Establishment of a memory B cell assay using recombinant Flavivirus protein for determinant of specific antiviral immunity

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Tick-borne encephalitis virus (TBEV) is a human pathogen of the genus Flavivirus, which includes closely related mosquito-borne viruses like Yellow fever (YF), West Nile (WN) and Dengue fever (DEN) viruses. Due to antigenic cross-reactivity the serological diagnosis is difficult, especially when whole virus is used as antigen. Moreover, an understanding of the frequencies of antigen specific memory B cells and their relationship with the antibodies in serum serological memory is likely to be critical to evaluate the long-term protective efficacy of vaccine. For he purpose to overcome the existing test-limitations, domain III of the envelope protein (ED3) from TBEV and WNV were expressed in *E. coli* to evaluate their use as antigen in ELISA using predefined sera positive for either TBEV or WNV and sera tested negative for flaviviruses. An Enzyme-Linked ImmunoSpot (ELISPOT) assay is used for detection of activated TBE-specific antibody secreting B cells (ASCs) in TBE vaccinated individuals. This ELISPOT-based technique follows same basic principles as ELISA, but it enables directly to identify antibody secreting progeny of memory B cells rather than only measuring the accumulated antibody that they produce. The results demonstrated that all sera from TBEV-infected patients were positive in rED3-based ELISA. This ELISA result was consistent with that displayed with commercial kit. In addition, ELISA using recombinant antigens showed no cross-reactivity against sera from WNV or DENV infected patients. Therefore, rED3 used in this study demonstrated significant potential for the development of anti-TBEV ELISA diagnostic assay for detection of specific IgG to TBEV in serum specimens. TBEV-rED3 specific ASCs were detected in previously vaccinated individuals with frequency of ASCs ranging 0.016-0.188% per total IgG ASCs. No statistical significant correlation between frequency of antigen specific ASCs and antibodies level in serum was found. In addition, these study findings also indicated TBE specific memory B cells and neutralizing antibodies have been maintained for long time since vaccination.

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LIST OF ABBREVIATIONS

Ab	antibody
Ag	antigen
ASC	antibody secreting cell
BSA	bovine serum albumin
BCR	B cell receptor
C	capsid protein
CD	cluster of differentiation
ddH_2O	double distilled water
DEN	Dengue
DENV	Dengue virus
DENV-1	Dengue virus type 1
DENV-2	Dengue virus type 2
DENV-3	Dengue virus type 3
DENV-4	Dengue virus type 4
DMEM	Dulbecco's modified essential media
E	envelope protein
ECL	enhanced chemiluminescence
ED1	envelope protein domain I
ED2	envelope protein domain II
ED3	envelope protein domain III
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme-Linked ImmunoSpot
FBS	fetal bovine serum
JEV	Japanese encephalitis virus
HBV	hepatitis B virus
HBsAg	hepatitis B virus (HBV) surface antigen
Ig	immunoglobulin
IPTG	isopropyl β-D-1-thiogalactopyranoside
kDa	kilodalton
LLPC	long-lived plasma cell
M	membrane protein
mAb	monoclonal antibody
MBC	memory B cell
MBP	maltose binding protein

ml	milliliter
mm	millimeter
MOI	multiplicity of infection
nM	nanomolar
ND_{50}	50% neutralizing dose
NMR	Nuclear magnetic resonance
NT	neutralisation test
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline with tween-20
PCR	polymerase chain reaction
PFU	plaque forming unit
PRNT	plaque reduction neutralization test
$PRNT_{50}$	plaque reduction neutralization test 50%
RBC	red blood cell
RdRp	RNA-dependent RNA polymerase
rED3	recombinant envelope protein domain III
rhIL-2	recombinant human interleukin-2
rpm	revolutions per minute
RT	reverse transcriptase
SDS	sodium dodecylsulfate
TBE	Tick-borne encephalitis
TBEV	Tick-borne encephalitis virus
TBEV-Eur	Tick-borne encephalitis virus, European subtype
TBEV-FE	Tick-borne encephalitis virus, Far Eastern subtype
TBEV-Sib	Tick-borne encephalitis virus, Siberian subtype
$TCID_{50}$	tissue culture infectious dose 50%
TLR	toll-like receptor
ul	microliter
uM	micromolar
UTR	untranslated region
WNV	West Nile virus
w/v	weight per volume
x g	x gravity
YF	yellow fever

CHAPTER 1. INTRODUCTION

1.1. Tick borne encephalitis virus (TBEV): Epidemiology and Structure

Tick-borne encephalitis virus (TBEV) is causing severe illness including infection of the central nervous system. It represents a major health problem in Central and Eastern European countries. Between 1990 and 2009 a total of 169,937 cases of TBE were recorded in Europe, i.e. an annual average of 8497 cases. Of these, 2815 cases (33.1%) occurred in Central and Eastern Europe excluding Russia (Heinz and Kunz, 2004; Kollaritsch et al., 2011; Suss, 2011). The reported cases over the last three decades correspond to an increase of 317.8% in Europe including Russia and 193.2% in Europe without Russia (Suss, 2011; Zavadska et al., 2013). As TBEV is transmitted by ticks, the reasons for the increase of TBE cases may be in association with the exceptional weather conditions in Central Europe. A continuous increase of the average temperatures and of the precipitations lead to increased humidity and improve the living conditions of ticks as a humidity >85% and temperature 6°C to 7°C by wild winter (Suss, 2008). These climate changes are affecting vector biology and pathogen transmission (Gray et al., 2009).

TBEV belongs to genus Flavivirus that comprises over 70 viruses, of which many are important human pathogens (Lindenbach et al., 2007). Although, some flaviviruses have no known vector, most members are either transmitted by mosquitoes e.g. WNV, DENV or ticks e.g. TBEV (Chambers et al., 1990).

Based on moderate differences in their nucleotide sequences and “signatures” on protein level, TBE viruses are grouped into three closely related subtypes: a central European subtype (TBEV-Eur), a Far-Eastern subtype (TBEV-FE) and a Siberian subtype (TBEV-Sib) (Ecker et al., 1999; Mukhopadhyay et al., 2005).

The virions of TBEV are spherical particles with approximately 50-60 nm in diameter (Slavik et al., 1967). Like all flaviviruses, the genome is a single-stranded positive-sense RNA genome of approximately 11 kb in length and has a 5’ type-1 cap structure (m^7G5’ppp5’A) but no 3’ polyadenylated tail (Chambers et al., 1990; Lindenbach et al., 2007; Linquist et al., 2008). The viral RNA contains a single open reading frame (ORF) that is flanked by 5’ and 3’ untranslated regions (UTRs) (Figure 1). The single ORF encodes a polyprotein that is cleaved co- and post-translationally by viral and cellular proteases into three structural and seven nonstructural proteins. While the structural proteins, the capsid (C), pre-membrane/membrane (prM/M), and the major envelope glycoprotein (E) are parts of the virion the non-structural proteins NS1 (glycoprotein), NS2A, NS2B (protease component),

NS3 (protease, helicase and NTPase activity), NS4A, NS4B and NS5 (RNA-dependent polymerase) play major role in virus replication and modulation of immune response (Lindenbach et al., 2007).

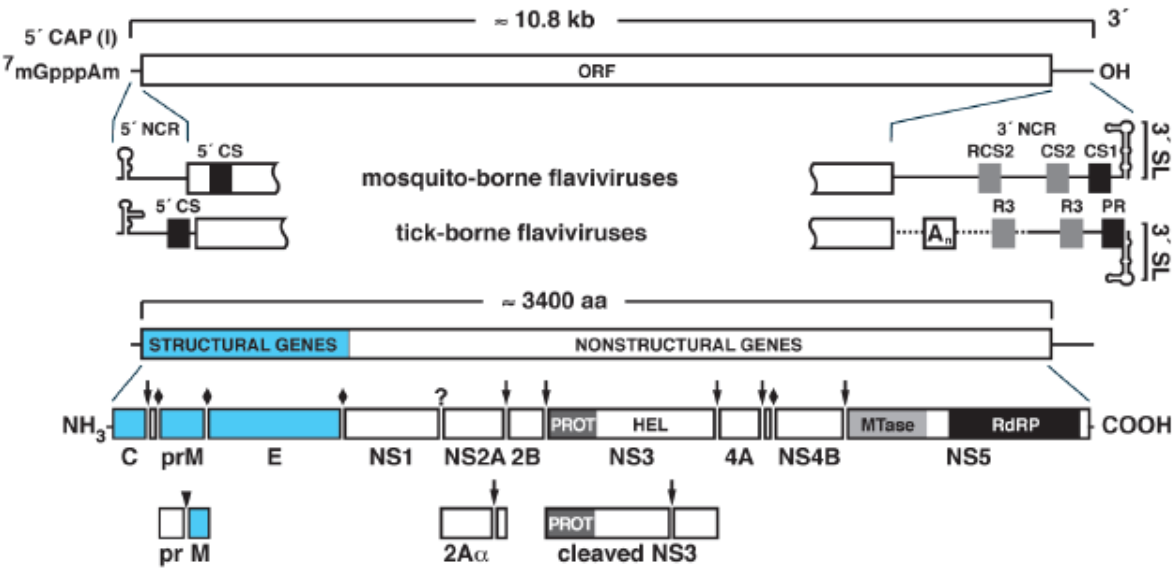


Figure 1. The schematic drawing of a Flavivirus full-length genome (Lindenbach et al., 2007)

1.2. Structure and function of virus envelope protein

The envelope protein of flaviviruses, E protein, carries the receptor-binding site for attachment and comprises the fusion peptide, which mediates uncoating by fusion of viral and cellular membranes. Additionally, the E protein comprises the major antigen and induces formation of protective antibodies (Chambers et al, 1990; Pletnev, 1986). Early studies used mAbs to identify the antigenic structure of the flavivirus E protein (Heinz et al., 1983; Roehrig et al., 2003). The atomic structure of E has been solved for TBEV, DENV-2, DENV-3, WNV and JEV by X-ray crystallography of recombinant proteins comprising the first approximately 400 residues of E, referred to as the soluble E ectodomain (sE) (Kanai et al., 2006; Modis et al., 2005). The E glycoprotein forms a head-to-tail homodimer and is composed of three distinct domains, referred to as ED1, ED2 and ED3 (Figure 2). ED1 is the central domain and links domain ED2 and ED3 (White et al., 2003). ED2 is called the dimerization domain, as it appears to be the principal region of interaction between monomers in E protein dimers on the virion surface.

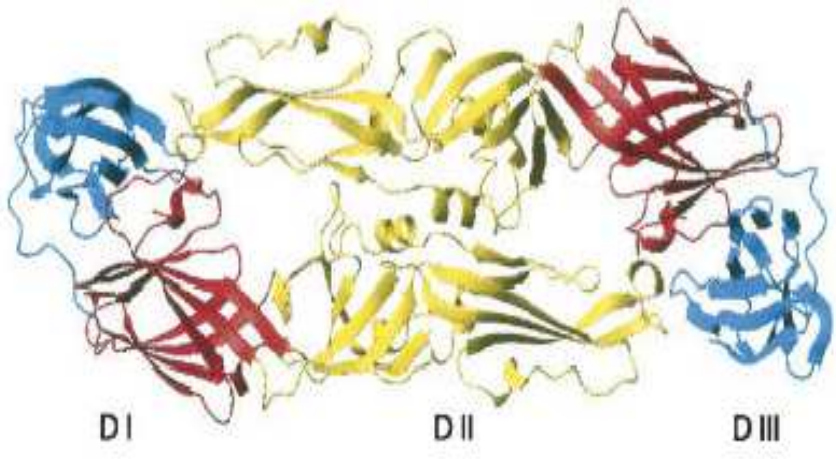


Figure 2. Crystal structure of the E protein domains (Mukherjee et al., 2006)

ED3 contains the receptor-binding site, and is the putative receptor-binding domain (Erb et al., 2010; Lee and Lobigs, 2002). ED3 is highly immunogenic with a number of virus-neutralizing antibody sites mapped to ED3 (Beasley et al., 2002; Crill et al., 2001; Thullier et al., 2001). In particular, ED3 contains many linear epitopes as determined using monoclonal antibodies (White et al., 2003). Correct presentation of ED3 epitopes was originally shown to depend on the single disulfide bridge that ED3 contains (Mason et al., 1989; Winkler et al., 1987). Many studies have verified the antigenic and biological authenticity of recombinant ED3 (rED3) from several different flaviviruses (Beasley et al., 2004; Bhardwaj et al., 2001). Some of these studies also demonstrate the application of ED3 for the specific serological diagnosis of flavivirus infection, like WNV infections (Davis et al., 2004; Wang et al., 2001) or DENV infections (Wahala et al., 2009). In animal model, mice immunized with virus specific ED3 resulted in high titres of neutralizing antibodies and thus protection against lethal infectious doses of WNV (Martina et al., 2008; Spohn et al., 2010), JEV (Wu et al., 2003) and DENV-2 (Simmons et al., 1998), respectively. Additionally, cross protection was observed in mice immunized with ED3 of JEV against lethal WNV infection (Li et al., 2011). NMR (Nuclear magnetic resonance)-derived solution structures of ED3s of the DENV-2 (Huang et al., 2008), DENV-4 (Volk et al., 2007), JEV (Wu et al., 2003), WNV (Volk et al., 2004), Omsk hemorrhagic fever virus (Volk et al., 2006), and Langat virus (Mukherjee et al., 2006) have been determined based on work with recombinant ED3s and indicate an overall similarity in structural fold. NMR structures are also very similar to the ED3 structures obtained by X-ray crystallography of full E protein ectodomains (Kanai et al., 2006; Modis et al., 2005; Rey et al., 1995).

