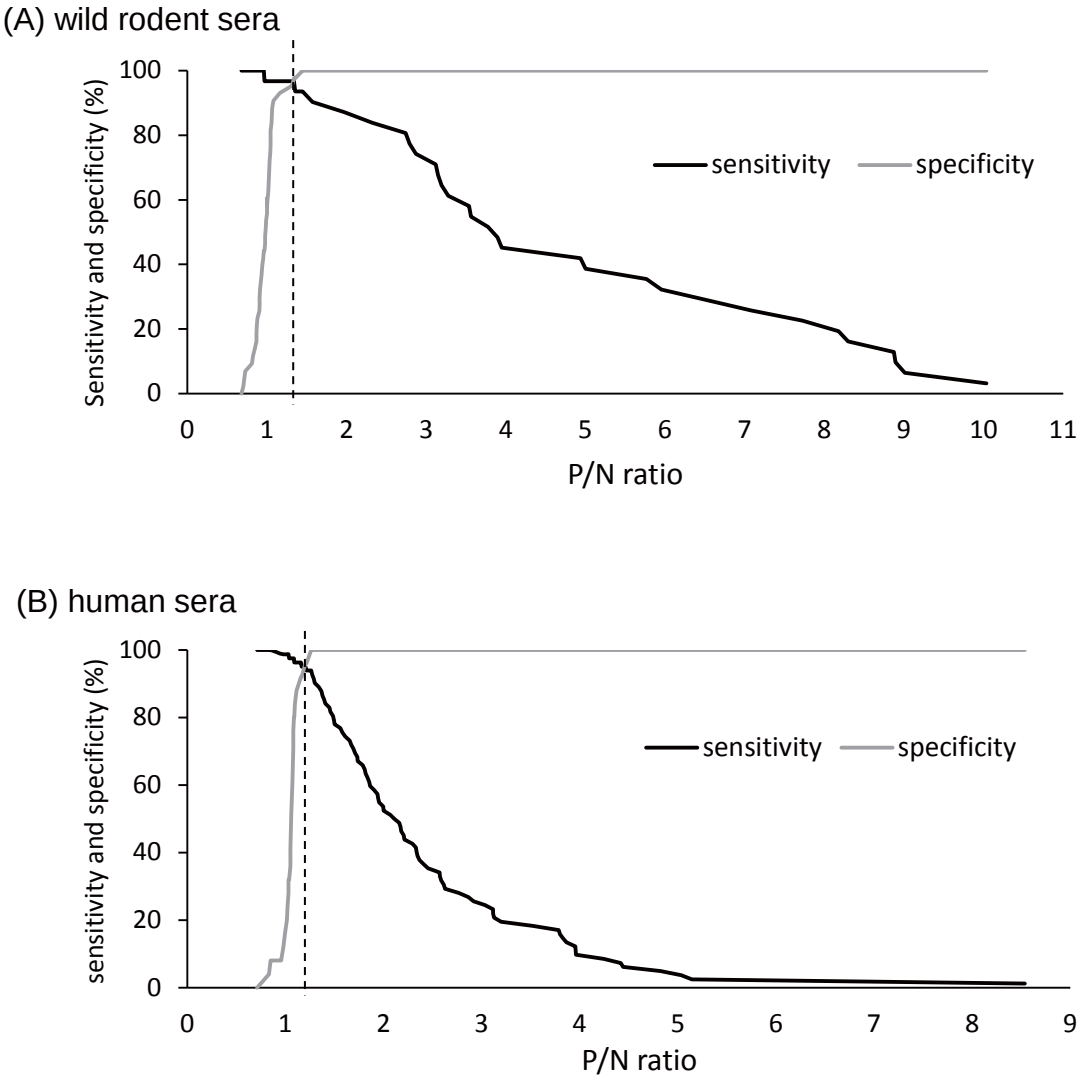


Table 1. Comparison of the results obtained by neutralization and Strep-SP ELISA in rodent serum.

Neutralization test	Strep-SP ELISA		Total
	Positive	Negative	
Positive	30	1	31
Negative	1	42	43

Table 2. Comparison of the results obtains by neutralization and Strep-SP ELISA in human serum.

Neutralization test	Strep-SP ELISA		Total
	Positive	Negative	
Positive	78	4	82
Negative	1	24	25

Table 3. Cross-reactivity of JE patient sera to TBEV by commercial ELISA and strep-SP ELISA

Patient no.	Commercial ELISA	strep-SP ELISA
1	±[a]	−
2	−	−
3	−	−
4	−	−
5	−	−
6	−	−
7	−	−
8	−	−
9	±	−
10	±	−

[a] boudary