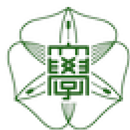
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# Studies on the antigenic characteristics of tick-borne flaviviruses and their application

**(**ダニ媒介性フラビウイルスの抗原性状の解析と応用**)**

Nozyechi Ngulube Chidumayo

Laboratory of Public Health

Department of Environmental Veterinary Sciences

Graduate School of Veterinary Medicine, Hokkaido University

Sapporo, Japan

Submitted 2014

**Contents**

Contents…………………………………………………………………………………..………..i

Abbreviations…………………………………………………………………………….…….iii

Preface……………………………………………………………………………………..….1

Chapter 1: Evaluation of the European tick-borne encephalitis vaccine against Omsk hemorrhagic fever virus……………….……………………………………………………..6

1. Introduction ……………..……………………………………………………………….…6

2. Methods…………………………………………………………………………...……..….9

  Viruses and cell lines…………………………………………………………...……….9

  Animals……………………………………………..………………………..……….....9

  Cross-neutralization tests……………….…………………………………………..10

  Virus neuralization assay………...……………….…………………………………...10

  Vaccination…….………………………………………….…………………………..11

  Vaccination and virus challenge…...……………………………………….……………11

3. Results …………….…………………………………………………………………………13

  Antigenic cross-reactivity between TBEV and OHFV…………….…………………………….13

  Efficacy of TBE vaccine against OHFV infection in mice…..…………………………..…15

  Efficacy of TBE vaccine against OHFV infection in humans…….……………………..18

4. Discussion………..……………………………………………………….………………….20

5. Summary………...………………………………………………………………………...24

Chapter 2: Development of a tick-borne encephalitis serodiagnostic ELISA using recombinant Fc-antigen fusion proteins…………………………………..……………………………25

6. Introduction…………………………………………………………………….……….25

7. Methods……………………………………………………………………………..……….28
Cells…………………………………………………………………..…………28
Serum……………………………………………………………………………….28
Preparation of recombinant antigens…………………………………………………29
Characterization of the E-Fc proteins………………….……………..……………31
ELISA………………………………...……………………………………31
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blotting………………………………………..………………………31
Immunoprecipitation……………………..……………………………32
E-Fc serodiagnostic ELISA……………………………..…………………………32
8. Results…………………………………………………………….……………………34
Characterization of the E-Fc proteins…………………….……………...………34
E-Fc serodiagnostic ELISA……………………………………..…………………39
9. Discussion…………………………………………………...…………………44
10. Summary………………………………………………………………………………..47
Conclusion………………………………………………………………………………48
Acknowledgements…………………………………………………...……………50
References……………………………………………………………………………..52
日本語要約…………………………………………………………………………………58

**Abbreviations**

| | |
|---|---|
| ALKV | Alkhurma virus |
| BHK | baby hamster kidney |
| BSA | bovine serum albumin |
| BSL 3 | biosafety Level 3 |
| C | capsid |
| E | envelope |
| ELISA | enzyme-linked immunosorbent assay |
| Ewt | envelope-wild type |
| Fc | fragment crystallizable |
| FCS | fetal calf serum |
| FFU | focus forming unit |
| GMT | Geometric mean titer |
| HRP | horseradish peroxidase |
| I-C | infectious clone |
| IgG | immunoglobulin G |
| JE | Japanese encephalitis |
| JEV | Japanese encephalitis virus |
| KFDV | Kyasanur Forest disease virus |
| LGV | Langat virus |
| LIV | Louping ill virus |
| mAb | monoclonal antibody |
| MEM | minimum essential media |

| | |
|---|---|
| NT | neutralization test |
| NS | nonstructural protein |
| OD | optical density |
| OHF | Omsk hemorrhagic fever |
| OHFV | Omsk hemorrhagic fever virus |
| OPD | o-phenylenediamine dihydrochloride |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate-buffed saline |
| PEG | polyethylene glycol |
| POWV | Powassan virus |
| prM/M | premembrane/membrane |
| PVDF | polyvinylidene fluoride |
| SDS | sodium dodecyl sulphate |
| TBE | tick-borne encephalitis |
| TBEV | tick-borne encephalitis virus |
| WNV | West Nile virus |

## Preface

The genus *Flavivirus* belongs to the family *Flaviviridae* [1]. The genus *Flavivirus* contains at least 70 viruses, several of which are of medical or veterinary importance. Flaviviruses are classified into three main groups based on the mode of transmission: vector-borne (mosquito-borne and tick-borne), no-known vector, and insect-specific flaviviruses [2-4]. Several members of the mosquito- and tick-borne flaviviruses are zoonotic pathogens that cause various diseases, including encephalitis and hemorrhagic fevers. The viruses within each group are subdivided into serocomplexes (Table 1) based on antigenic cross-reactivity [3, 5]. The medically important serocomplexes are the tick-borne encephalitis serocomplex (tick-borne encephalitis virus, Langat virus, Powassan virus, louping ill virus, Alkhurma virus, and Kyasanur Forest disease virus), the Japanese encephalitis serocomplex (Japanese encephalitis virus, West Nile virus, St Louis encephalitis virus, and Murray valley encephalitis virus), the yellow fever virus serocomplex (yellow fever virus), and the dengue virus serocomplex (dengue virus serotype 1-4) [5-9]. These viruses are distributed widely throughout the world, and some regions have more than one endemic flavivirus. This thesis focuses on the tick-borne encephalitis serocomplex, in particular tick-borne encephalitis virus (TBEV) and Omsk hemorrhagic fever virus (OHFV).

Flaviviruses are small (40-60 nm in diameter), spherical, single-stranded RNA viruses [10-12]. A lipid envelope surrounds a nucleocapsid core containing the viral genome of

Table 1 Flavivirus serocomplexes of medical and veterinary importance

| Serocomplex group | Viruses | Vector | Geographic distribution |
|---|---|---|---|
| Tick-borne encephalitis | Tick-borne encephalitis | Tick | Europe and Asia |
| | Louping ill | Tick | British Isles, Ireland |
| | Langat | Tick | Asia |
| | Omsk hemorrhagic fever | Tick | Omsk, Novosirbirsk oblast |
| | Alkhurma | Tick | Saudi Arabia, Egypt |
| | Kyasanur Forest disease | Tick | Asia |
| | Powassan | Tick | North America |
| Japanese encephalitis | Japanese encephalitis | Mosquito | Southeastern Asia, Far eastern Russia, Japan, Korea, India |
| | St. Loius encephalitis | Mosquito | America |
| | Murray Valley encephalitis | Mosquito | Australia, New Guinea |
| | Kunjin | Mosquito | Australia |
| | West Nile | Mosquito | Africa, Europe, America |
| Dengue | Dengue type 1<br>Dengue type 2<br>Dengue type 3<br>Dengue type 4 | Mosquito | Tropical areas of Asia, Australia, Oceania, Africa, America |
| Yellow fever | Yellow fever | Mosquito | Africa, South America |

approximately 11 kb [12]. The genome encodes for three structural proteins: the capsid (C), membrane (prM/M), and the envelope (E) proteins [13]. The E protein is the largest surface protein and plays important roles in virus attachment, receptor-mediated virus entry and initiates membrane fusion following virus uptake into the cell [14-17]. In the immature virion, the prM and E proteins form heterodimers [18]. 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 [18-21].

The envelope protein is highly antigenic and contains important epitopes that are targeted by antibodies [16, 22]. All flaviviruses are antigenically related with the minimum amino acid homology among viruses in different serocomplexes ranging from 40-44% while homologies within a serocomplex range from 62-98% [14, 15, 22]. Due to their antigenic similarities, flaviviruses induce the production of two functionally distinct populations of antibodies [17]. The first group of antibodies (virus species-specific antibodies), have the potential to neutralize the homologous and closely related serocomplex viruses [22]. These neutralizing antibodies are important mediators of immunity against flaviviruses. The second group of antibodies (flavivirus group antibodies) are broadly cross-reactive but lack significant neutralizing activity [22]. The extent of cross-neutralization within a serocomplex is strongly dependent on the degree of amino acid similarity.