1.3. Clinical Symptom and immune response to TBEV

Serological surveys suggest that 72-87% of human TBE virus infections develop a typical biphasic course of disease with “flu-like illness” (Holzmann, 2003; Kaiser and Holzmann, 2000). The incubation period is generally 7–14 days and during a typical biphasic infection, symptoms during the initial short febrile period (Figure 3) can include fatigue, headache and pain in the neck, shoulders and lower back, accompanied by high fever and vomiting (Gritsun et al., 2003). This is often followed by an asymptomatic period lasting 2–10 days and if the disease progresses to neurological involvement, this leads to the second phase, characterized by acute central nervous system (CNS) symptoms with a high fever. CNS infection can manifest in the meninges, the brain parenchyma, the spinal cord and/or the nerve roots. Thus the second phase of disease is characterized by encephalitic symptoms in 45–56% of patients (Haglund and Gunther, 2003) with high fever (often >39°C) accompanied by signs of meningitis, encephalitis, or radiculitis or mixed neurological forms characterized by severe headache, stiffness of the neck, nausea, vomiting and vertigo. Cerebellar signs and symptoms typically including ataxia dominate encephalitis caused by TBEV (Kaiser and Holzmann, 2000). However, TBEV may induce a variety of neurological symptoms such as disturbed consciousness, convulsions, speech disorder, and vertigo. Affection of cranial nerves with associated symptoms may occur as well.

IgM antibodies appear during early stage of the disease, and persist for at least 6 weeks (up to several months), while IgG antibodies tend to appear a little later and to reach maximum concentration in the convalescent phase, around 6 weeks after onset of the disease. IgG antibodies confer immunity and persist for lifetime and provide a level of immunity that prevents reinfection (Holzmann, 2003). Figure 3 illustrates the antibody dynamics.

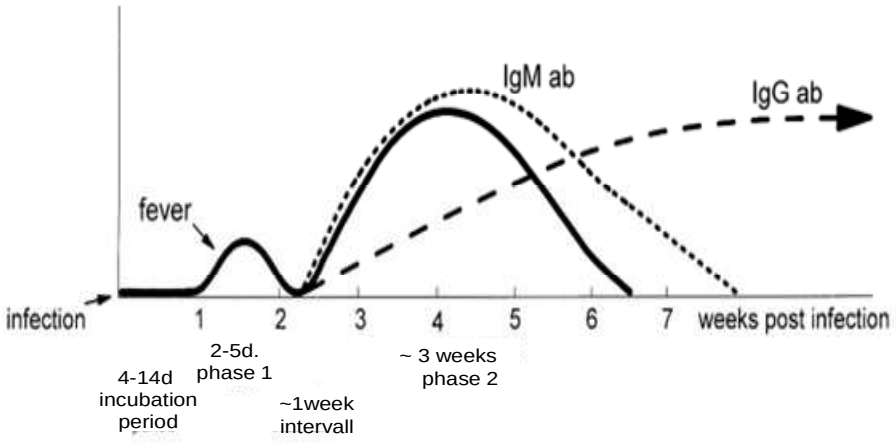


Figure 3. Immune response in TBE with development of specific antibodies (ab) in serum and cerebrospinal fluid (CSF) (Holzmann, 2003)

1.4. Vaccination and humoral immune response

The goal of vaccination is to stimulate the primary adaptive response to a specific agent including the development of immunologic memory so that upon natural exposure to the same agent, the adaptive immune system will be primed to deal effectively and swiftly with the pathogen.

Active immunization is currently the only option for prophylaxis against TBE and is highly recommended for all persons in high-risk areas when pursuing outdoor activities. Encepur® and TBE-Immun®, the two vaccines that are manufactured in Western Europe, are based on cell cultured and inactivated TBEV, adjuvanted to aluminum hydroxide (Zent and Broker, 2005). Both vaccines are widely used in TBE-endemic EU-countries and have been proved to be highly effective (Barrett et al., 2003; Zent et al., 2003). Two Russian vaccines are on the market: TBE Moscow Vaccine® and EnceVir® (Leonova and Pavlenko, 2009; Vorob'eva et al., 2007). Like the Western TBE vaccines, they are based on primary cell culture of chicken fibroblasts and are using aluminum hydroxide as adjuvant (Leonova and Pavlenko, 2009). The Western vaccines use strains of European (TBEV-Eu) subtype (Neudörfl and K23) which are almost identical in amino acid sequence, while the Russian vaccines are derived from the Sofjin and 205 strains, both belonging to the Far Eastern (TBEV-FE) subtype (Leonova and Pavlenko, 2009; Vorob'eva et al., 2007). However, the degree of variation between TBEV subtypes is low with a maximum difference of 5.6% at the amino acid level (Ecker et al., 1999; Lindquist and Vapalahti, 2008). Both Western vaccines show cross protection in mice against the other TBEV subtypes, and vice versa (Holzmann et al., 1992; Leonova et al., 2007).

The conventional vaccination schedule is 0, 21 days to 3 months, and 9-12 months with regularly boosters after 3 years. Several studies have investigated the persistence of protective immunity following vaccination using ELISA for serum antibody titre determination or neutralisation tests and found a longer protection than originally assumed (Holzmann et al., 1996; Rendi-Wagner et al., 2004; Rendi-Wagner et al., 2007). Thus, requirements of booster immunizations and their time interval are questioned regularly.

After natural infection or vaccination, naive antigen-specific B cells bind the soluble antigen by their B-cell receptor (BCR), internalize the complex, degrade the antigen into peptide pieces in endosomal compartments, and present those pieces in complex with MHC II molecules to T cells (Parham, 2005; Tulp et al., 1994). Are the presented antigens recognized by CD4+ T cells it comes to mutual activation steps between the B and T cells and a primary focus in which B cells proliferate and differentiate to IgM-secreting plasma cells is formed. 3-4 days after primary immunization or infection, antigen-specific IgM antibodies are detected in the serum. These molecules have only a low affinity to their

antigen (Berek, 1992). The short-lived IgM secreting plasma cells can't maintain long time immunity. Some of these proliferating B cells, in cognate interaction with T cells, migrate into primary follicles to form a germinal centre (GC) (Liu and Arpin, 1997). At the GC, proliferating B cells undergo massive proliferation, isotype switching to other Ig classes (IgG, IgE, IgA), somatic hypermutation and affinity maturation (Parham, 2005). In the second phase of the primary immune response plasma cells producing high-affinity IgG molecules and long-lived memory B cells with high BCR-affinity to the specific antigen are formed (Kalia et al., 2006) (Figure 4).

Prolonged antibody production lasting for years after infection or vaccination provides the first line of defense against pathogens and is the key to humoral protection. Immunological memory in the B-cell compartment consists of two distinct cell types: Memory B cells (MBCs) and Long-lived plasma cells (LLPCs). While LLPCs mainly reside in the bone marrow and continuously produce large amounts of Abs without the need for re-stimulation by antigen (Radbruch et al., 2006; Tangye, 2011) the MBCs are re-activated by antigen contact (Gray, 1993). MBCs express a high-affinity BCR and are mainly localized in proximity to secondary lymphoid organs and especially in the spleen (Liu et al., 1989; Mamani-Matsuda et al., 2008) at much higher frequencies relative to naïve B cells specific for the same antigen. MBCs express a reduced activation threshold coupled with an increased expression of co-stimulatory molecules and activation markers compared to naïve B cells and they can therefore quickly react to antigen challenge. Upon re-encounter with the cognate antigen MBCs respond by proliferating and differentiating into short-lived antibody-secreting cells (ASCs) that produce large amounts of high affinity Abs (mainly IgG) (Ahmed and Gray, 1996; Rajewsky, 1996).

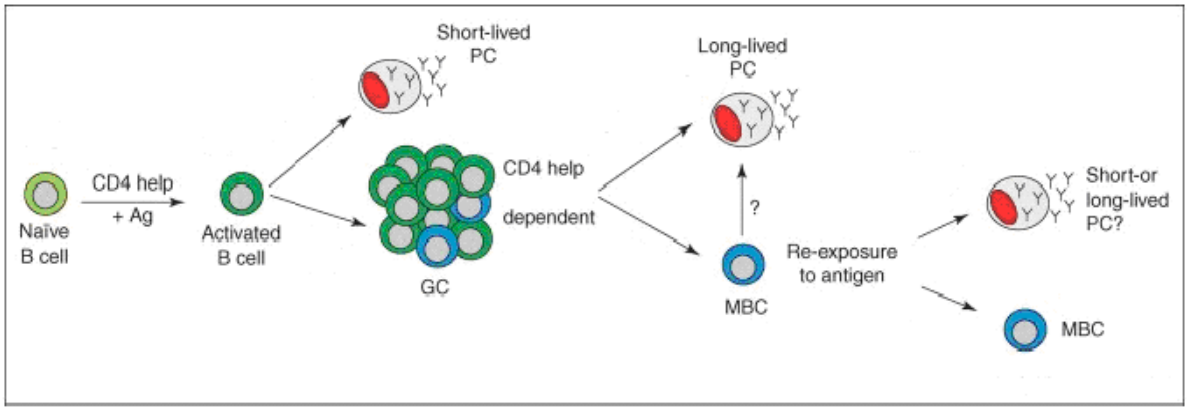


Figure 4. Differentiation of memory B and T cells (modified, Kalia et al., 2006). Ag: antigen; PC: plasma cell; GC: germinal centre; MBC: memory B cell.

While antibody titres decrease over the years memory B-cells might be still present. Thus, for analysis of immune status of vaccinees the examination of the presence or absence of memory B cells might be of interest. A technique that allows the investigation of immunological memory of the humoral immune response is the B-cell ELISPOT.

The B-cell ELISPOT was first described in 1983 (Czerkinsky et al., 1983) and has been an important method for the detection of IgG-producing B cells. Memory B cells require pre-stimulation in order to differentiate into detectable antibody secreting cells (ASCs). Various methods have previously been described for inducing the differentiation of human memory B cells. These methods involve the culture of peripheral blood mononuclear cells (PBMCs) or purified B cells for 5 to 6 days in the presence of polyclonal stimuli and cytokines, such as *Staphylococcus aureus* Cowan I (SAC) and interleukin (IL)-2 (Kelly et al., 2006), pokeweed mitogen (PWM), unmethylated CpG oligodeoxynucleotides (CpG ODN) and SAC (Crotty et al., 2004), CD40L transfected CDw32L mouse fibroblasts plus IL-2 and IL-10 (Tuaillon et al., 2006), CpG and IL-15 (Bernasconi et al., 2002) or PWM, *Staphylococcus aureus* lysate, IL-2, IL-10 and phosphorothioated CpG ODN (Bussmann et al., 2010). Recently, evaluated methods for the activation of memory B cells and found that using the TLR7/TLR8 agonist R848 plus IL-2 was more efficient compared to PWM, CpG and SAC (Jahnmatz et al., 2013; Pinna et al., 2009; Walsh et al., 2013). While, interferon-γ ELISPOT assay relies on the visualization of cytokine secretion by individual T cells following in vitro stimulation with antigen and does not require in vitro expansion of specific T cells before testing (Yang et al., 2000).

Nevertheless, data exist insufficiently which role memory B cells play for long-term immunity in humans e.g. after vaccinations and whether immunity is more reflected by the antibody titre or the memory B cells.

2. Objectives of this work

Tick-borne encephalitis virus (TBEV) is the most important tick-transmitted neurological disease in Central and Eastern European countries and in Russia. Due to antigenic cross-reactivity between TBE and other flaviviruses the serological diagnosis is difficult, especially when whole virus is used as antigen. Domain III of the E protein (ED3) is highly antigenic, and produced antibodies are highly neutralizing and specific for the different flaviviruses.

Thus, this thesis aimed the establishment of a recombinant protein based ELISA and of a memory B cell assay for analysis of specific antiviral immunity after vaccination. In detail this included the following two objectives.

2.1. Expression and purification of recombinant envelope protein domain 3 (rED3) and verification of its application as antigen in ELISA for TBEV diagnosis.

The aim of this subproject is to express domain III of the envelope protein (ED3) from TBEV and WNV in *E. coli* to evaluate their use as antigen in ELISA for specific TBEV and WNV diagnosis. This includes cloning of expression vectors using *E. coli* as expression system and subsequently expression and purification of rED3 with N-terminal MBP and C-terminal histidine tag by affinity chromatography. Purified rED3 was tested for its applicability as ELISA antigen using predefined sera positive for either TBEV or WNV and sera tested negative for flaviviruses.

2.2. Establishment of a memory B cells assay using rED3 for determination of frequency of rED3 specific antibody-secreting cells (ASCs) in individuals vaccinated against TBE.

Memory B cells are the principal determinant of long-term humoral immunity elicited by vaccination. Thus, assessing quantitative parameters other than serum antibody levels after immunisation, such as memory B-cells may provide helpful insight into major determinants of persistent immunity. For this purpose, PBMCs were isolated from blood donors with different vaccination history. Antigen specific B memory cells were stimulated to proliferate and differentiate into antibody secreting cells (ASCs) and were finally quantified using an Enzyme-Linked Immunospot (ELISPOT) assay. Plasma of donors was analysed using ELISA and neutralisation test.

CHAPTER 2. MATERIALS AND METHODS

2.1. Materials

Table 1. Cell lines

Cell line	Kind of cell	ATCC-Nr.	Source
Vero-Cells	African green monkey kidney cells	CCL-81	Institute of Virology, Leipzig University
PS-cells	porcine kidney cells	not available	RKI, Berlin

Table 2. Bacteria strains

Bacteria strain	Genotype	Source
E. coli K12 DH5α	*huA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Institute of Virology, Leipzig University
E. coli BL21 (DE 3)	*fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5*	Institute of Virology, Leipzig University

Table 3. Plasmids

Name	Characteristics
pMAL-c2x	Empty expression plasmid for expression of cloned fragments as MBP fusion proteins in bacteria (New England Biolabs)
WNV-D3pMAlTag2	Bacterial expression plasmid for expression of WNV ED3 as MBP fusion protein with C-terminal histidine tag, provided by Dr. Ulbert, Fraunhofer Institute, Leipzig.

Table 4. Virus strains

Type	strain	Accession number	Source
WNV	WNV New York 2000-crow 3356	AF404756.1	Bernhard-Nocht-Institute, Hamburg
TBEV	Hypr 9BMP	U39292.1	F.X. Heinz and C. Mandl/Vienna

Table 5. Kits

Name	Source	Order number
Big Dye® Terminator v1.1 Cycle Sequencing Kit	Applied Biosystems	4337450
Bio Rad Protein Assay	Bio-Rad	500-0006
Nucleospin® Plasmid	Machery-Nagel	740588.250
QIAprep® Spin Miniprep Kit	Qiagen	72106
ELISpot^{PLUS} for Human IgG Kit	Mabtech	3850-2AW-Plus
Wizard SV Gel and PCR clean up system	Promega	A9282
QIAquick Gel Extraction Kit	Qiagen	28704
recomWell FSME/TBE Virus IgG	Mikrogen	6504

Table 6. Standards

Name	Source	Order number
Gene Ruler 1 kb DNA-Ladder	Fermentas	SM0311
Gene Ruler 50 bp DNA-Ladder	Fermentas	SM0371
50 bp DNA-Ladder	New England Biolabs	N0467G
Gene Ruler LR DNA-Ladder	Fermentas	SM1191
Page Ruler Prestained Protein Ladder	Fermentas	SM0671
Bovine Gamma Globuline (2 mg/ml)	Bio-Rad	500-0208

Table 7. Enzymes

Name	Source	Order Number
Platinum-Taq-Polymerase	Invitrogen Life Technologies	10966034
PfuUltra™ II Fusion HS DNA Polymerase	Stratagene	600670
Restriction enzymes + buffers	Fermentas	
T4 DNA-Ligase (5 Weiss U/µl)	Fermentas	EL0331

Table 8. Antibodies

Name	Source
Mouse-anti-$(His)_6$-Tag (monoclonal, unconjugated); 0.2 mg/ml	Dianova (Dia900)
Rabbit anti-human IgG (HRP conjugated)	Dako Cytomation (P0214)
Rabbit-anti-mouse immunglobuline (HRP-conjugated); 1.3 g/l	Dako Cytomation (P0260)

Table 9. Chemicals and reagents

Name	Source	Order number
Acetic acid	Roth	3738
Acetone	Roth/Applichem	9372 / A1582
Acrylamide (Rotiphorese® Gel 30)	Roth	3029
Agarose: SeaKem®LEAgarose	Biozym	840004
Agar-Agar	Roth	5210
Ammonium persulfate (APS)	AppliChem	A2941
Ampicillin sodium salt	Roth	K029.1
Amylose resin	NEB	E8021S
Aqua ad injectabilia (dH_2O_B)	Braun	PZN3113087
Bovine serum albumine (BSA)	Sigma	A4503
Bromphenol blue	Fluka	18040
dNTP-Set (ATP, CTP, GTP TTP each with 100 mM)	Fermentas	R0186
Citric acid	Roth	1818

Name	Source	Order number
p-Coumaric acid	Sigma	C9008
3,3'-Diaminobenzidine-tetrahydrochloride (DAB)	AppliChem	A0596
1,4-Dithiothreitol (DTT)	AppliChem	A1101
DMEM (+4500 mg/l, +Glutamax™I, -Pyruvat)	Gibco	61965
Ethanol absolut	J.T.Baker	8228
Ethidiumbromide	Sigma	E8751
Ethylenediaminetetraacetic acid(EDTA)	Roth	8043
Fetal Calf Serum (FCS)	Biochrom	S0115/0446K
Formaldehyde (37%)	Roth	4979
D-(+)-Glucose	Roth/AppliChem	X997 / A3594
L-Glutamine (200 mM)	Gibco	25030
Glycine	Roth	3790
Glycerol	Roth	7530
Hi-Di-Formamide	Applied Biosystems	4311320
HPLC-Water	Roth	A511
Hydrogen peroxide (30%, H_2O_2)	Sigma	216763
Hydrochloric acid (37%ig; HCl)	Applichem	A0659
Imidazole	AppliChem	A3635
Isopropanol (2-Propanol)	J.T.Baker	8067
Loading Dye (6x)	Fermentas	R0611
Luminol Sodium Salt	Sigma	A4685
Low-fat milk powder	Vitalia	
Isopropyl -β- D- thiogalactopyranosid (IPTG)	Applichem	A1008
D-(+)-Maltose	Roth	8951.1
MEM NEAA (100x)	Gibco	11140
Methanol	Roth	CP43
Neutral red	Merck	1.01369.0025
Ni-NTA Agarose	Qiagen	30210
PBS 10x (without Ca^{2+} and Mg^{2+})	Gibco	70013
Pen/Strep (10000 U/ml Penicillin, 10000 µg/ml Streptomycin)	Gibco	15140
Peptone	Roth	8986
Potassium acetate (CH_3COOK)	Roth	T874

Name	Source	Order number
Potassium dihydrogen phosphate (KH_2PO_4)	AppliChem	A2946
di-potassium hydrogen phosphate (K_2HPO_4)	Roth	687
Potassium chloride (KCl)	AppliChem	A2939
Potassium hydroxide (KOH)	Roth	6751
Potassium sulfate (K_2SO_4)	Roth	CN79
RPMI Medium 1640+Glutamax-I	Gibco	72400
Sodium acetate (CH_3COONa)	AppliChem	A4555
Sodium hydrogen carbonate ($NaHCO_3$)	AppliChem	A1352
Sodium chloride (NaCl)	Roth	P029
Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$)	AppliChem	A7261
di-sodium hydrogen phosphate (Na_2HPO_4)	AppliChem/Roth	A2943 / P030
Sodium hydroxide (NaOH)	Roth	6771
Sodiumthiosulfat pentahydrate	AppliChem	A3822
Sodium dodecylsulfate (SDS)	Roth/AppliChem	2326 / A1112
Silver nitrate ($AgNO_3$)	Roth	9370
Sodium pyruvate 100 mM	Gibco	11360
Trichloroacetic acid (TCA)	Merck	3744
Tris(hydroxymethyl)-aminomethane (TRIS)	Roth	5429
TRIS-hydrochloride (TRIS-HCl)	AppliChem/Roth	A1087 / 9090
Trypsine-EDTA (0,05 %)	Gibco	25300
Tween 20	AppliChem	A4974
Yeast extract	Roth	2363

Table 10. Buffers and solutions

Name	Composition
D-Glucose-Solution (20%)	Dilute 20 % (w/v) d-glucose in dH_2O, autoclave
1 M potassium phosphate buffer, pH 6	13.2 % (v/v) 1 M K_2HPO_4, 86.8 % (v/v) 1 M KH_2PO_4, pH set up with phosphoric acid or KOH, autoclave
10x PBS	1.37 M NaCl, 27 mM KCl, 80 mM $Na_2HPO_4 \cdot 2H_2O$, 18 mM KH_2PO_4 in dH_2O, pH 7.4.

Name	Composition
	For application: 1x
50x TRIS-acetate-EDTA-buffer (TAE)	2 M TRIS; 5.71 % (v/v) acetic acid, 100 mM EDTA in dH_2O, pH 8.5
1.2 M TRIS-HCl-buffer, pH 8.3	1.2 M TRIS-HCl in dH_2O, pH 8.3; autoclave
High Salt LB Medium	1 % (w/v) peptone, 0.5 % (w/v) yeast extract, 1 % (w/v) NaCl; pH 7.0
Supplemented RPMI	10 %FCS, 1 mM Sodium Pyruvate, 1x MEM NEAA, 2 mM L-Glutamine, 100 units/ml Penicillin, 100µg/ml Streptomycin in RPMI1640+Glutamax I

Table 11. PCR primers

Name	Sequence (5' → 3')	T_M (°C)
TBEVc2x-ED3 s	AAA GGT CTT ACG TAC ACA ATG TGT GAC	65.3
TBEVc2x-ED3 as	TAA TAA GCT TTT AAT GAT GAT GAT GAT GAT GTT TTT GGA ACC ATT GAT AAC TCA GTT C	76.7
RT-TBEV-E	AACCGAAGTCCCAGGCGTGC	64.4
pMAL-Kolonie s	ACG CGC AGA CTA ATT CGA GC	60.5
pMAL-Kolonie as	TAA CGC CAG GGT TTT CCC AG	60.5

2.2. Methods

2.2.1. Molecular biological methods

2.2.1.1. Reverse transcription of viral RNA

Isolated positive stranded TBEV-RNA (obtained from Dr. Petra Fiebig) was reverse transcribed using AMV reverse transcriptase for 1 hour at 58°C. Therefore 5 µl RNA were denatured by incubation at 65°C for 1 minute, afterwards the reaction mix (Table 12) was added.

Table 12. Reaction mix composition for reverse transcription

Component	Volume in µl
5x Buffer	4
dNTP	2.5
Primer (RT-TBEV-E)	3
RNAse inhibitor	0.5
H_2O	4
AMV (10 U/µl)	1

2.2.1.2. Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a technique to amplify defined DNA regions by means of specific oligo-nucleotides (primers). Thermal cycling of repeated heating and cooling for DNA denaturation, annealing and extension leads in production of millions of copies of a particular DNA sequence. In this assignment the PCR reactions were composed as shown in Table 13. Amplification of gene sections coding for TBEV-ED3 was carried out with *pfu* –polymerase which exhibits a proof reading function using cDNA as template and Primers TBEVc2x-ED3 s and TBEVc2x-ED3 as. The following PCR programme was used: 95°C 2’; 6x (95°C 20’’, 61°C 20’’, 72°C 30’’); 29x (95°C 20’’, 65°C 20’’, 72°C 30’’); 72°C 3’; 4°C forever. It’s a two step PCR-programme with lower annealing temperature during first 6 cycles as gene specific primer TBEVc2x-ED3 as contained a 5’ overhang and do not bind to template completely.

Table 13. Composition of PCR reaction mixtures

Component	Volume using	
	***pfu*-polymerase (2.5U/µl)**	**Platinum-*Taq* polymerase (5 U/µl)**
10x PCR buffer	5 µl	2 µl
$MgCl_2$	-	1.4 µl
dNTP (10 mM each)	1 µl	0.5 µl
primer forward (10 µm)	1 µl	0.5 µl
primer reverse (10 µM)	1 µl	0.5 µl
Polymerase	1 µl	0.2 µl
Template	5 µl cDNA	10-50 ng DNA
dH_2O	36 µl	Up to 20 µl

Colony PCR is a method to control ligation and to verify the insert of the desired DNA fragments in transformants i.e. *E. coli* transformed with pMAl-c2x-TBEV-ED3.

A portion of a colony was used as template DNA for the colony PCR and was applied directly to the PCR master mix. For colony PCR the primer pMAL-Kolonie s and pMAL-Kolonie as were used, as well as Platinum-*Taq* Polymerase. PCR programme was the following: 95°C 2'; 30x (95°C 30'', 55°C 10'', 72°C 30''); 72°C 3'.

2.2.1.3. DNA Sequencing

For sequencing, the Big Dye Terminator Sequencing Kit was used, which is based on the chain-termination method by Frederick Sanger (Sanger et al., 1975). For automated sequencing each dideoxynucleotide is labelled with a different fluorochrome, so the chain terminated polynucleotides are distinguished as they pass by the detector (Brown, 2006). The reaction mixture was composed of 4 µl Big Dye Premix, 2 µl primer (10 µM), HPLC-H_2O and 10-100 ng of the PCR-fragments or 200-500 ng plasmids DNA in a total volume of 10 µl. The sequencing reaction programme was: 96°C 2'; 25x (96°C 10'', 55°C 15'', 60°C 4'), at which the annealing temperature was adapted to the annealing temperature of the used primers. At the end of the reaction an ethanol-precipitation was carried out, with 70 µl HPLC-H_2O, 10 µl 3 M sodium acetate pH 4.6-6.8 and 250 µl ethanol (100%) added to the sequencing mixture. After centrifugation for 35 minutes at 20800 x g at 22°C the pellet was washed with 250 µl ethanol (70%) and centrifuged at 20800 x g for 15 minutes. Afterwards pellet was dried in a vacuum centrifuge for 10 minutes at 35°C. Before analysis with the automatic sequencing apparatus ABI Prism 310, pellet was solved in 20 µl Hi-Di-formamide in the dark for at least 30 minutes. The data was then imported in Chromas Lite and analysed manually.

2.2.1.4. Agarose gel electrophoresis

Agarose gel electrophoresis is a method to separate DNA fragments by length and was used to analyse products of PCR reactions or restriction digests. Addicted to the expected length of the fragments the concentration of agarose in the gel varies between 0.8% (1.0-3.6kb) and 2% (0.2-0.6kb) in 1xTAE buffer. Finally ethidium bromide, a dye which fluoresces under UV light after incorporating into the DNA, was added to the gel-solution ($4.5x10^{-4}$ mg/ml). The PCR or digestion samples were mixed with 6x loading dye (1 µl loading dye + 5 µl sample) and the electrophoresis was carried out in 1xTAE at an electric tension of 100 volt. Depending on the expected fragment length Gene Ruler 1kb DNA ladder or Gene Ruler 50bp DNA ladder (Fermentas) was used as marker. The gels were photographed with the MultiImage Light Cabinet of Alpha Innotech and the software ChemiImager 4400.

2.2.1.5. DNA Purification

High-quality and purified DNA, separated from enzymes, nucleotides, reaction buffers, primers and proteins, was important for further DNA applications such as cloning and sequencing. The Promega Wizard SV Gel and PCR Clean Up System or QIAquick® gel extraction kit was used to purify DNA fragments from amplification reactions or after restriction digestion according to instruction manual. The procedure is based on the ability of DNA to bind to silica in the presence of chaotropic salts.

2.2.1.6. Determination of DNA concentration

The concentration of the purified DNA was determined spectrophotometric with NanoDrop® spectrometer (PeqLab), which allowed the analysis of 2.0 µl samples. To estimate DNA concentration the absorbance of a DNA sample was measured at a wavelength of 260 nm against the elution buffer. DNA purity was estimated by determination of the A_{260}/A_{280} ratio. An A_{260}/A_{280} ratio between 1.7 and 2.0 represented a high-quality DNA sample.

2.2.1.7. Restriction Digestion

The restriction digestion procedure was required to cleave DNA molecules at specific sites using particular restriction endonucleases.

Restriction digestion of PCR products and vector for ligation:

Vector pMAL-C2X-ED3 was digested with *XmnI* and *Hind III* in a total volume of 30 µl in a recommended buffer system (buffer orange, Fermentas) with 1 µl of each enzyme, while PCR products were digested by *Hind III* only. After incubation period of 16 hours at 37°C enzymes were inactivated at 80°C for 20 minutes. Fragments were purified as described before (2.2.1.5).