Vaccination is the most important preventative measure against flaviviruses. Despite the high public health significance of flaviviruses, commercial vaccines are only available for tick-borne encephalitis virus, Japanese encephalitis virus and yellow fever virus [15]. Due to the antigenic similarity of flaviviruses within a serocomplex, it is possible for a flavivirus vaccine to confer cross-protection against closely related viruses [22]. In chapter 1, the feasibility of cross-protective vaccination against flaviviruses of the tick-borne encephalitis serocomplex was investigated. TBEV and OHFV are both members of tick-borne encephalitis serocomplex and are the causative agents of tick-borne encephalitis (TBE) and Omsk hemorrhagic fever (OHF), respectively. Despite their difference in disease manifestation, the viruses have over 80% E protein homology. Neutralization tests and mouse protection tests were performed to evaluate the efficacy of the commercial TBE vaccine to cross-protect against OHF.

TBEV is one of the most important vector-borne viruses in Europe and Asia [23]. The risk of the disease has been increasing with the expansion of the geographic range of the tick vectors as a result of climate change [24]. Integrated surveillance of the vector and the virus in reservoir hosts and humans can identify endemic areas and reveal epidemiological trends of TBE. Reliable surveillance of TBEV reservoirs and TBE cases is important for implementing effective disease prevention and control measures. The diagnosis of TBE is based on the detection of antibodies in serum or cerebral spinal fluid [25]. Enzyme-linked immunosorbent assay (ELISA) is the most commonly used diagnostic test for flavivirus diagnosis. Commercial TBE ELISA kits use

inactivated whole virus as antigens. However, the production of the inactivated whole virus antigens is expensive and requires high biosafely facilities. Since the E protein is the major immunogen of flaviviviruses and thus the major target for antibodies, recombinant proteins produced from the E protein can provide an alternative method of producing antigens for use in flavivirus diagnosis. In chapter 2, the applicability of E-Fc fusion proteins as antigens for the diagnosis of TBE was investigated. Recombinant E-Fc fusion proteins were produced by replacing the E protein C-terminus with the Fc domain of rabbit immunoglobulin G (IgG). The sensitivity and specificity of an ELISA using the E-Fc antigen was assessed and the results were compared with those of neutralization tests.

## Chapter 1: Evaluation of the European tick-borne encephalitis vaccine against Omsk hemorrhagic fever virus

### 1. Introduction

TBEV and OHFV are tick-borne flaviviruses belonging to the TBE serocomplex [7, 8]. This serocomplex consists of tick-borne flaviviruses that are cross-reactive in serological tests [26]. Other members of this group include Langat virus (LGV), Powassan virus (POWV), Louping ill virus (LIV), Alkhurma virus (ALKV) and Kyasanur Forest disease virus (KFDV) [8, 27, 28]. The majority of the viruses in the serocomplex cause encephalitis; however OHFV, ALKV and KFDV are known to cause hemorrhagic fever syndrome [8, 27]. TBEV is an important human pathogen, causing over 10,000 cases of encephalitis annually [29]. There are three subtypes of the TBEV; European, Far-Eastern and Siberian [30-35]. Mortality rates vary depending on the TBEV subtype. The Far-Eastern subtype is the most fatal with a mortality rate of 5-20% [36]. TBEV has a wide geographic range that extends Europe and Asia [37-40]. The virus is transmitted by *Ixode*s ticks, with *Ixodes ricinus* and *Ixodes persulcatus* being the principle vectors in Europe and Asia, respectively [41]. In contrast, OHFV is endemic in the Omsk and Novosibirsk regions of Russia. The Siberian subtype of TBEV is also endemic in these regions [41]. In these regions, *Dermacentor* ticks are thought to be the principle vectors of OHFV [7, 41]. The incidence of OHF is unclear, but most outbreaks are seasonal and coincide with an

increase in tick populations or muskrat *(Ondatra zibethicus)* hunting [7, 41]. Muskrats are highly susceptible to the virus and are thought to be the amplifying host [41].

Tick-borne flaviviruses are zoonotic arthropod-borne viruses belonging to the genus *Flavivirus*, in the family *Flaviviridae*. The flavivirus genome contains a single open reading frame that encodes 10 proteins. Three of these, C, prM/M and E, are structural proteins. The remaining seven are non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) [13, 42]. Despite the difference in disease manifestations, phylogenetic analysis has revealed that OHFV is related more closely to TBEV than to the other two hemorrhagic fever viruses [8].

Vaccination is the most important method of reducing the risk of flavivirus infection. Four vaccines against TBE are currently available on the global market. Two of these, Encepur® (Chiron Behring GmbH & Co KG, Marburg, Germany) and FSME-IMMUN® (Baxter AG, Vienna, Austria), are based on strains of the European subtype of TBEV. The other two, EnceVir® (Microgen , Tomsk, Russia) and TBE-Moscow vaccine® (Chumakov Institute of poliomyelitis and Viral encephalitides, Moscow, Russia), are based on strains of the Far-Eastern subtype [37, 43]. All of these vaccines have shown themselves to be highly immunogenic against each of the viral subtypes [37, 43-47]. The cross-protection amongst the subtypes is thought to be due to the E protein having an amino acid homology in excess of 95% [8, 48]. Protection against flaviviruses is mediated primarily by neutralizing antibodies against the E

protein, and to a lesser extent the prM and NS1 proteins [49-53]. The E protein forms three structural domains, which are designated I, II, and III [18, 27]. Epitopes recognized by neutralizing antibodies have been identified in all three domains [10, 54]. Domain III is thought to be highly immunogenic—most serotype- and virus-type- specific neutralizing antibodies target this region [10, 54, 55]. In addition, many cross-reactive antibodies recognize the fusion loop of domain II [56, 57].

Despite the availability of several TBE vaccines, no vaccine for OHFV exists. It is therefore necessary to determine if the available TBE vaccines can be used for the prevention of OHFV infection. Although OHFV and TBEV are cross-reactive in serological tests, limited data regarding whether this cross-reactivity is protective are available. Analysis of TBEV Neudoerfl (the prototype of the European subtype of TBEV used in the production of FSME-IMMUN® vaccine) found an 81.8% similarity with OHFV [8] in the gene encoding the E protein. Because of the similarities between the E proteins of TBEV and OHFV, the available TBE vaccines could provide effective protection against OHFV. Therefore, the aim of this study was to evaluate the antigenic cross-reactivity of these two viruses and determine if the commercially available TBE vaccines, which are based on viruses of the European subtype of TBEV, can provide sufficient cross-protection against OHFV infection.

## 2. Methods

### Viruses and cell lines

The TBEV-Sofin-HO strain (accession No. 062064) and Omsk hemorrhagic fever infectious clone (OHF-IC) were used for neutralization tests and mouse inoculation. The OHF-IC strain was derived from the Guriev strain of OHFV (accession No. AB507860) [58]. The Sofin-HO strain was first isolated from the brain of a TBE patient in Khabarovsk in 1937. This virus (of unknown passage history) was provided by Dr. Ohya (National Institute of Infectious Disease, Tokyo, Japan) in 1967, and further passaged seven times in suckling mouse brain and twice in Baby Hamster Kidney (BHK) cells. The BHK cell line was grown at 37°C in Eagles Minimum Essential Medium (MEM, Gibco®) and supplemented with fetal calf serum (FCS).

### Animals

Four-to-five-week-old female C57BL/6J mice (Charles River Laboratories Japan, Inc. Yokohama, Japan) were used throughout the experiment. Water and food pellets were supplied ad libitum. Animals were handled under BSL3 containment conditions. All experiments were approved (experiment number 13025) following review by the Animal Care and Use Committee and President of Hokkaido University.

**Cross-neutralization tests**

For cross-neutralization tests, mice were inoculated subcutaneously with 1,000 ffu of either TBEV or OHFV. Sera were collected at the onset of clinical signs, which occurred around days 8 and 9 for OHFV and TBEV, respectively.

**Virus neutralization assay**

An equal volume of serially diluted serum was mixed with stock virus and incubated at 37°C for 1 h. Monolayers of BHK cells grown in 12-well plates were then inoculated with the mixtures (100 µl/well) and incubated at 37°C for 1 h. After the viral inocula were removed, the cells were rinsed once with 2% FCS-MEM. The cells were then layered with 1-ml MEM containing 1.5% carboxyl methylcellulose (CMC) and 2% FCS. These monolayers were next incubated in a $CO_2$ incubator at 37°C for 4 days for TBEV and 5 days for OHFV. Following incubation the mixed CMC-MEM medium was removed and the cells were fixed and stained with formalin-crystal violet. Neutralizing antibody titers were determined as the reciprocal of the highest serum dilution that reduced the virus counts by at least 50%. Results were considered to be positive at a titre ≥20.