2.2.1.8. Ligation

DNA ligation was used to join together insert DNA of ED3 with the plasmid pMAL-C2X, to generate the expression plasmid. It involved creating a phosphodiester bond between the 3' hydroxyl bond of one nucleotide and the 5' phosphate of another. The reaction was catalyzed by the enzyme T4 DNA ligase.

The ligation mix contained digested vector and insert in a molar ratio of 1:6, 1 µl T4 DNA-Ligase (5 U/µl), 1 µl of 10x T4 ligase buffer in a total volume of 10 µl. The solution was incubated at 20°C for 2 hours and the DNA ligase was denatured at 60°C afterwards.

2.2.1.9. Transformation of *E. coli*

During chemical transformation of the expression plasmid was introduced into competent *E. coli* BL21 (DE 3) cells. 5 µl of the ligation mix or 2 µl plasmid were added to 50 µl of competent BL21 (DE 3) cells. After incubation on ice for 30 minutes, followed by heat shock at 42°C for 30 seconds and additional incubation on ice for 2 minutes, 300 µl SOC medium were added and the mixture was incubated at 37°C for 1 hour. 50-200 µl cell suspension was spread on a plate with LB agar containing 50 µg/ml of ampicillin, which served as a selective antibiotic. The plate incubated overnight at 37°C. In cells, which lack the ampicillin resistance gene, ampicillin caused cell death by intercalating into DNA and cleaving it. By colony PCR plasmids were checked for correct integration of PCR product.

2.2.1.10. Purification of plasmids

Transformed *E. coli* cells were grown in liquid LB media containing ampicillin for 16 hours at 37°C and 220 rpm. Isolation of plasmid DNA from *E. coli* was performed using the QIAprep® Spin Miniprep Kit. The procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt. Isolation of plasmid DNA was done as recommended, except for DNA elution. Here nuclease free water was used instead of Tris buffer.

2.2.2. Biochemical methods

2.2.2.1. Expression of TBEV-rED3 and WNV-rED3 protein

The expression plasmids pMal-c2x-TBEV-ED3 and WNV-D3pMAlTag2 were transformed into *E. coli* BL21 (DE 3); respectively.

For expression a 5 ml overnight bacterial culture was inoculated into one litre of LB broth containing 2% glucose and ampicillin (100 µg/ml). The culture was grown in a shaker at 37°C and 200 rpm to an OD_{600} of approximately 0.6. By addition of IPTG with a final concentration of 0.3 mM protein expression was induced. After 3 hours shaking at 37°C 200 rpm, bacteria were harvested by centrifugation at 9,000 rpm for 15 minutes at 4°C, and pellet was re-suspended in 50 ml of column buffer for amylose affinity chromatography (20 mM Tris-HCl, 200 mM NaCl), and frozen at -20°C overnight.

2.2.2.2. Purification of the fusion protein

2.2.2.2.1. Fusion protein purification by amylose affinity chromatography (MBP-tag)

The MBP-fusion protein is purified from bacterial lysate by binding to an amylose resin and eluted off the amylose using maltose. Briefly, a 1 litre expression cell pellet was re-suspended in 50 mL washing buffer. The resulting solution was subjected to sonication (Branson 450 Sonifier) 3 times for 3 minutes with 2 minute intervals between sonications in order to avoid protein degradation due to heat produced by the sonicator's sound waves. The solution was then centrifuged at 9,000 rpm for 15 minutes at 4°C. The supernatant was subsequently loaded on amylose resin filled columns. Unbound protein was removed by washing of affinity columns with 50 mL washing buffer. By addition of 25 ml elution buffer (20 mM Tris-HCL, 200 mM NaCl, 10 mM Maltose), target protein was eluted and fractions were collected for analysis by SDS-PAGE. Fractions containing the target protein were pooled and concentrated by dialysis against 20% PEG6000 in PBS for several hours (depending on the volume) using dialysis tubing with a 14 kDa molecular weight cutoff (MWCO) membrane. Subsequently, concentrated solution was dialyzed extensively against PBS to remove salts and other small molecules.

2.2.2.2.2. Fusion protein purification by nickel affinity chromatography (6x-his-tag)

In order to improve protein purity, amylose purified-protein was subsequently subjected to nickel affinity chromatography (6x-his-tag) using Imidazole gradient among 400 mM-1.2 M. Nickel-NTA agarose beads (1 ml) were added to the column and equilibrated by addition of washing buffer (50 mM Tris-Cl, 300 mM NaCl, 20 mM imidazole). Subsequently, amylose purified protein was loaded onto the column. Unbound protein was removed by washing with washing buffer and target protein was eluted using a stepwise of increasing imidazole concentrations 0.4 M, 0.6 M, 0.8 M, 1 M and 1.2 M in buffer (50 mM Tris-Cl, 300 mM NaCl). Fractions were collected and analysed by SDS-PAGE. Fractions containing the target protein were combined and dialyzed against PBS over night at 4°C using a dialysis tube with a molecular weight cutoff of 1400 Da to remove imidazole and low molecular weight molecules.

2.2.2.3. Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used to separate proteins according to their molecular weight. In an electric field the proteins, negatively charged by treating with the denaturing agent SDS, migrated through the gel towards the anode. Depending on their size, each protein moved differently and covered another distance in the gel, with the largest proteins travelling the slowest. In this thesis a Tris-Tricin buffers were used (Schagger and von Jagow, 1987).

Table 14. Composition of SDS-gels

Reagent	10% running gel	4% stacking gel
30% Acryl-/Bisacrylamide	4.9 ml	0.8 ml
Tris/Cl/SDS (pH 8.45)	5.0 ml	1.55 ml
dH_2O	3.51 ml	3.89 ml
Glycerol	1.58 ml	-
10% APS	0.05 ml	0.025 ml
TEMED	0.01 ml	0.005 ml

The electrophoresis apparatus was set up with cathode buffer covering the gels and the anode buffer outside of the gel cassette. 10 to 15 µl sample were mixed with 7 µl probe buffer each, were loaded on the gel after incubation at 95°C for five minutes together with 1 µl prestained protein ladder. The common running conditions were 150 volt for 70 minutes. The gels could then be used for further analysis by western blot (protein extracts) or silver staining.

2.2.2.4. Silver staining

To detect proteins after electrophoresis separation on polyacrylamide gels silver staining was carried out according to the protocol of Nesterenko et al., 1994 shown in Table 15. The procedure is based on binding of silver ions to certain amino acid residues (i.e. aspartate, glutamate, histidine, cysteine and lysine) and subsequent reduction of silver ions to metallic silver by formaldehyde. All these steps were carried out at permanent agitation and room temperature. After staining, the gels were documented with Multi-Image Light Cabinet (Alpha Innotech).

Table 15. Protocol for silver staining

Steps	Solutions	Time
Fixation	60ml acetone (50%), 1.5ml TCA (50%), 25µl formaldehyde (37%)	5 min
Washing	dH_2O	3 x 5s
Pre-treatment	60ml acetone (50%)	5 min
Pre-treatment	0.1ml sodiumthiosulfate-pentahydrate (10%) in 60ml dH_2O	1 min
Washing	dH_2O	3 x 5s
Impregnating	0.8ml silver nitrate, 0.6ml formaldehyde in 60ml dH_2O	8min
Washing	dH_2O	2 x 5s
Developing solution	1.2g sodium carbonate, 25µl formaldehyde, 25µl sodiumthiosulfate-pentahydrate in 60ml dH_2O	10-30s
Stop solution	60ml acetic acid (1%)	30s
Washing	dH_2O	10s

2.2.2.5. Western Blot

After electrophoresis, the separated molecules are transferred or blotted onto polyvinylidene difluoride (PVDF) membrane. The PVDF membranes containing transferred proteins were blocked for 30 minutes with 5% skimmed milk solubilized in PBS buffer, and incubated overnight at 4°C with primary antibodies diluted in 5% skimmed milk in PBS. Immunoreactive bands were detected with an appropriate secondary antibody conjugated to HRP diluted in PBS for 90 minutes. After each step, the membranes were washed three times with PBS and were developed with enhanced chemiluninescence ECL substrate (Luminol Enhancer and Peroxide solutions). Labeled proteins were visualized using the chemiluminescence imaging system Intelligent Dark Box II and LAS-1000 camera.

2.2.2.6. Bradford Protein Assay

The evaluation of the protein concentration was carried out using the method of Bradford, 1976. This method is based on the complexion of Coomassie-Brilliant-Blue G250 with proteins. The red-brown form of the dye is converted into its blue form. This absorbance shift can be measured spectroscopic at 595 nm.

To obtain a standard curve dilution series of bovine y-globulin (2 mg/ml) from 0-1000 µg/ml were made in Millipore H_2O and assayed in triplicate. Sample which protein concentration had to be determined was diluted in Millipore H_2O, too. Bradford concentrate was diluted 1:4 in H_2O. 240 µl Bradford-reagent were pipetted into a well of a 96 well-plate and 10 µl samples were added and mixed. 10 µl Millipore H_2O in 240 µl Bradford reagent served as blank values. After incubation of 30 minutes at room temperature in the dark, the colour change could be measured with Tecan Sunrise ELISA reader.

2.2.2.7. Indirect ELISA

The analysis of sera from infected people for TBEV and WNV antibodies was carried out by ELISA. High binding microtiter plates (Greiner Bio-one) were coated with 500 ng/well recombinant protein in 0.1 M sodium carbonate buffer (pH 9.6) at 4^0C overnight. The plates were washed with PBS (pH 7.4) containing 0.05% (v/v) Tween 20 (PBS-T). Remaining sites were blocked with 5% skimmed milk in PBS/0.05% Tween 20 for 1 hour at 37^0C. Plates were then incubated with the test sera diluted in blocking buffer for 2 hours at 37^0C at a 1:200 dilution (50 µl/well). The secondary antibody was a peroxidase-conjugated rabbit anti-human IgG diluted in blocking solution (1:4000) and incubated for 90 minutes at 37^0C. The 3,3′,5,5′-Tetramethylbenzidine (TMB) peroxidase substrate system was used to visualize TBEV and WNV-specific antibody (100 µl/well). Then, the reaction was stopped with 50 µL of 1N H_2SO_4. The samples were measured at 450/650nm wavelength with Tecan Sunrise ELISA reader. Between each step, plates were washed five times using washing buffer. Wells filled with blocking buffer instead of serum sample functioned as blank, which was subtracted from each OD-value. Predefined sera tested negative for flaviviruses were used as negative controls to determine the cut off value as follows: cut off = geometric mean + 3*standard deviations.

2.2.3. Cell culture and Virological Methods

2.2.3.1. Cell culture of adherent growing cells

Cell culture is the process in which cells grow under defined conditions. In this assignment Vero cells were used for propagation of WNV and PS cells for TBEV neutralisation test. Cells were cultivated in tissue culture flask with DMEM supplemented with 5% FCS for Vero cells or 10 % FCS for PS cells at 37°C, 5% CO_2 and 90% humidity. Two times per week cells were splitted. Medium was removed, cells were washed with PBS and trypsine was added to loose the cells from the ground. After incubation for 2-5 minutes reaction was stopped by addition of DMEM containing FCS, cells were resuspended by pipetting up and down and splitted 1:10.

2.2.3.2. Cell count

For determination of the cell count of living cells, staining of dead cells with trypan blue was carried out. 20 µl cell suspension was mixed with 20 µl trypan blue solution (0.4%). 10 µl of this mixture was placed into a NEUBAUER-microscope counting chamber. The unstained living cells were counted in two large squares. The living cell count could be calculated in cells/ml with the arithmetic average of these squares (Formula 1).

Formula 1. Calculation of cell count

$$Cellcount = \frac{arithmetic\ average\ of\ counted\ squares}{A \cdot T \cdot f} \cdot 10^3$$

A	area of one large square (1 mm²)
T	chamber depth (0.1 mm)
f	dilution grade

2.2.3.3. Tissue Culture Infection Dose 50 ($TCID_{50}$)

The tissue culture infection dose ($TCID_{50}$) assay, an endpoint dilution assay, was used to quantify the amount of virus required to produce a cytopathic effect in 50% of inoculated tissue culture cells.

The procedure was adapted to the micro-neutralisation test (2.2.3.4.) and was carried out in a 96 well plate. PS cells were used for TBEV and Vero cells for WNV quantification. Therefore, 50 µl of DMEM were added to each well and virus was diluted serial from 10^{-1} to 10^{-10} in DMEM without supplements to be assayed fourfold. 50 µl of virus dilution were added to each well. As negative control DMEM without FCS was added to the wells. After 1 hour incubation at 37°C, 100 µl with $1x10^4$ cells in DMEM supplemented with 5 % FCS were added per well. Plates were further incubated at 37°C and 5% CO_2 and 90% humidity for five days and stained for colorimetric results with neutral red as described by Taketa-Graham et al., 2010. Therefore the supernatant was removed and 100 µl neutral red dye (prepared as a 1:25 dilution of a 0.33% stock in dH_2O) was added and plates were incubated for 75 minutes at 37°C and 5% CO_2. Afterwards the dye was removed and cells washed twice with 100 µl PBS per well. After a final aspiration cycle 100 µl of acid alcohol (50% ethanol and 1% acetic acid in dH_2O) was added to all wells. After 30 minutes of incubation in which the neutral red retained by remaining living cells is solubilised plates were read on Tecan Sunrise ELISA reader at wavelengths of 540/0nm. For quantification the geometric mean of the OD values obtained for cell control (CC) was calculated and of the lowest virus dilution (V) used. The endpoint OD value (cut off) was calculated using the following formula: cut off value = 0.5*(CC-V) + V.

Wells with OD values above the cut off are regarded as negative (living cells), wells with OD values below the cut off are positive, i.e. virus infection and CPE. $TCID_{50}$ was calculated according to the formula (Formula 2) of Spearman and Kaerber (Kaerber, 1931). Working steps, containing virus, were carried out in a bio-safety level 3 laboratory.

Formula 2. Calculation of $TCID_{50}$

$\frac{\log TCID_{50}}{Volumen} = X_0 - \frac{d}{2} + d \cdot \frac{r}{n}$	X_0	Logarithm of the reciprocal value of the lowest dilution, at which all test units are positive (disrupted cell layer)
	d	Logarithm of dilution interval (10x-dilution: d = 1)
	n	Number of test units per dilution
	r	Amount of positive test units per dilution level from and including X_0

2.2.3.4. Micro-neutralisation test

The micro-neutralisation test for determination of highest serum dilution resulting in 50% protection of inoculated tissue culture with a determined amount of virus was performed according to the determination of $TCID_{50}$.

Serum was diluted in a two fold serial dilution in DMEM, starting at 1:25. 50 µl of serum dilution was added to each well to be assayed four fold. 50 µl virus dilution with a concentration of 100 $TCID_{50}$ were added to 3 of the 4 serum dilution containing wells. To the fourth 50 µl DMEM without supplements was added as control. After incubation of this mixture for 1 hour at 37°C 100 µl with $1x10^4$ cells in DMEM supplemented with 5% FCS were added per well. Plates were further incubated at 37°C and 5% CO_2 and 90% humidity for five days and stained for colorimetric results with neutral red as described above (2.2.3.3). In each test, used virus stock was titrated again by diluting 10^{-1} to 10^{-8} and the used virus dilution was also back titrated using it undiluted to 10^{-6}. Those performing too low or too high virus doses were not accepted and neutralisation test was repeated.

Neutralizing antibody titres were expressed as neutralizing dose that protects 50% of the cell cultures (ND_{50}) calculated according to Behrens & Kaerber (Taketa-Graham et al., 2010).

Formula 3. Calculation of ND_{50}

$ND_{50} = V[d*(S-0,5)]$	*V*	Logarithm of the reciprocal value of the serum dilution, at which all test units are positive (disrupted cell layer)
	d	Logarithm of dilution interval (10x-dilution: d = 1)
	S	amount of all positive test units from 0% to 100% divided by the number of test units per dilution

ND_{50} ≥10 were considered protective antibodies. Evidence of neutralizing antibodies was used as a surrogate parameter for this purpose since their formation is of decisive importance for virus elimination in TBE infection and results in protection against disease (Rendi-Wagner et al., 2004).

2.2.3.5. Purification of virus

Vero cells were grown and infected with TBEV and WNV at a multiplicity of infection (MOI) of approximately 0.01. The infected cell culture supernatant was harvested on day 7 post infection. Cell debris was removed by centrifugation at 4,000 rpm for 30 minutes at 4ºC. The supernatant was layered on top of a 5 ml sucrose cushion (25 % sucrose in PBS). The virus was pelleted by ultracentrifugation at 100 000 x g for 4 hours at 4ºC to remove low molecular weight contaminants such as soluble proteins. The supernatant was poured off and the tubes were briefly left upside down on chromatography paper in order to remove excess liquid from the side of the tubes and virus pellet was resuspended in 1 ml PBS.

2.2.3.6. Virus inactivation by H_2O_2

Inactivation was performed by addition of H_2O_2 as described by Amanna et al., 2002. Briefly, H_2O_2 with a final concentration of 3% was added to the concentrated virus and was incubated at room temperature for 2 hours with vortexing every 30 minutes. Adjacent sample was dialysed extensively against PBS to remove H_2O_2. Inactivation was verified by infection of cell culture before virus was used as antigen in ELISPOT or ELISA. Concentration was determined using Bradford assay.

2.2.4. ELISPOT Assay

To quantify antibody secreting B-cells, a B-cell ELISPOT assay (Enzyme-linked immunospot assay) is performed. This method based on the detection of antibody secreted by B cells and was published in 1983 by Czerkinsky et al. ELISPOT was performed using ELISpot^{PLUS} for Human IgG Kit from MABTECH according to the manufacturer's instructions.

Performance

Isolation of peripheral blood mononuclear cells (PBMCs)

15 ml heparinized venous blood was diluted with an equal volume of PBS and was carefully layered on to 15 ml Histopaque (Sigma). After centrifugation at 1000 x g for 20 minutes at 20°C, PBMCs were remained at the interface of plasma and Histopaque. Red blood cells (RBCs) and other cell debris were precipitated at the bottom of the tube. The mononuclear cells were then collected carefully with a Pasteur pipette and were washed twice in PBS at 400 x g for 15 minutes at 20°C.

Finally, the PBMCs were resuspended in 5 ml of supplemented RPMI medium (Table 10) and counted. Therefore, 10 µl of cell suspension was added to 50 µl PBS and 40 µl trypanblue.

Activation of memory B-cells

For activation $1x10^6$ cells were plated per well of a 24 well plate in supplemented RPMI which contained 1 µg/ml R484 and 10 ng/ml rhIL-2. Cells were incubated for 3 days at 37°C, 5% CO_2 in a humidified incubator. About $1x10^6$ cells were placed in a well of a 96 well plate without stimulation agent and incubated likewise.

Preparation of ELISPOT plates

To test for antigen specific memory B-cells sterile 96-well Multiscreen-IP filter plates with PVDF membranes (Millipore) provided in the kit were pretreated with 50 µl 70% ethanol for 45 seconds and then washed five times with sterile water before coating. Coating antigens including TBEV-rED3, WNV-rED3, inactivated TBEV, Influenza virus nucleoprotein (NP) and MBP were diluted to 1 µg/well in sterile phosphate buffered saline (PBS) and anti-IgG coating mAbs were diluted to 10 µg/ml in PBS and added to the plate. The plates were then incubated overnight at 4°C.

Incubation of cells in ELISPOT plate

On day of ELISPOT performance plates were washed and blocked with supplemented RPMI for 30 minutes at 37°C. After the 72 hour pre-activation, activated PBMCs were harvested and washed three times with RPMI 1640 before counting. The cells were resuspended and added to the plates with 100 µl cell suspension/well with concentration of $1x10^5$ and $3x10^5$ cells/well in antigen coated wells while 500 and 1000 cells/well were placed in anti-IgG coated wells (Table 16). Each cell concentration was tested in duplicate. Plates were incubated absolutely vibration-free for 20-24 hours at 37°C, 5% CO_2 in a humidified incubator.

Table 16. Antigens and cell concentrations for ELISPOT coating

Antigen	Function	Plated number of activated cells		Plated number of non activated cells
TBEV-rED3	To be tested specific antigen	100 000	300 000	-
WNV-rED3	To be tested specific antigen	100 000	300 000	-
inactivated TBE whole virus	To be tested specific antigen	100 000	300 000	-
MBP	Control antigen for unspecific reactions with MBP moiety of fusion proteins	100 000	300 000	-
Influenza virus Nucleoprotein	Positive control for ELISPOT	100 000	300 000	-
Anti-IgG-immunoglobuline	Activation control	500	1000	1000

Detection of Spots

After 20 hours of incubation, the cells were discarded and the plates were washed with PBS (5×200 µl/well). Biotinylated anti-IgG detection mAbs diluted to a concentration of 1 µg/ml in PBS/0.5% FCS were added to the plate wells and incubated for 2 hours at 37°C. The plates were washed with PBS (5×200 µl/well) before Streptavidin conjugated with Alkaline-Phosphatase (SA–ALP) (Mabtech) diluted 1/1000 in PBS/ 0.5% FCS was added and incubated for 1 hour at 37°C. Unbound conjugate was washed away with PBS (5×200 µl/well). BCIP/NBT-plus substrate (Mabtech) was filtered through a 0.45 µm-filter and 100 µl/well was added to the wells and incubated for 15 minutes at room temperature. The reaction was stopped by rinsing the plates with tap water. The plates were then left to dry overnight in darkness.

Plates were counted on an ELISPOT reader (AID ELISpot 04 plate reader, Autoimmun Diagnostika GmbH). Spots in wells with control protein (MBP) were subtracted from the number of spots in wells coated with specific antigen (TBEV-rED3, WNV-rED3 and NP).

Calculation of the memory B-cell frequencies

The frequency of antigen-specific cells was calculated as the percentage of total antibody-secreting cells according to the Formula 4 (Bussmann et al., 2010).

Formula 4. Calculation of memory B-cell frequencies

$$\%\ \text{Ag specific spots} = \frac{\text{cells at Ig coated wells}}{\text{Ig spots}} \text{ x } \frac{\text{Ag spots} - \text{control spots}}{\text{cells at Ag coated wells}} \text{x } 100$$

2.3. Study design: study subjects and statistical analysis

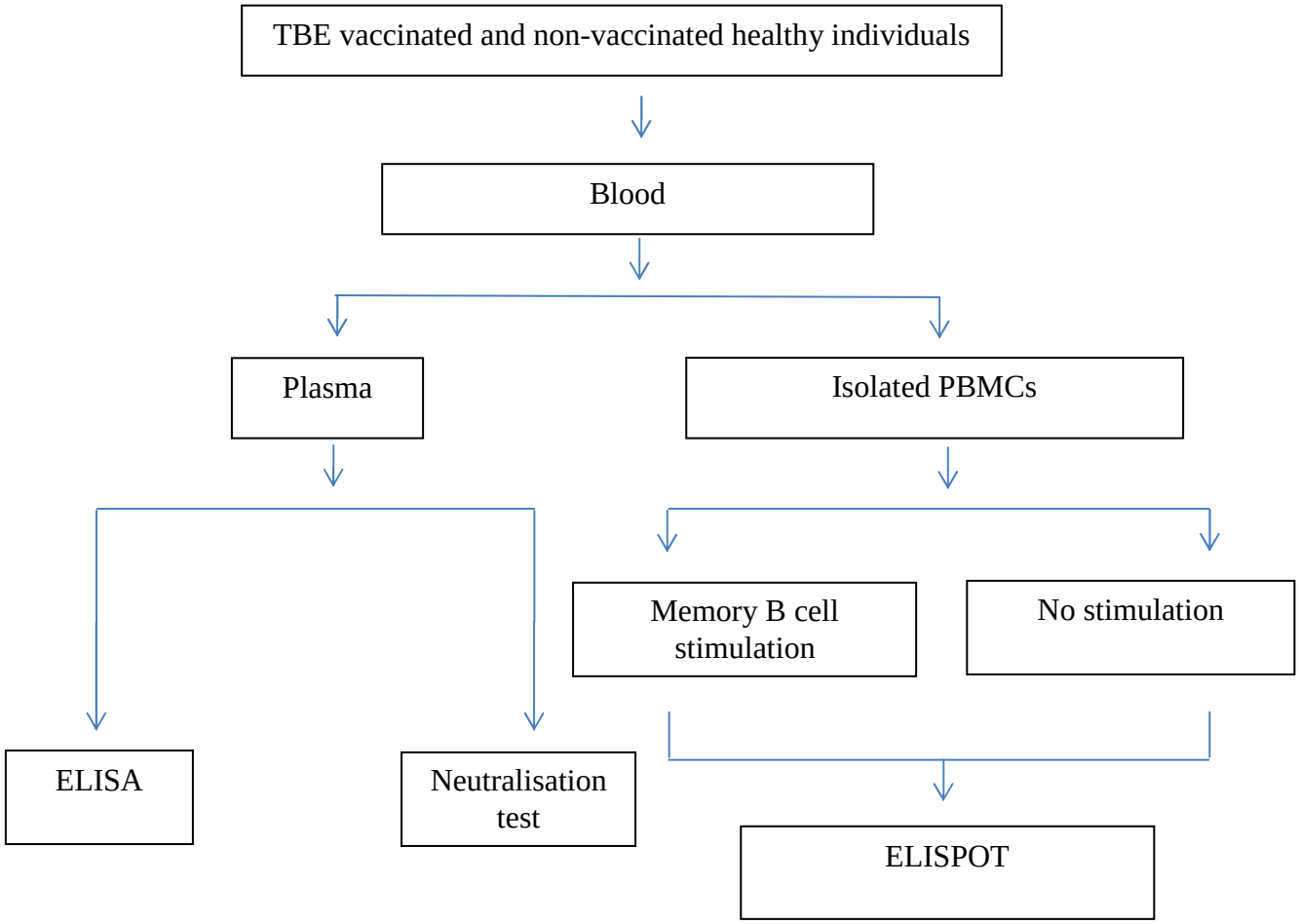
Nineteen normal adult healthy volunteers were enrolled in the analysis (7 males; 12 females). Twelve of 19 individuals had get a basis and booster immunization against TBE, in which, six of those individuals vaccinated less than 5 years ago received multiple vaccinations and six individuals vaccinated more than 10 years ago at time of blood draw. The year of the most recent vaccination was used to establish the year's post-vaccination of the vaccine in all data analysis. Seven TBE unvaccinated individuals were also determined for TBE specific memory B cells as negative control; four of those individuals were naturally infected with Dengue virus.

Since the data were not distributed normally, a Mann–Whitney test was performed to compare values between groups. Pearsons's correlation as well as linear regression was done to analyse the correlation between variables.

Geometric mean titers (GMT) were calculated with 95% confidence intervals (CI). Confidence intervals of GMTs were calculated based on the assumption of a log-normal distribution of titres.

Analyses were performed with SPSS (SPSS Inc. Version 18) and GraphPad Prism (GraphPad Software, Inc. Version 5.0). For all statistical analyses, p values less than 0.05 were considered statistically significant.

The following flow chart summarizes the study



CHAPTER 3. RESULTS

3.1. Expression and purification of recombinant envelope domain 3 protein (rED3) and evaluation of its use for TBE diagnosis by rED3-based ELISA

3.1.1. Construction of the pMAL-ED3 expression vector

In order to express the TBEV rED3 in *E. coli* (Figure 5) the vector pMAl-c2x was used which contains the *malE* gene that encodes the maltose-binding protein (MBP) under the control of the promoter P_{tac}. Additionally, P_{tac} bears an operator region negatively regulated by the *lacI* repressor. Therefore, the induction of gene expression from such operon could be controlled by IPTG. The ED3 coding sequence was cloned downstream from *malE* gene using the *XmnI* and *HindIII* site to ensure a directed insertion of the fragment and produce the pMAL-TBEV-ED3 expression vector.