**Vaccination**

Tick-borne encephalitis vaccines Encepur® Adult (Chiron Behring GmbH & Co) and FSME-IMMUN® inject (Baxter AG) were used in human studies. Only FSME-IMMUN® inject was used in the mouse experiments. All vaccinated volunteers belonged to the Laboratory of Public Health, Hokkaido University, Graduate School of Veterinary Medicine and received the vaccinations to prevent laboratory infections with TBEV.

Human volunteers received a total of either two or three intramuscular injections performed on day 0 and 28 or on days 0 and 28 followed by a booster after 12 months. Sera were collected 4 weeks after the second or third vaccination.

Mice were immunized subcutaneously on days 0 and 10 with 0.2 ml of a fourfold dilution of the TBE vaccine containing 0.2% Al $(OH)_3$. Mice in the control group were immunized with an equal volume of 0.2% Al $(OH)_3$ in phosphate-buffered saline (PBS). Blood samples were collected 10 days after the second vaccination. Sera were heat-inactivated at 56°C for 30 min.

**Vaccination and virus challenge**

For vaccine protection, mice were immunized with the TBE vaccine or PBS as described above. Groups of ten mice were challenged with 100 $LD_{50}$ of TBEV (68 ffu/mouse) or 100 $LD_{50}$ OHFV (209 ffu/mouse) 10 days after the second vaccination. The mice were observed for 24 days after the virus challenge.

## Statistical analysis

Statistical analyses were performed using Student's *t*-test (Microsoft Office Excel). A value of $P \leq 0.05$ was considered to indicate statistical significance.

## 3. Results

### Antigenic cross-reactivity between TBEV and OHFV

To determine if antigenic differences existed between TBEV and OHFV, cross-neutralization tests were performed using sera from mice infected with the TBEV-Sofin strain and the OHFV-Gruiev strain. Neutralizing antibody titers of mouse sera can be seen in Figures 1a and 1b. All animals showed detectable antibody levels to both TBEV and OHFV. Sera from those infected with TBEV exhibited consistently high antibody titers to both TBEV and OHFV with no significant difference in geometric mean titers (GMT). However, there was a significant difference ($P=0.002$) in antibody titers of sera from OHFV-infected mice, exhibiting high titers of antibody to OHFV but only moderate titers to TBEV.

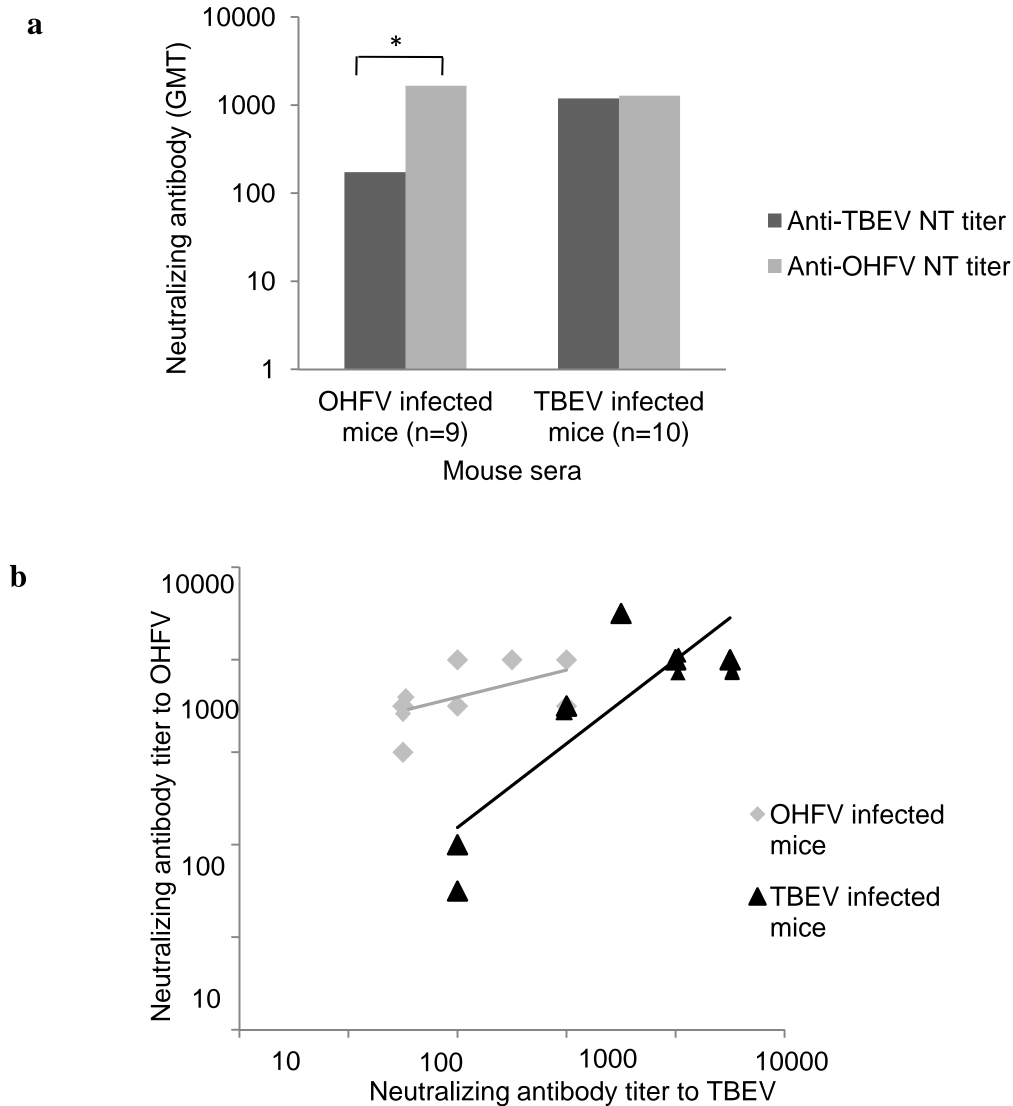



Figure 1. Cross-neutralization test of sera from OHFV and TBEV infected mice (a) GMT of neutralizing antibodies to TBEV and OHFV from sera of OHFV- or TBEV-infected mice. (b) Correlation between levels of neutralizing antibodies to TBEV and OHFV from OHFV- and TBEV-infected mice. Serum was collected from OHFV- and TBEV-infected mice 8 and 9 days after infection. Neutralizing antibody titers were determined as the reciprocal of the highest serum dilution that reduced the virus focus counts by 50%. * Significant difference ($P<0.05$).

**Efficacy of the TBE vaccine against OHFV infection in mice**

To determine if the commercial TBE vaccine could protect mice against OHFV infection, mice were vaccinated subcutaneously with FSME-IMMUN® inject and re-vaccinated 10 days later. Seventy-one percent of vaccinated mice had detectable titers of TBEV antibodies and 57% had antibodies to OHFV (Table 2). There was no significant difference in the GMTs of neutralizing antibody to either of the two viruses. To further evaluate the efficacy of the commercial TBE vaccine in protecting against OHFV, vaccinated mice were challenged with a lethal dose of OHFV. Survival rates of vaccinated mice challenged with 100 $LD_{50}$ of OHFV are shown in Figure 2. As the results show, the TBE vaccine provided 100% protection against a lethal dose of OHFV in these animals. In addition, those vaccinated did not show any clinical signs during the observation period. Mice in the control group (administered an equal dosage of PBS) exhibited a 100% mortality rate. Therefore, the TBE vaccine induced a protective immune response against OHFV infection.

Table 2. Seroconversion rates and neutralizing antibody titers in mice against TBEV and OHFV after vaccination with the TBE vaccine.

| Neutralizing antibodies | Seroconversion rate (%) | Geometric mean titer[a] |
|---|---|---|
| Anti-OHFV | 57 (4/7)[b] | 40 |
| Anti-TBEV | 71 (5/7) | 46 |

Mice were immunized with 0.2 ml of a four-fold dilution of the TBE vaccine on day 0 and 10. Sera was collected from mice 10 days after the second vaccination.