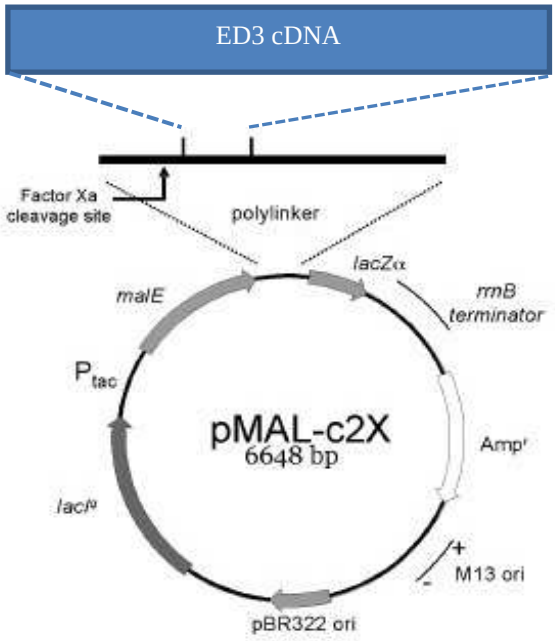


Figure 5. The diagram illustrates the strategy employed to construct the pMAL-ED3 vector. The cDNA fragment was cloned into the polylinker of the expression vector pMAL-c2X, as described in Materials and Methods. Features of the pMAL-c2X expression vector, such as the factor Xa cleavage site, the *malE*, *lacI*^{q}, *lacZα* and *Amp*^{r} genes, the polylinker region, the replication origins of M13 phage, the P_{tac} promoter, and the rrnB terminator, are indicated.

The first step to clone pMAL-TBEV-ED3 was the amplification of the coding region of domain 3 of TBEV structural gene E by PCR using gene specific primers TBEVc2x-ED3 s and TBEVc2x-ED3 as. cDNA derived by reverse transcription of viral RNA was used as template. Primer TBEVc2x-ED3 as includes a 5' extension with a *HindIII* site to facilitate directed integration into restricted pMAL-c2x. Additionally, it contained the coding sequence for a histidine tag for easier purification of the recombinant protein. Corresponding to the length of ED3 the PCR reaction produced a 319 base pair (bp)-long fragment as verified by electrophoresis (Figure 6)

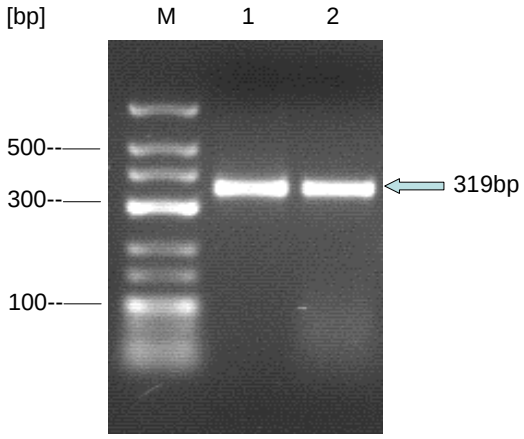


Figure 6. PCR products showed a successful amplification of gene coding for TBEV-ED3. A corresponding band of 319 bp was revealed (lane 1 and 2), M: molecular weight marker (GeneRuler Low range DNA ladder).

For the construction of pMAL-TBEV-ED3 the PCR products were purified and digested with *Xmnl* and *HindIII*, as was pMAL-c2x vector. Amplicons were ligated into the vector using T4-DNA-Ligase.

The cloned expression plasmids were transformed into *E. coli* BL21 (DE3) cells. Positive selected clones were confirmed by colony PCR using pMAL-kolonie s and pMAL-kolonie as primers. As shown in Figure 7, using DNA from clone 1(cl1), clone 2 (cl2), clone 3 (cl3) and clone 4 (cl4) as template fragments of 436 bp were obtained as visualized by electrophoresis. In the control reaction with pMAL-c2x vector as template the fragments are smaller, 165 bp. Thus the clones 1, 2, 3 and 4 contain the ED3 gene.

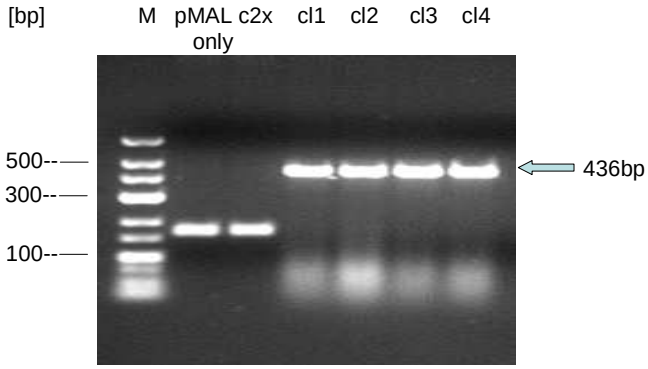


Figure 7. Transformed *E.coli* containing the pMAL-ED3 vector was confirmed by colony PCR in comparison to empty vector pMA-c2x. PCR with DNA of recombinant clones cl1, cl2, cl3, cl4 resulted in a 436 bp sized fragment. M: molecular weight marker (GeneRuler Low range DNA ladder).

Overnight cultured *E. coli* transformants were then used in subsequent screening experiments. The plasmids were isolated from the bacterial cells and digested with restriction enzymes *HindIII* and *BglII*. Gel electrophoresis analysis shows that a double restriction produced the expected fragments of 5837 and 1080 bp; respectively (Figure 8).

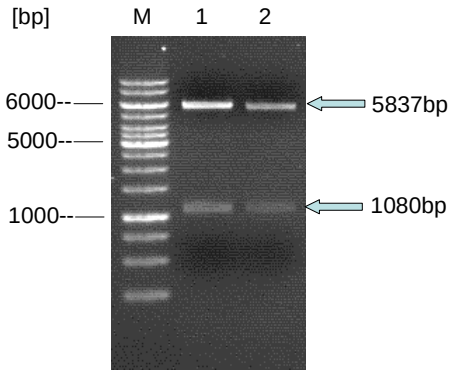


Figure 8. pMAL-ED3 was characterized by enzymatic restriction. Lane: 1, 2, *BglII* and *HindIII* digestion. The expected size of digested fragments is indicated on the left. M: molecular weight marker (GeneRuler 1kb DNA ladder).

The next screening step for recombinants was DNA sequencing using primer *pMAL-klonie s* to confirm a correct sequence and insertion of ED3 coding DNA fragment.

For expression, one clone was chosen, which inserted fragment showed 100% concordance with the corresponding region of ED3 cDNA, i.e. clone 2.

3.1.2. Expression and purification of TBEV-rED3 and WNV-rED3 protein in *E. coli*

According to the used cloning strategy the vector pMAL-c2x-TBEV-ED3 encodes a MBP-fusion protein of 489 amino acids (aa). The N-terminal 387 aa encode for MBP with amino acids of the polylinker, whereas 102 residues correspond to TBEV-ED3 with a C-terminal $(His)_6$-tag. The vector pMAL-WNV-ED3, which was obtained from Dr. Ulbert (Fraunhofer Institute, Leipzig), was cloned by *BamHI* and *XhoI*. Thus, it encodes a protein of 504 amino acids with 393 residues comprising the N-terminal MBP with polylinker and 111 residues comprising the WNV-ED3 with C-terminal $(His)_6$-tag. Theoretical molecular weights are shown in Table 17.

Table 17. Theoretical molecular weight of recombinant proteins

Protein	TBEV-rED3	WNV-rED3
Molecular weight of MBP-fusion protein [kDa]	53.9	55.0
Molecular weight of rED3 [kDa]	10.61	10.81

Cytoplasmic expression of both proteins was successful, as shown in Figure 9 representative for the expression of TBEV-rED3. Three hours after induction of protein expression with IPTG, dominant bands were detected in lane AI at 54 kDa, which are not present in the cell lysate of non induced cells (lane BI).

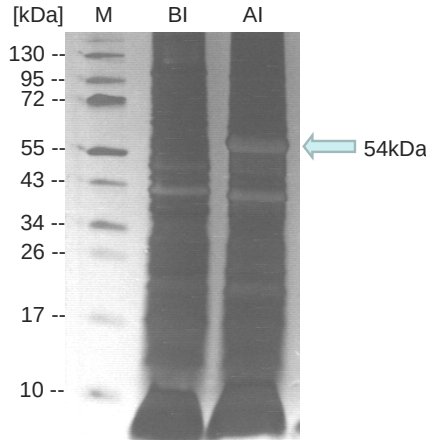


Figure 9. Analysis of MBP-ED3 fusion protein expression after addition of final 0.3 mM IPTG. Cell lysates were obtained before (BI) and after 3 hours of IPTG induction (AI). Proteins were fractioned by SDS–PAGE analysis and silver stain. The bands matching the expected size of 54 kDa for TBEV-rED3.

To determine whether these bands corresponding to the fusion protein, a Western blot analysis of IPTG induced cultures was performed. As shown representative in Figure 10 for MBP-TBEV-ED3 by using his tag specific antibody, a band with apparent molecular mass of 54 kDa was revealed. Overall, the immunoblot analysis corroborated that the protein with apparent molecular weight of 54 kDa, induced after IPTG treatment corresponds to the fusion MBP-ED3 protein. The smaller bands detected by the antibody probably represent N-terminal degraded MBP-fusion proteins. No bands are visible before IPTG induction.

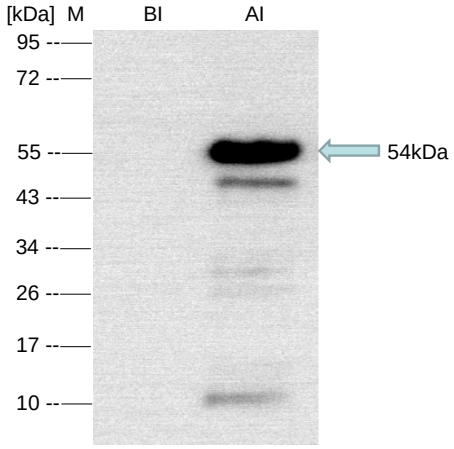


Figure 10. Analysis of protein expression before (BI) and after (AI) IPTG induction by Western blot using histag specific antibody.

The protein purification process was started using amylose affinity chromatography. Purification results are shown representative for protein MBP-TBEV-rED3 in Figure 11. A single prominent band migrating at the 54 kDa marker visible in the silver-stained gel was detected in the elution fractions 1-4 (Figure 11A) indicating the successful purification of the MBP-ED3 fusion protein. Anyway, a band of the same size is visible in the flow through (F), which indicates insufficient binding of the protein to the amylose. Eluted fractions containing the fusion protein were pooled and purified further by nickel affinity chromatography using an imidazole gradient. Again the flow through contains the target protein (Figure 11B). Bound protein was eluted by imidazole at concentrations from 0.4 M to 1.4 M.

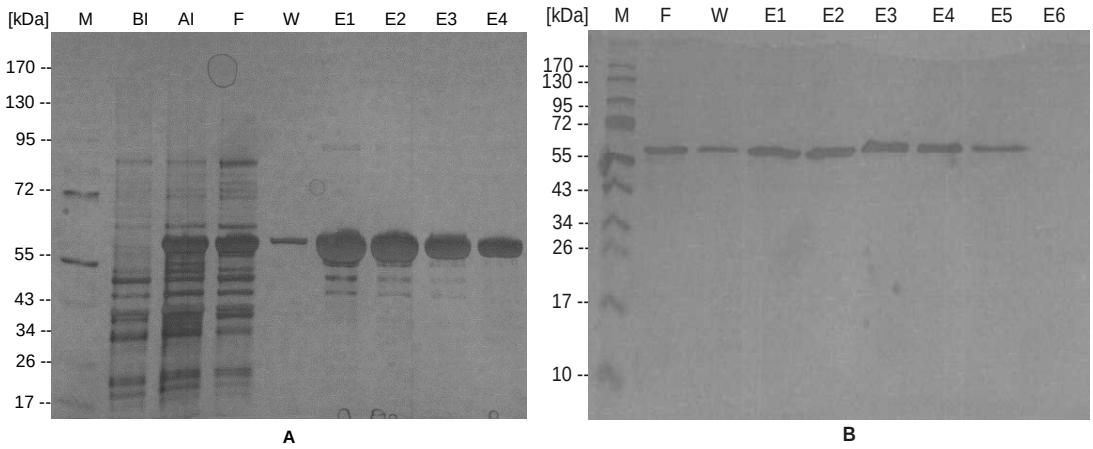


Figure 11. Purification of fusion protein 3 hours after induction with IPTG The protein was purified by amylose resin column (A) followed by nickel affinity chromatography (B). Purification was analysed by 10% SDS-PAGE and stained with silver.

BI: before IPTG induction; AI: after IPTG induction; F: flow through; W: wash fraction.

E1-E4: eluted fractions of amylose resin column by 10 mM amylose (A)

E1-E6: eluted fractions of nickel column purification by increased imidazole concentration (400 mM, 600 mM, 800 mM, 1 M, 1.2 M, 1.4 M imidazole; respectively (B)

Finally, fractions containing the target protein were pooled and dialyzed in PBS (pH 7.4) overnight in order to remove the high imidazole concentration and small weight molecules.

3.1.3. Analysis of proteins as antigens by Western Blot

In order to analyse antigen specificity of TBEV-rED3 and WNV-rED3 predefined sera were analysed on blotted purified rED3 proteins (1µg per lane). By the TBEV and WNV positive serum, rED3s were detected as proteins with an apparent molecular weight of approximately 55 kDa. However, application of WNV positive sera or TBEV positive sera on WNV- or TBEV-rED3 protein resulted in a reaction with both proteins and similar results were obtained when negative serum was applied (Figure 12A, 12B and 12C). Moreover, the reactivity between antibodies and MBP in all positive and negative sera was observed. Thus, in Western Blot a discrimination of WNV positive sera and TBEV positive sera using rED3 proteins is not possible, probably due to either linearization of the protein and reaction of cross reactive antibodies with conserved linear epitopes or reactivity with MBP moiety of fusion proteins.