[a] Neutralizing antibody titers were determined as the reciprocal of the highest serum dilution that reduced the virus focus counts by 50%.

[b] Number of positive sera/ total number of sera examined

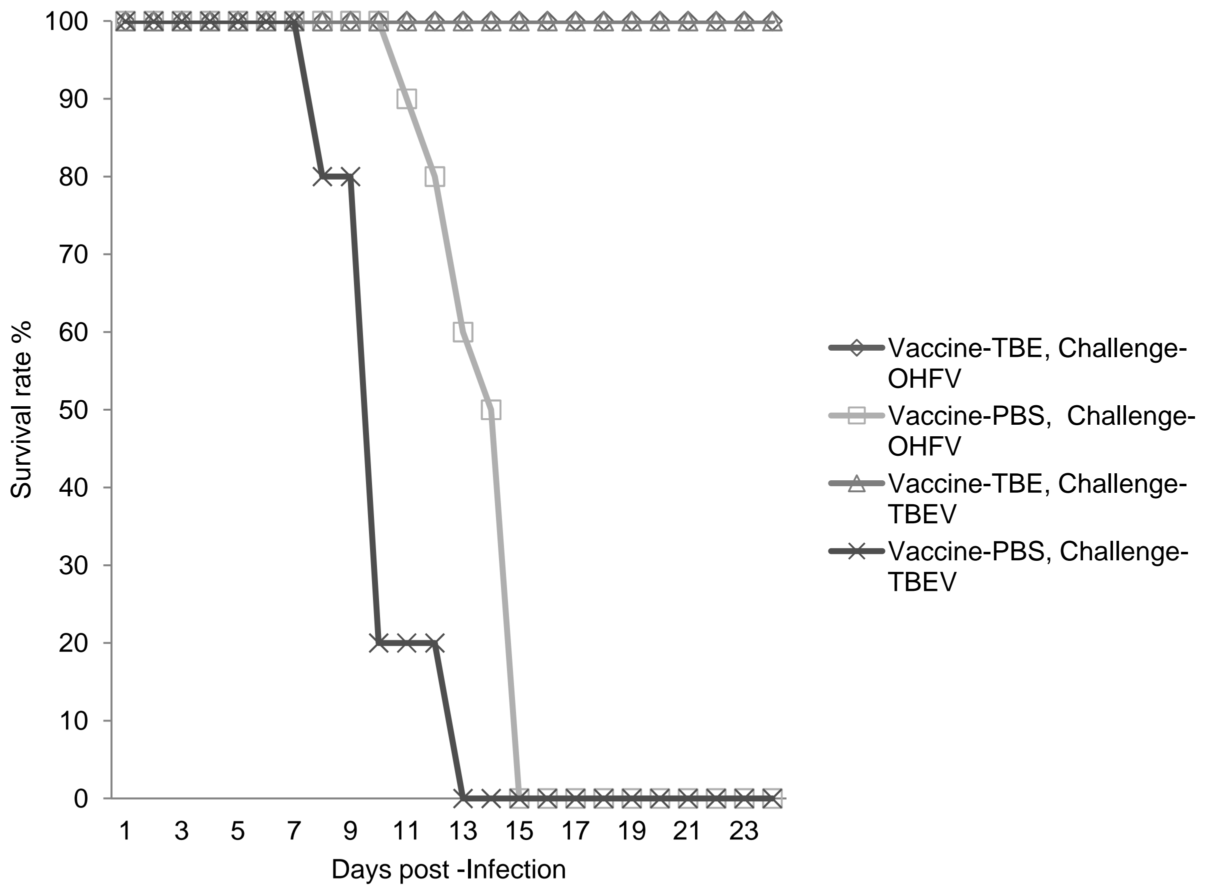



Figure 2. Percentage of mice surviving after being vaccinated and challenged with 100 $LD_{50}$ of OHFV or TBEV.

Mice were immunized subcutaneously on days 0 and 10 with 0.2 ml of a fourfold dilution of the TBE vaccine, or PBS, and challenged with a lethal dose of TBEV or OHFV. Survival was recorded every day for 24 days after viral challenge.

### Efficacy of the TBE vaccine against OHFV infection in humans

To evaluate the efficacy of the commercial TBE vaccines against human OHFV infection, antibody responses to the vaccines were assessed. The resulting neutralizing antibody titers against TBEV and OHFV are shown in Table 3. Seroconversion rates to TBEV and OHFV after the second vaccination were 87% and 79% respectively, with no significant differences in neutralizing antibody GMT. After the third vaccination, seroconversion rates increased to 100% for TBEV and 86% for OHFV. Statistical analysis showed no significant difference in the GMT between the two viruses. Therefore, the commercial TBE vaccine was able to induce an efficient antibody response against OHFV in humans.

Table 3. Percentage of positive human sera for antibodies to TBEV or OHFV after vaccination with TBE vaccine

| Number of vaccinations [a] | Neutralizing antibodies | Seroconversion rate (%)[b] | Geometric mean titer [c] |
|---|---|---|---|
| 2 | Anti-TBEV | 87 (33/38) | 83 |
| | Anti-OHFV | 79 (30/38) | 100 |
| 3 | Anti-TBEV | 100 (14/14) | 819 |
| | Anti-OHFV | 86 (12/14) | 1015 |

[a] Vaccinees received two immunizations on day 0 and 28 or three immunizations on day 0, 28 and 12 months. Sera was collected from vaccinees 4 weeks after each vaccination.

[b] Number of positive sera/ total number of sera examined

[c] Neutralizing titer was determined as the reciprocal of the highest serum dilution that reduced the virus focus counts by 50%.

## 4. Discussion

The antigenic cross-reactivity of TBEV and OHFV was investigated and assessed to determine the efficacy of the European-subtype-based commercial TBE vaccines. Neutralizing antibodies from mice infected with the virus showed that TBEV and OHFV are cross-reactive. However, significant differences in antibody titers were found in these animals. Sera from TBEV-infected mice had equally high titers of neutralizing antibodies against both TBEV and OHFV. In contrast, sera from animals infected with OHFV had high neutralizing antibodies against OHFV, but only moderate neutralizing antibodies against TBEV. This difference in neutralizing activity may be due to differences in amino acid residues in the E protein that could have an effect on epitope accessibility and tertiary structure [8, 27, 57, 59-61]. Domain III of the OHFV E protein differs from other TBE complex viruses at 18 distinct residues [27]. Domain II has one amino acid residue that is specific to the three hemorrhagic viruses but is absent in TBEV [8]. It is thought that these amino acid variations play a role in protein conformation and antigenic variation [8, 27]. Other factors such as differences in virus cell tropism and induction of immune response may contribute to the difference in cross-neutralization activity. Studies in mice demonstrated that TBEV primarily targets the brain while OHFV targets visceral organs [62]. The NS1 protein of flaviviruses interacts with various components of the host immune system and some of these interactions are thought to be virus-specific [63]. Furthermore, the NS 1 protein may also contribute to the pathogenesis of hemorrhagic fever flaviviruses [64, 65].

In the vaccine efficacy study, 57% of vaccinated mice had neutralizing antibodies against OHFV. No difference was found in the geometric mean titre of anti-OHFV and anti-TBEV neutralizing antibodies in vaccinated animals. These results correspond with the cross neutralization results that were seen in the TBEV-infected mice. Efficient protection was observed in the vaccinated animals challenged with a lethal dose of OHFV. The TBE vaccinated mice that survived the OHFV challenge had significantly lower neutralizing antibodies against OHFV compared to unvaccinated OHFV-infected mice (data not shown). This suggests that viral replication was inhibited in the vaccinated mice. Although the vaccine showed moderate seroconversion rates, it still provided complete protection against a lethal dose of OHFV. This may be due to protection by non-neutralizing antibodies. Prior studies have demonstrated that poorly neutralizing or non-neutralizing antibodies are able to protect against flavivirus infection via Fc-receptor-mediated mechanisms and complement-mediated cell lysis [66].