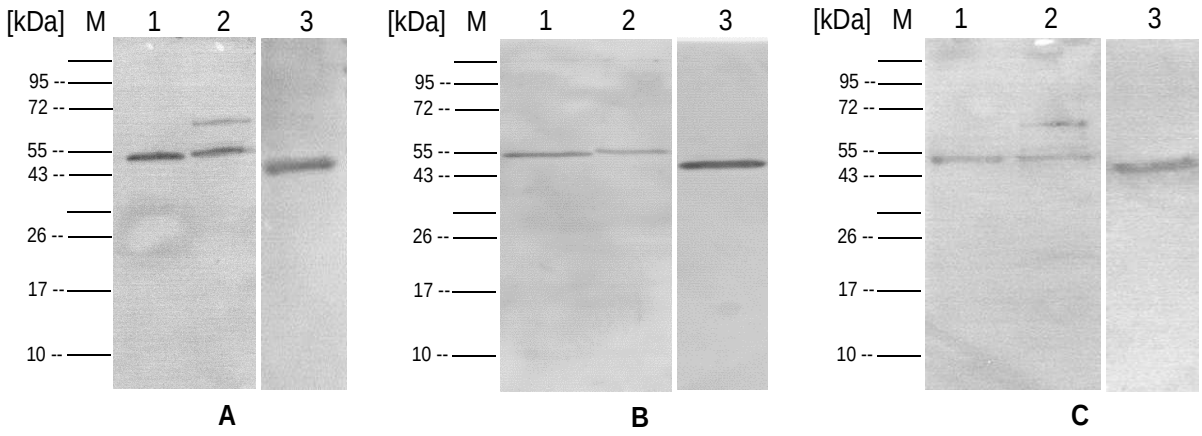
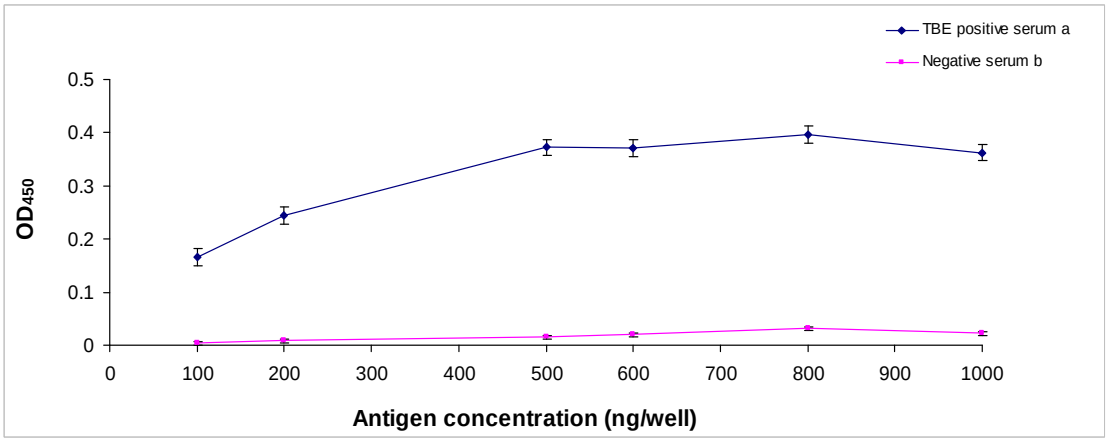


Figure 12. Immunoassay based on recombinant ED3 protein, immobilized on PVDF membrane. Following sera were used (A) TBEV positive serum; (B) WNV positive serum; (C) Negative serum. All sera were diluted 1:20. 1: TBEV-rED3; 2: WNV-rED3; 3: MBP.

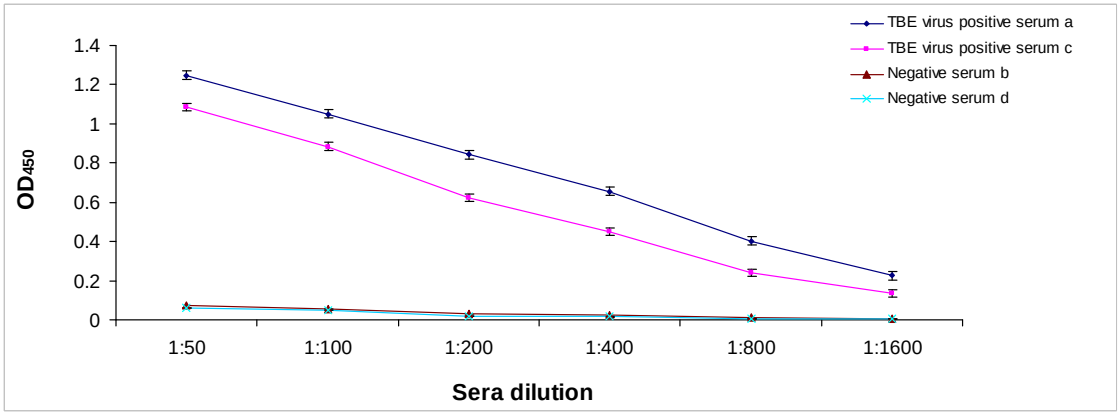
3.1.4. Analysis of proteins as antigens by Enzyme-linked immunosorbent assay (ELISA)

The first step was to determine the specificity of sera for the immobilized antigen. Therefore, soluble rED3 protein at concentrations from 100 ng to 1.0 µg per well was coated in ELISA plate. TBE positive and negative sera were tested to identify optimal antigen concentration. This result proved that binding of TBE virus-specific serum antibodies with the best signal-to-noise ratio was obtained at 500 ng/well (10 µg/ml). An increased concentration of antigen did not result in higher OD values (Figure 13A). Thus, 500 ng/well of rED3 was selected for overall ELISA coating.

Next, in order to determine minimum background and maximum sensitivity, TBE virus-positive and negative anti-sera were serially diluted from 1:50 to 1:1600 in 5% non-fat milk. The results showed that at a dilution of 1:50, the background of the negative sera was relatively high. At a dilution of 1:200 the positive sera produced an absorbance reading that was 6-fold higher than the negative serum reading. Additionally, OD_{450nm} value at this dilution showed a minimum background reading of 0.153 (Figure 13B). Thus, the sera were used in 1:200 dilutions for further ELISAs.



A



B

Figure 13. Titration curves for antigen concentration (A) and serum (B) for minimum background and maximum sensitivity in ELISA. All sera were tested in duplicate against TBEV-rED3 protein (values are the mean OD_{450nm} with standard deviation (SD)). Optimum values with the best signal-to-noise ratio were obtained at antigen concentration of 500 ng/well and at a serum dilution of 1:200. Error bars present as standard deviation.

To evaluate whether the rED3 based ELISA was applicable for serological diagnosis, 28 serum samples from TBE post-infected and vaccinated individuals were tested. The results were compared with those obtained using commercial ELISA kit (Figure 14 and Table 18). Of the 28 serum samples examined, 23 were positive, 5 were negative on commercial IgG ELISA. The 23 serum samples shown to be positive by commercial IgG ELISA, were also positive by IgG-ELISA using rED3. Five serum samples that were negative by commercial IgG ELISA were also negative by rED3-based IgG ELISA.

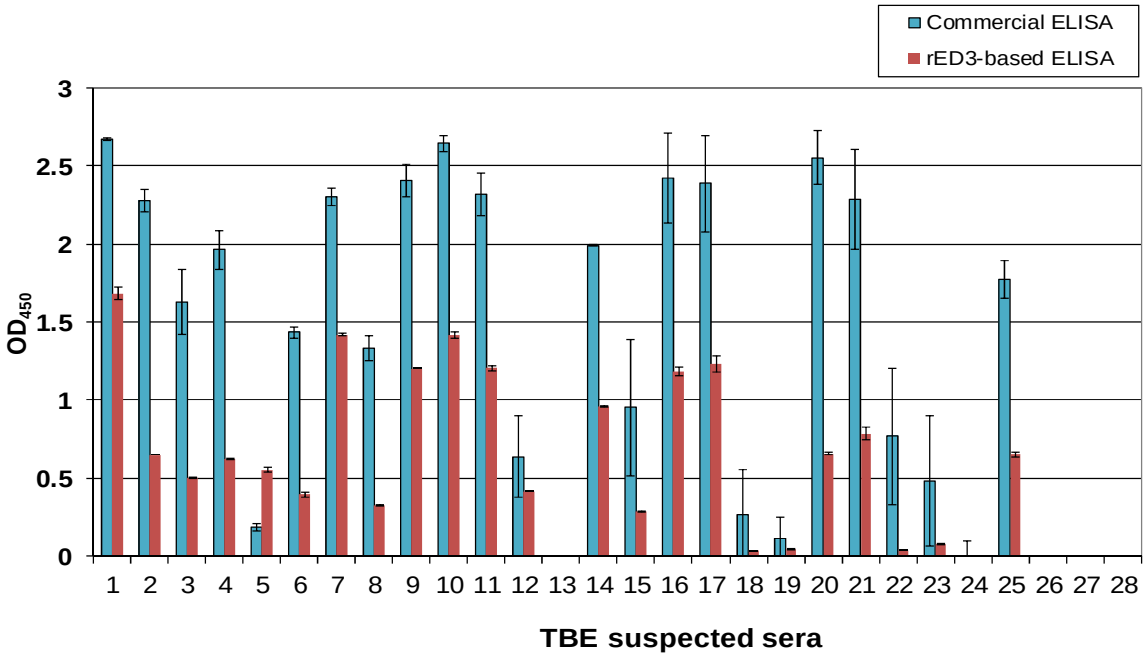


Figure 14. Comparison of rED3-based ELISA and commercial ELISA kit in TBE diagnosis (column values shown as OD_{450nm} value of sample – cut off value). Error bars present as standard deviation.

Table 18. Comparison of the results obtained in commercial IgG ELISA and ELISA using rED3 antigen

Commercial IgG ELISA	**IgG ELISA using recombinant antigen (rED3)**		
	Positive	**Negative**	**Total**
Positive	23	0	23
Negative	5	5	5
Total	28	5	28

It has been reported that antibodies against flaviviruses show cross-reactivity with other flavivirus antigens (Dobler et al., 1996, Holzmann et al., 1996; Litzba et al., 2013; Niedrig et al., 2001). Therefore, 14 serum samples from WNV and 4 DENV infected donors were tested using commercial and rED3-based ELISA. All 18 of these serum samples were negative by the rED3 ELISA, while up to 11 WNV (80%) and all 4 DENV samples showed cross-reactivity with commercial IgG ELISA (Figure 15). Cut-off values for commercial IgG and rED3-based ELISA were 0.297 and 0.146, respectively.

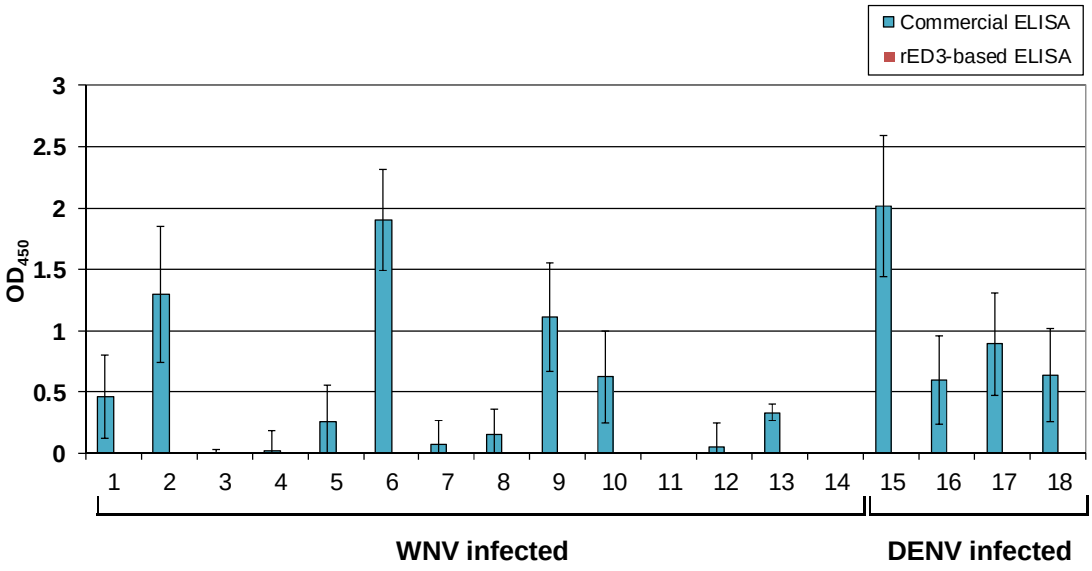


Figure 15. Analysis for cross-reactivity with other flaviviruses by rED3-based ELISA and commercial IgG ELISA using WNV positive human sera and DENV positive sera (column values shown as OD_{450nm} value of sample – cut off value). Error bars present as standard deviation.

In conclusion, the TBEV-rED3 demonstrated significant potential for the development of rED3-based ELISA diagnostic assay for detection of specific IgG to TBEV in serum specimens and for differentiation of infections caused by TBE serogroup and mosquito-borne flaviviruses.

3.2. Determination of antigen-specific memory B cells in TBE vaccinated individuals by Enzyme-linked immunospot (ELISPOT)

To better characterize human B cell responses after vaccination, an ELISPOT-based assay to quantitate Ag-specific memory B cells in human blood was applied. In ELISPOT, TBEV-rED3, inactivated whole TBEV, Influenza virus nucleoprotein (NP) and WNV-rED3 were used as antigens to evaluate specificity for identification of memory B cells against TBEV. To be able to measure antibody secreting cells (ASCs), the B-cells within the isolated PBMCs were activated by incubation with a combination of R848 (Resiquimod) and rhIL-2 for 3 days. A representative culture is shown in Figure 16. Stimulation of PBMCs with R848 and rhIL-2 for 3 days resulted in a marginal proliferation of cells visible as proliferation clusters.