The human sera of vaccinated subjects were collected from 1995 to 2013. During this period, Encepur® Adult or FSME-IMMUN® inject vaccines were used depending on vaccine availability. Both vaccines are produced from closely related European subtype TBE viruses and the vaccines have been shown to be highly immunogenic with comparable efficacy [43, 67, 68]. Neutralization tests performed on human sera showed that complete vaccination (vaccine administered on Days 0, 28 and a booster after 12 months) induced production of anti-TBEV and anti-OHFV neutralizing antibodies in 100% and 86% of subjects, respectively. These results

suggest that high levels of neutralizing antibodies against OHFV are achieved after complete vaccination. Furthermore, there was no significant difference in the GMT of anti-TBEV and anti-OHFV neutralizing antibodies, similar to the neutralization test results in the vaccinated mice and unvaccinated TBEV-infected mice. The seroconversion rate of TBEV following complete vaccination is similar to that reported previously [48, 69]. A study assessing the efficacy of the European subtype TBE vaccine using chimeric viruses consisting of a consensus backbone derived from West Nile virus (WNV) and encoding the prM and E proteins of the three TBEV subtypes and OHFV reported 100% seroconversion against the WNV-TBEV chimeric viruses and 98% seroconversion against the WNV-OHFV chimera, suggesting that the TBE vaccine could protect against OHFV infection [70]. Our study supports these findings although the seroconversion rate against OHFV is lower compared to the WNV-OHFV chimera. Several studies have reported on the 'original antigenic sin phenomenon', where pre-existing immunity to one or more flaviviruses can affect the immune response following vaccination [71-74]. The majority of the TBE-vaccinated human subjects had previously been immunized against Japanese encephalitis. However, the findings indicate that the TBE vaccine is effective despite any prior immunization to Japanese encephalitis virus.

In 1991, the Russian government authorized the use of the TBE vaccine as a preventative measures during an OHF outbreak despite lack of conclusive evidence on the cross-protective potential of the vaccine [41]. These results show that the TBE vaccine can provide effective

protection against OHFV infection. Moreover, it is likely that recovered TBEV patients also have cross-protective antibodies against OHFV.

In conclusion, complete vaccination with the TBE vaccine based on the European subtype of the virus provides effective protection against OHFV infection, and therefore has potential for the prevention of Omsk hemorrhagic fever.

## 5. Summary

This study focused on the antigenic cross-reactivity between TBEV and OHFV to assess the efficacy of the commercial tick-borne encephalitis vaccine against OHFV infection. Neutralization tests performed on sera from OHFV and TBEV-infected mice showed that neutralizing antibodies were cross-reactive. The GMT of antibodies against TBEV and OHFV from TBEV-infected mice were similar. However, the levels of anti-TBEV antibodies in OHFV-infected mice were significantly lower than the levels of anti-OHFV antibodies in the same animals. In mouse vaccination and challenge tests, the TBE vaccine provided 100% protection against OHFV infection. In addition, eighty-six percent of vaccinees seroconverted against OHFV following complete vaccination, and the GMT of neutralizing antibodies against OHFV were comparable to those against TBEV. These data suggest that the tick-borne encephalitis vaccine can prevent Omsk hemorrhagic fever virus infection.

## Chapter 2: Development of a tick-borne encephalitis serodiagnostic ELISA using recombinant Fc-antigen fusion proteins

### 6. Introduction

TBEV causes over 10,000 cases of encephalitis annually [29]. 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 [38, 75, 76]. 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) [38, 75, 76]. Diagnosis of flavivirus infections is challenging due to the similar clinical presentation and the cross-reactive nature of flaviviruses in serological tests [10]. Laboratory diagnosis of flavivirus infections is based primarily on the identification of antibodies in neutralization tests and ELISA [11, 13]. The neutralization test (NT) 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 [13]. The ELISA kits use inactivated whole virus antigens to detect TBEV-specific antibodies, but cross-reactivity with

other flaviviruses is a concern [6, 11, 13]. Commercial TBEV diagnostic kits have a sensitivity of 73-99% and specificity of 14-81% [77]. Cross-reactive antibodies from yellow fever, dengue virus, and Japanese encephalitis positive sera have all produced false positive results in these ELISA kits [77, 78].

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 [6, 10], reducing the number of false positive results in areas endemic to more than one flavivirus. ELISA diagnostic tests using viral proteins exhibited high sensitivity and specificity for anti-TBEV antibodies, and no cross-reactivity with mosquito-borne flaviviruses [10, 11, 13, 26, 78].

Flaviviruses contain two surface proteins, E and prM/M [13]. In the immature virion, the prM and E proteins form heterodimers [18]. 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 [18-21]. The E protein is the major surface protein and has been shown to be useful in TBEV diagnostics and vaccine development [10]. The ectodomain (which is exposed on the viral surface) represents the N-terminal 80% of the E protein [79]. The C-terminal 20% of the molecule consists of the transmembrane domains (which

anchor the protein to the lipid envelope) [18, 79]. X-ray crystallographic studies have revealed that approximately 90% of the ectodomain forms three structural domains designated I, II, and III [10, 11, 18]. These domains are highly immunogenic, and are the major target for neutralizing antibodies [10]. Previous studies demonstrated that recombinant proteins expressing prM and C-terminal truncated E protein (lacking the anchor region) are secreted as soluble E proteins [18], making them suitable candidates for immunodiagnostic studies [80-84]. This study describes the application of TBEV E-Fc proteins for TBEV diagnosis. The prM and E protein ectodomain were genetically linked to the fragment crystallizable (Fc) domain of rabbit IgG, and expressed in mammalian cells.

# 7. Materials and Methods

## Cells

The 293T cells were cultured at 37°C in high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, Paisley, United Kingdom) supplemented with 10% FCS, L-glutamine, and penicillin/streptomycin.

## Serum

Ninety-seven serum samples from suspected TBE patients were tested. The serum samples were obtained from the Far Eastern Medical Center, Khabarosvsk, 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) [85]. 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.

**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 3) using the pCAGprME plasmid containing the genes of TBEV Oshima 5-10 strain as the template [13]. 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 pCAGprME plasmid expressing prM and E proteins (wild-type E/Ewt) was used as a positive control. The plasmids were transfected into 293T cells using TransIT-LT1 reagent ( Mirus bio, Madison, Wisconsin, USA ) 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.

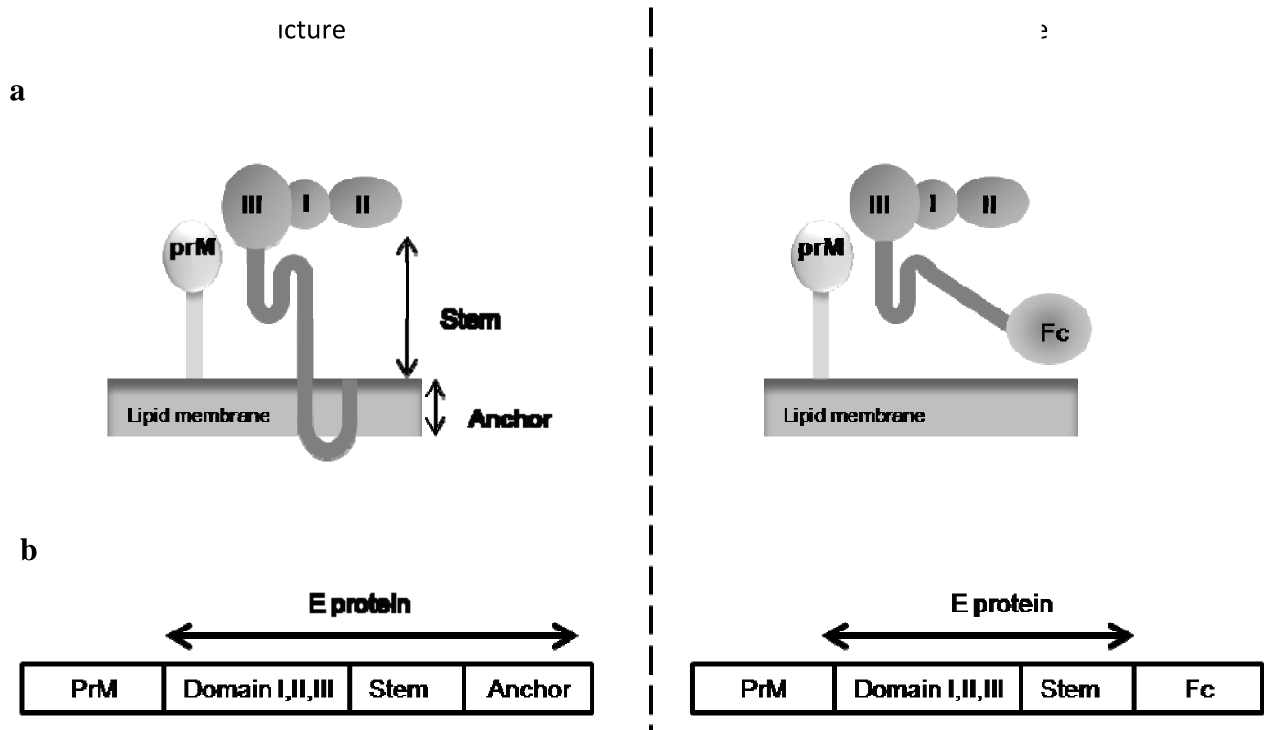



Figure 3. Construction and expression of TBEV E-Fc fusion proteins. (a) Schematic representation of TBEV E protein and E-Fc fusion proteins. (b) The anchor region of the E protein was replaced with the Fc region of rabbit IgG.

**Characterization of the E-Fc proteins**

**ELISA**

The TBEV E-Fc proteins were detected by sandwich ELISA using the monoclonal antibodies (mAb) IH4 and 4H8 to recognize the conformational epitopes of E-protein [86] . To prepare the samples, the cells and supernatants were treated with 1% Triton X-100 in ELISA buffer (0.3% bovine serum albumin in PBS 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 1H4 or 4H8 and horseradish peroxidase (HRP)-conjugated streptavidin ( Sigma-Aldrich, St. Louis, Missouri, USA). HRP activity was detected by the addition of o-phenylenediamine dihydrochloride (OPD; Sigma) in the presence of 0.03% (v/v) $H_20_2$, and absorbance was measured at 450-620 nm.

**Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (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 [11, 87]. Membranes were then incubated

with alkaline phosphatase-conjugated secondary antibody (1:6,000) or HRP-conjugated anti-mouse IgG antibody (1:2,000). Bands were visualized using an alkaline phosphatase kit (Merck, Darmstad, Germany) or chemiluminescent detection reagent (Ge healthcare, Buckinghamshire, United kingdom) following the manufacturer's instructions. To detect the Fc domain, PVDF membranes were incubated with HRP-conjugated anti-rabbit IgG (1:2,000), and signals were detected using chemiluminescent detection reagent (GE Healthcare, United Kingdom).

**Immunoprecipitation**

Cell lysates were immunoprecipitated using mAb 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 (1:10,000), respectively, followed by alkaline phosphatase-conjugated secondary antibody (1:6,000). Protein bands were visualized using an alkaline phosphatase detection kit (Merck Darmstadt Germany) following the manufacturer's instructions.

**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 Immuneresearch, West Grove, Pennsylvania, USA) and blocked with

Block Ace. Rodent or human sera (1:200) were added, followed by peroxidase-conjugated anti-mouse IgG (1:7,000; Jackson Immunoresearch) or anti-human IgG (1:20,000; 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.

# 8. Results

## Characterization of the 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 3), and transfected into 293T cells. The E-Fc proteins were detected in cell lysates and supernatants (Figure 4a), indicating that the proteins were successfully expressed and secreted into the culture medium. Reactivity with anti-E (Figure 4b) and anti-rabbit IgG (Figure 4c) 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 [18]. 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 5). The E proteins were efficiently immunoprecipitated with mAb IH4, and the wild-type E protein (Ewt) and E-Fc protein-prM heterodimers were co-immunoprecipitated. The thicker intensity of E and prM proteins in the E-Fc homodimer is due

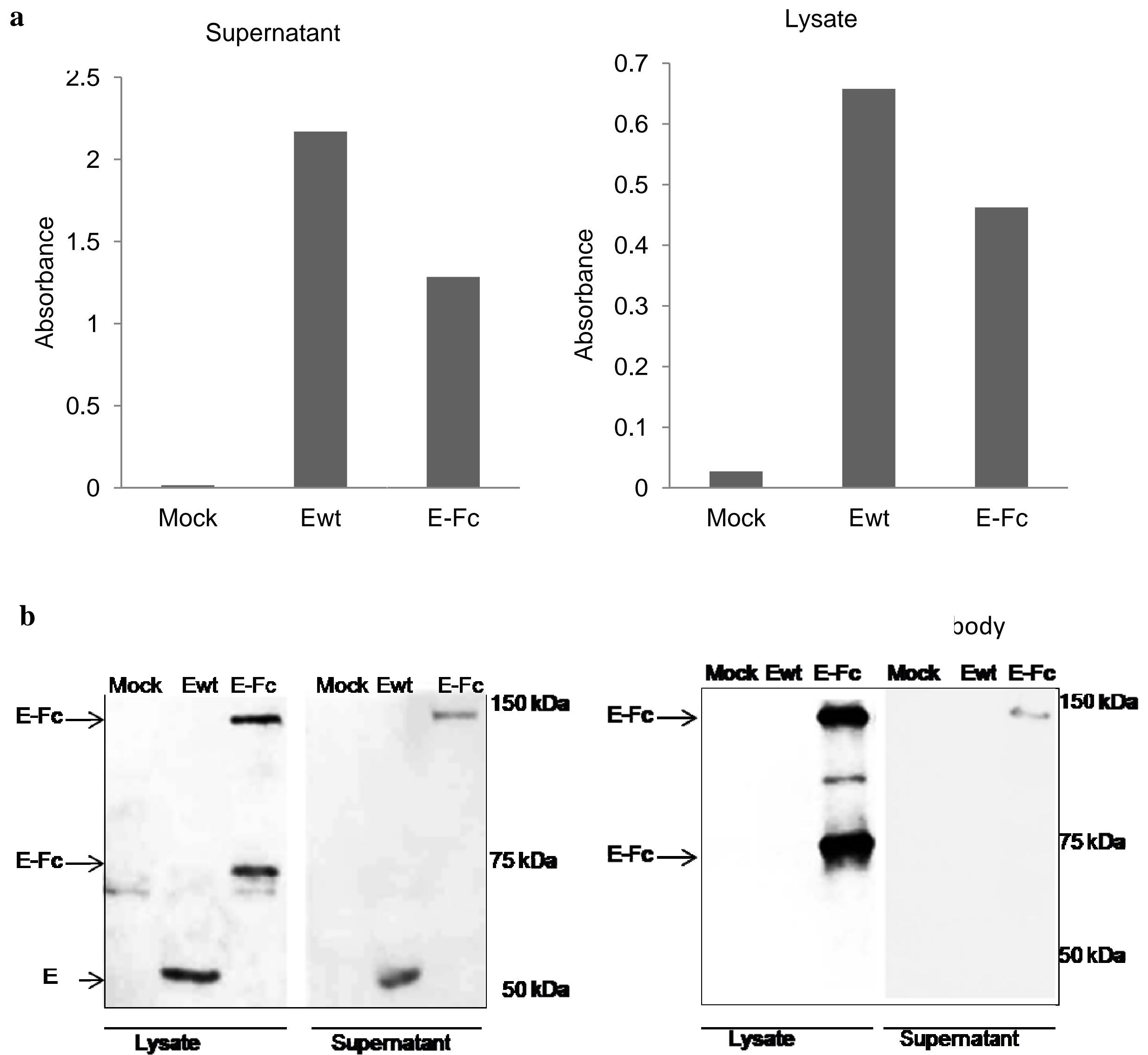



Figure 4. Expression and reactivity of TBEV E-Fc protein. (a) Expression of E-Fc proteins. 293T cells were transfected with pCAGprME (expressing wild-type E protein/Ewt), pCAGprME449-Fc (expressing E-Fc protein), or no plasmid (mock). Forty-eight hours after transfection, E proteins were detected in 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.

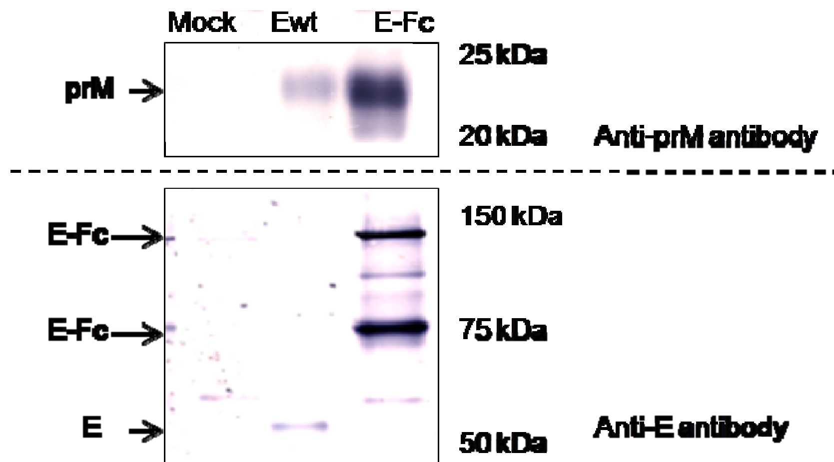
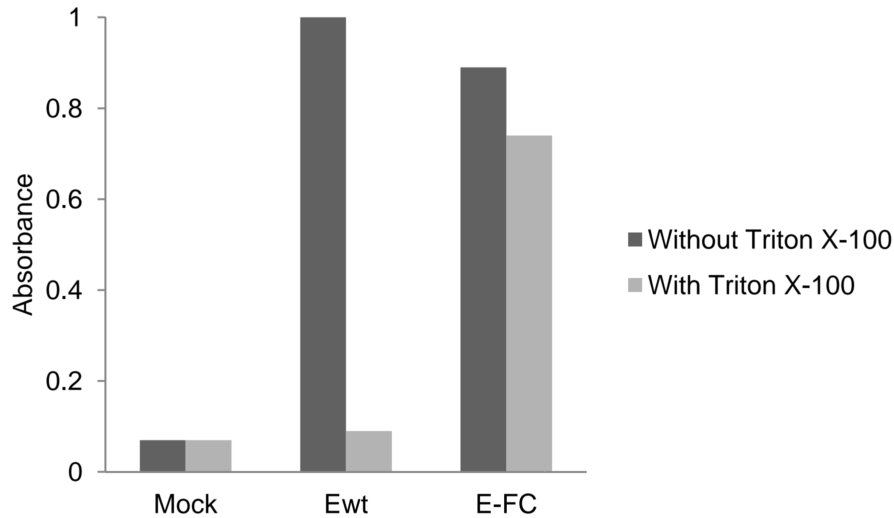



Figure 5. Heterodimer formation of prM and E proteins. Cell lysates were immunoprecipitated using anti-E mAb 1H4, and separated by SDS-PAGE. Bands corresponding to proteins were detected using anti-prM and anti-E antibodies.

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 [18]. 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 6a). 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 [84]. 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 6b).



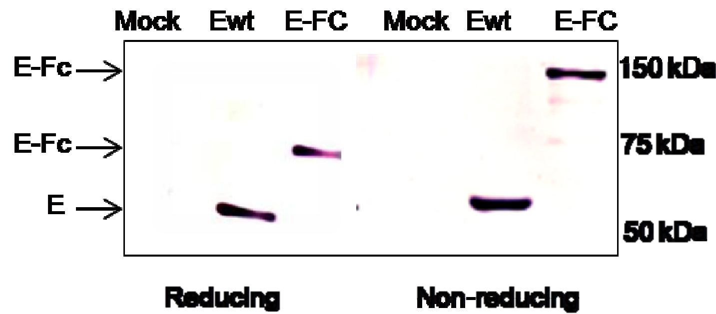




Figure 6. Analysis of physical characteristics of the homodimer form of E-Fc proteins. (a) Comparison of the reactivity of E-Fc proteins with membrane-bound E protein homodimers (without Triton X-100) and E protein monomers (with Triton X-100). Supernatants from transfected 293T cells were treated with 1% Triton X-100. The amount of E-protein homodimers was determined before and after solubilization by ELISA using mAb IH4 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.

**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 7). 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 4).

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 1.1 (Figure 8). 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 5). Of the 12 samples negative in neutralization test, 10 samples were also negative using the E-Fc ELISA (Table 5). 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.

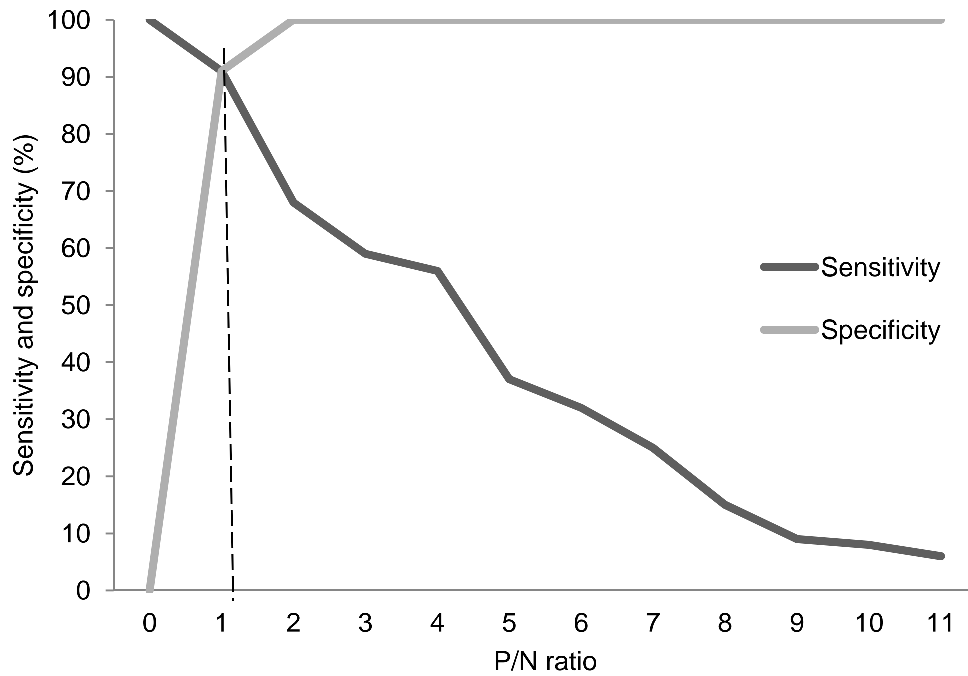



Figure 7. Relationship between cut-off value, sensitivity, and specificity in the E-Fc ELISA. Sixty-six rodent samples were tested for TBEV antibodies. The cut-off value (broken line) was set as the point at which the difference between the sensitivity and the specificity was minimal.

Table 4. Comparison of the results obtained by neutralization test and E-Fc ELISA (66 rodent serum samples were tested for anti-TBEV antibodies)

| | E-Fc ELISA | | Total |
|---|---|---|---|
| | Positive | Negative | |
| NT positive | 29 (90.6%) | 3 (8.8%) | 32 |
| NT negative | 3 (9.4%) | 31 (91.2%) | 34 |
| Total | 32 | 34 | 66 |

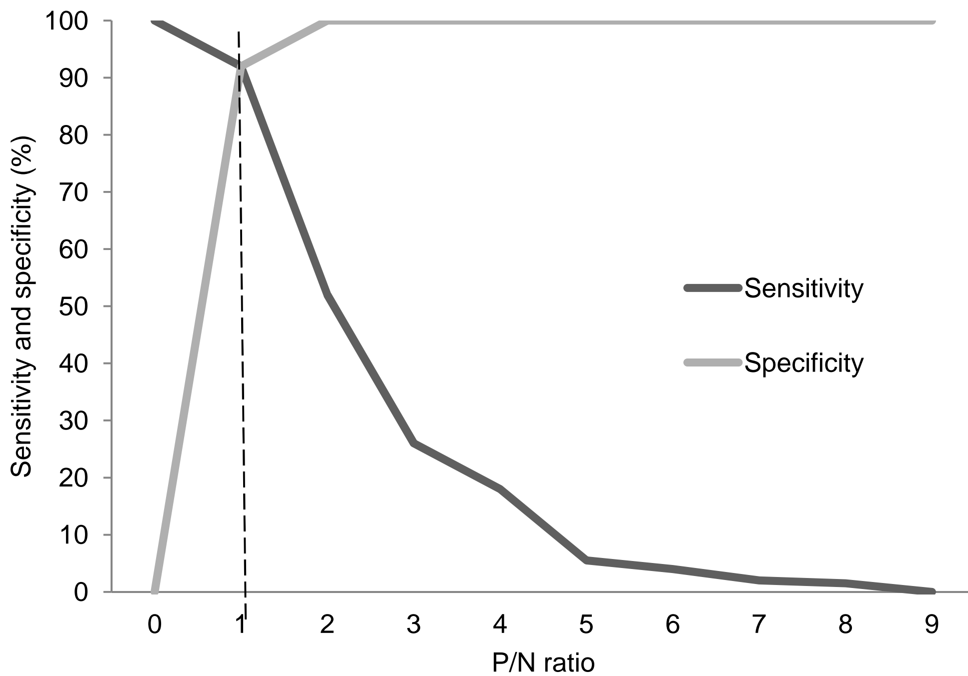



Figure 8. Relationship between cut-off value, sensitivity, and specificity for the E-Fc ELISA. One hundred and seventeen human samples were tested for TBEV antibodies. The cut-off value (broken line) was set as the point at which the difference between the sensitivity and the specificity was minimal.

Table 5. Comparison of the results obtained by neutralization test and E-Fc ELISA (97 serum samples from TBE suspected patients were tested for anti-TBEV antibodies)

| | E-Fc ELISA | | Total |
|---|---|---|---|
| | Positive | Negative | |
| NT positive | 83 (97.6%) | 2 (16.7%) | 85 |
| NT negative | 2 (2.4%) | 10 (83.3%) | 12 |
| Total | 85 | 12 | 97 |

## 9. Discussion

Rapid and accurate flavivirus diagnostic tests are important for surveillance and proper medical management. The neutralization test is the most specific flavivirus 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-TBEV 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 [26, 88, 89]. However, purification of the secreted proteins is cumbersome. Fusion partners are used to purify tagged E-proteins easily by affinity chromatography providing an alternative for the production of viral proteins [26, 90]. The 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 [91, 92].

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 5), 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 [84, 93, 94]. Importantly, the E-Fc protein had no cross-reactivity with anti-human and anti-mouse IgG antibodies, 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 the E-Fc ELISA. The E-Fc ELISA could therefore be used to distinguish between JEV and TBEV infections. Previous studies using Ewt derived from TBEV Oshima 5-10 as antigens for TBEV diagnostic ELISAs reported over 90% sensitivity and

specificity [11, 78]. Our findings correlate well with Ewt ELISA results, indicating that the E-Fc protein has comparable antigenicity.

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

## 10. Summary

Current diagnostic tests for 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 prM/M and 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 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 antibodies to Japanese encephalitis virus. These findings suggest that the E-Fc ELISA may be a useful tool for TBEV serodiagnosis.

**Conclusion**

TBEV and OHFV are both members of the TBE serocomplex although TBEV causes encephalitis while OHFV causes hemorrhagic fever syndrome. TBEV causes over 10,000 cases of encephalitis annually, with an endemic area extending from Western Europe to East Asia. The high public health significance of the virus necessitated the development of TBE vaccines. The development of a vaccine against OHF is economically unfeasible due to the lower prevalence of the disease. However, effective protection against OHF is required during outbreaks. The feasibility of a cross-protective flavivirus vaccine provides a cost effective method for OHF prevention.

The E protein of flavivirus is highly antigenic and is the major target for antibodies. The cross-neutralization of flaviviruses within a serocomplex is dependent on the degree of E protein similarity. Despite the obvious differences in disease manifestation, the two viruses share over 80% E protein homology. The high amino acid similarity suggested that a vaccine against TBE could cross-protect against OHF. In the chapter 1, the potential of commercially available TBE vaccines to protect against OHFV infection was investigated. Sera collected from vaccinated mice and humans demonstrated that the vaccine induces cross-neutralizing antibodies against OHFV. In addition, the vaccine provided 100% protection from a lethal challenge of OHFV in

mice. The findings of this thesis demonstrated that TBE vaccine could be used for OHF prevention.

TBEV is one of the most important vector-borne viral pathogens and the incidence of TBE has been increasing. Integrated surveillance of the vector and the virus in reservoir hosts and humans can identify TBE endemic risk areas and provide information for efficient prevention and control measures. Surveillance of the virus in reservoir species and humans requires cheap, reliable, and quick diagnostic tests. However, the production of commercial TBE ELISA kits is expensive as it requires the high biosafety facilities for the production of the inactivated whole virus antigen. The E protein is highly immunogenic and is therefore an important antigen for flavivirus diagnosis and vaccine development. In chapter 2, the applicability of E-Fc fusion proteins as antigens for TBE serodiagnosis was investigated. The E protein ectodomain was fused to the Fc domain of rabbit IgG and the recombinant protein was expressed in mammalian cells. The recombinant E-Fc protein retained reactivity with both anti-TBEV and rabbit anti-IgG antibodies. The lack of cross-reactivity of the E-Fc antigen with mouse and human anti-IgG antibodies suggested that the antigen could be useful in detecting anti-TBEV antibodies in multiple species. The E-Fc proteins were then used to develop an ELISA to detect TBEV antibodies. The E-Fc ELISA had high sensitivity and specificity in detecting TBEV antibodies in rodent and human sera. The results suggest this recombinant protein would be a good alternative to inactivated whole virus antigens in TBEV surveillance.

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http://onlinelibrary.wiley.com/doi/10.1111/1348-0421.12122/abstract

Chapter 2 is the pre-peer reviewed version of the following article: Chidumayo, N.N., Yoshii, K., Saasa, N., Sakai, M. & Kariwa, H. (2014), Development of a tick-borne encephalitis serodiagnostic ELISA using recombinant Fc-antigen fusion proteins. Diagnostic Microbiology and Infectious Disease, 78: 373-78, which has been published in final form at:

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日本語要約

フラビウイルスには、脳炎や出血などの重篤な臨床症状を引き起こす、節足動物媒介性の人獣共通感染症が属しており、その中でもダニ媒介性フラビウイルスであるダニ媒介性脳炎血清型群には、ダニ媒介性脳炎ウイルス（TBEV）やオムスク出血ウイルス(OHFV)といった医学・獣医学上、重要な病原体が含まれている。フラビウイルスのエンベロープ(E)蛋白は、ウイルス粒子表面を構成する蛋白であり、中和抗体の主要な標的となる。ダニ媒介性血清型群のウイルスは E 蛋白に高い相同性があり、中和抗体の交差反応性が指摘されている。また、その高い免疫原性から血清学的診断において重要な抗原蛋白として研究がされている。本研究では、この E 蛋白に着目してダニ媒介性フラビウイルスの予防法及び診断法に関する研究を行った。

ダニ媒介性フラビウイルスにおいて、一般に利用できるワクチンは TBEV に対してのみであり、他のウイルスに対しては開発が進んでいない。そこで、第一章では TBEV に対するワクチンが他のダニ媒介性フラビウイルス感染症に応用が可能か検討するため、TBEV と OHFV の抗原交差性の解析を行い、市販の TBEV ワクチンが OHFV 感染に対して有効であるか評価を行った。TBEV、OHFV にそれぞれ感染したマウスから採取した血清中の抗体は、中和試験において双方ともに高い交差反応性を示すことが示された。さらに TBEV ワクチンの接種により、マウスにおいては致死量の OHFV 感染から防御され、またヒトにおいては OHFV に対す中和抗体価の陽転が 86%のワクチン接種者において認められた。これらの成績により、TBEV ワクチンは OHFV の感染を予防する上で有効であることが示された。

現在の TBEV の診断においては、ウイルスを扱うための物理的封じ込め施設の必要性や、他のフラビウイルスに対する交差反応性が問題となっている。そこで、第 2 章では安全で特異性の高い診断法開発を目的として、IgG 抗体 Fc 領域(Fc)を融合させた組み換え発現 E 蛋白(E-Fc)の診断用抗原としての応用を試みた。発現させた E-Fc 蛋白はホモ 2 量体で分泌されており、TBEV に対する抗体への反応性を有していた。E-Fc 蛋白を抗原とした ELISA 系を構築し、TBE 流行地で捕獲された野鼠及び TBEV 感染を疑われた患者の血清を用いて中和試験における判定結果と比較した所、90%以上の特異性及び敏感度を示した。さらに、日本脳炎患者血清とは全く交差反応を示さなかった。これらの成績により E-Fc 蛋白を利用した ELISA は TBEV の血清学的診断において有効に活用できる可能性が示された。