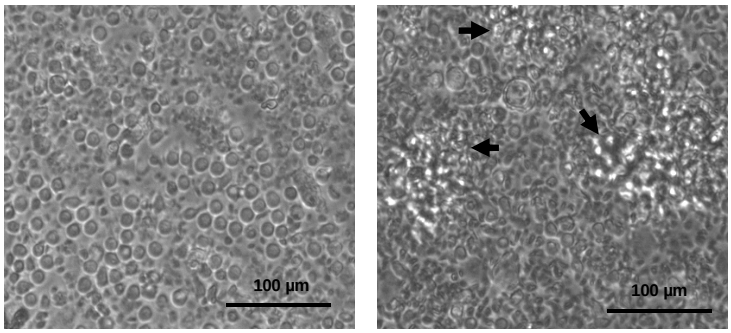


Figure 16. Bright field microscopy of non-stimulated PBMCs (A) and R848 and rhIL-2 stimulated PBMCs after 3 days of incubation (B), arrows indicated proliferating memory B cells.

Figure 17 shows the ELISPOT results of TBE vaccinated and non-vaccinated blood donor. In each ELISPOT-assay 1000 cells were plated on anti-IgG coated wells to control activation of B-cells. So in both samples spots were detected. On anti-IgG wells 10^5 non-activated cells were plated to screen for ASCs within the PBMC preparation. If spots would have been detected they were subtracted from spots on IgG. MBP coated wells functioned as control as specific antigens were MBP-fusion proteins. In this example no spots were detected on MBP in both samples. If so, spots were subtracted from antigen specific spots. For Influenza NP, which was used as another control, spots were detected in both samples but TBEV-rED3 specific memory B cells were detected in the blood of TBE vaccine recipient only. Moreover, Ag specific ASCs were only detected in TBEV-rED3 coated wells, not on inactivated whole TBEV or in WNV-rED3 coated wells (Figure 17). This demonstrated that TBEV-rED3 was superior to inactivated whole TBEV for determination of memory immunity against TBE, so inactivated virus was not used for further experiments.

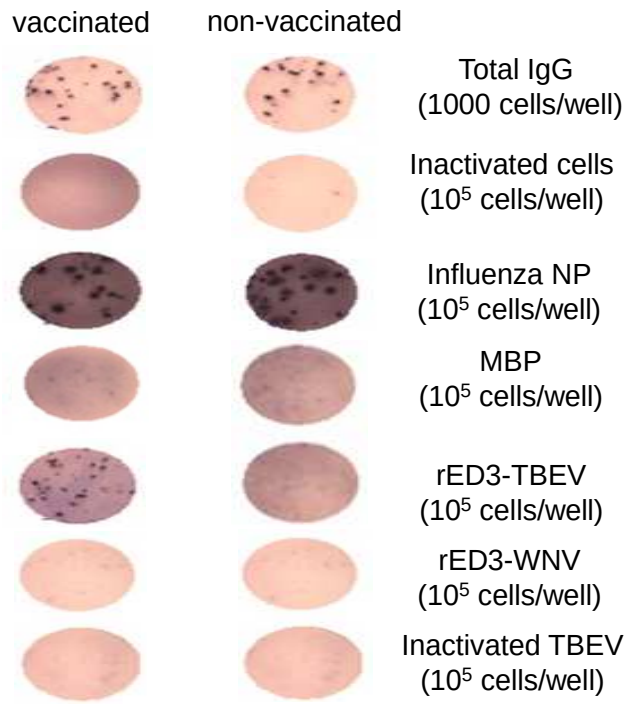


Figure 17. A representative ELISPOT of a vaccinated and a non-vaccinated donor

3.2.1. Analysis of vaccine status by ELISPOT

To analyse applicability of ELISPOT for evaluation of vaccine status 19 blood samples were investigated. From these 12 donors were vaccinated against TBEV at different time points in the past. TBEV-rED3 specific memory B cells presenting as ASCs were detected in eleven of 12 TBE vaccinated individuals ranging from 5 to 50 ASCs per 10^6 PBMCs (median=10 with a 25% percentile of 5, a 75% percentile of 20.25). In seven unvaccinated individuals no TBE-specific memory B cells were detected. Additionally, no cross-reactivity in persons infected with Dengue virus was detected by this assay (Appendix 1). There was a significant difference in TBE-rED3 specific ASCs (p=0.0012) between the TBE-vaccinated and TBE-non-vaccinated group (Figure 18)

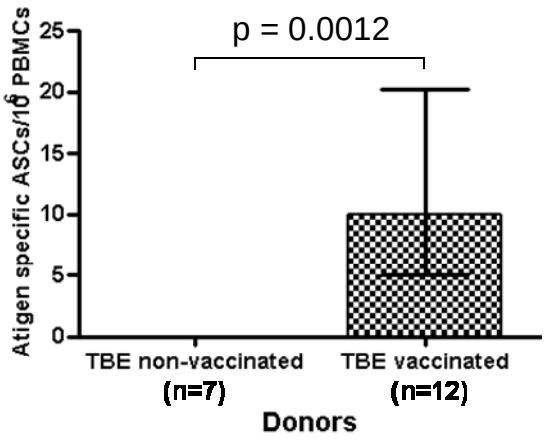


Figure 18. Specific memory B cells in group of vaccinated individuals compared to non-vaccinated group. There was significant difference between TBE vaccinated and non-vaccinated group (p=0.0012)

A frequency of antigen-specific ASCs was calculated as the numbers of antigen specific IgG ASCs per total IgG ASCs (Appendix 1). The result showed that frequency of TBVE-rED3 specific IgG ASCs ranged from 0.016% to 0.188% (median=0.050 with a 25% percentile of 0.025, a 75% percentile of 0.072) per total IgG producing cells. Thus, it is lower than frequency of NP specific IgG ASCs between 0.012% and 0.510% (median=0.104 with a 25% percentile of 0.060, a 75% percentile of 0.180) (Figure 19).

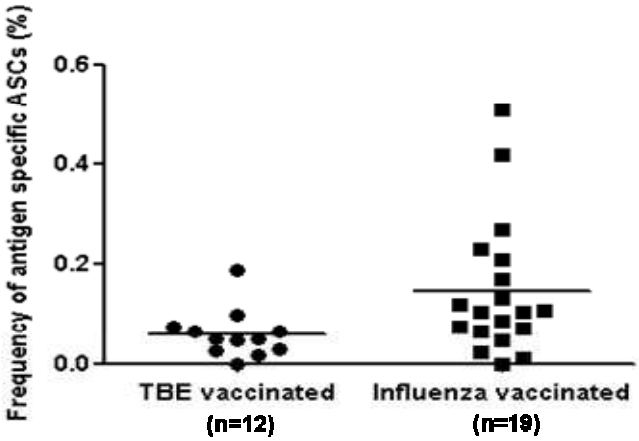


Figure 19. The frequency of antigen specific ASCs were detected in TBE and Influenza vaccinated individuals. Median values are represented by lines

The TBE vaccinated individual number 4 who got a TBE booster 4 years ago obtained the highest frequency of antigen specific memory B cells (0.188%). Other vaccinees who obtained the last booster 11 years and 23 years ago (donor #7 and #12) exhibited lower antigen specific ASC frequencies of 0.06% and 0.1%; respectively. Lower ASC frequency was also observed in vaccinee who obtained one booster 1 year ago (donor #1). Donor #8 was found TBEV-rED3 specific IgG ASCs with only single dose of primary vaccination past 11 years. Donor #9, who received two doses of primary vaccination 17 years ago, was both negative with anti-TBE antibodies in plasma and undetectable any TBEV-rED3 specific IgG ASC (Figure 20)

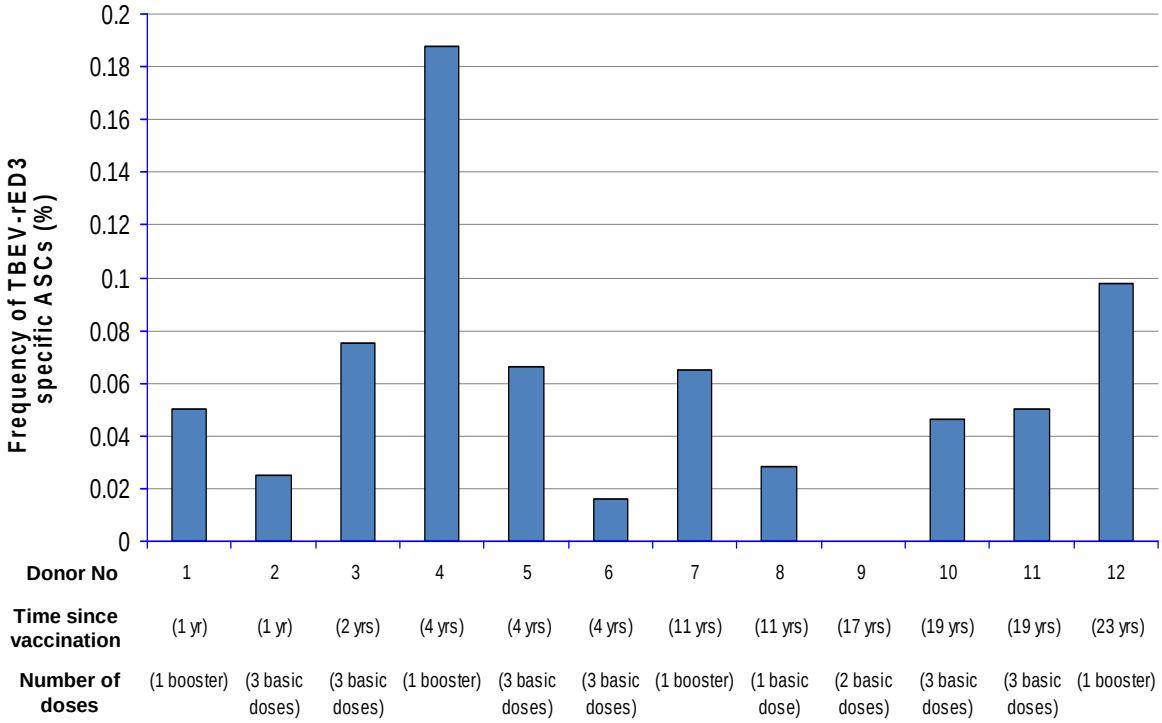


Figure 20. Frequency of antigen specific ASCs regarded time since vaccination by years and number of primary and booster doses in TBE vaccinated individuals

Antigen-specific IgG ASCs were analysed in groups with primary and booster vaccination. As shown in Figure 21 frequency of TBEV-rED3 specific ASCs was slightly higher in a booster group (median=0.081 with a 25% percentile of 0.053, a 75% percentile of 0.165) compared to primary vaccination group (median=0.037 with a 25% percentile of 0.018, a 75% percentile of 0.062). A significant difference between two groups was found ($p<0.05$). Therefore, the accumulation of antigen specific ASCs depended on the booster immunization given previously.

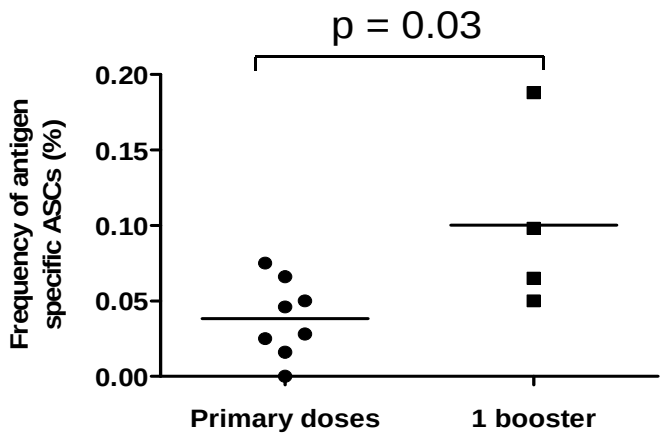


Figure 21. Frequency of antigen specific ASCs between groups with primary doses and booster. Median values are represented by lines.

Conclusion: longevity differs, depends on immune response of the individual. Nevertheless, the donors were grouped into a group of individuals vaccinated 1-5 years ago (mean of 2.5 years) and a group of individuals vaccinated more than 10 years ago (mean of 16.5 years). The frequency of TBEV-rED3 specific IgG ASCs in immunized group with 1-5 years of post-vaccination (median=0.058 with a 25% percentile of 0.022, a 75% percentile of 0.103) was only slightly increased than that in group with longer time of post vaccination (median=0.048 with a 25% percentile of 0.021, a 75% percentile of 0.073). However, statistical analysis showed no significant difference (p >0.05) in the frequency between the two compared groups (Figure 22).

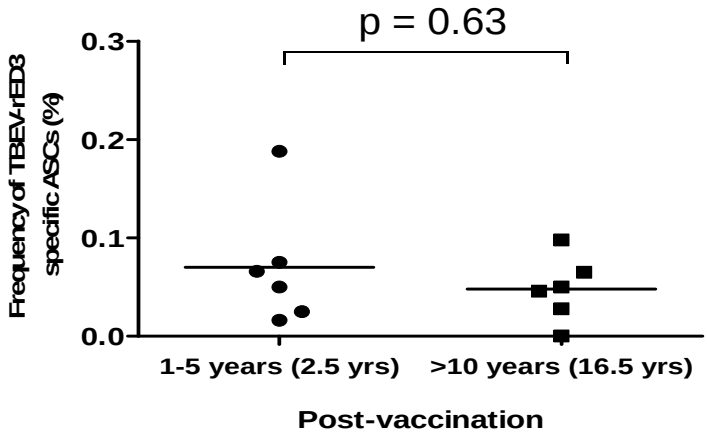


Figure 22. Comparison of longevity of TBE vaccine-specific B cell memory in individuals vaccinated 1-5 years (mean 2.5 yrs) and >10 years (mean 16.5 yrs). Individuals were tested for TBE-specific memory B cells using rED3 as the ELISPOT coating Ag and mean of frequency of TBEV-rED3 specific memory B cell levels were compared. Median values are represented by lines.

3.2.2. Determination of plasma antibody and neutralizing antibody titres

Additionally, plasma of all donors was analysed by commercial and rED3-based ELISAs and neutralisation test. TBEV-specific antibodies were detected in 11 of 12 TBE vaccinated individuals by rED3-based ELISA (Table 19). One TBE vaccinee was negative by rED3-based ELISA, but tested positive by commercial kit. The antibody negative donor was also found negative in the ELISPOT (Appendix 2). Interestingly, by neutralisation test in all 12 serum samples protective neutralizing antibodies were detected with a Geometric mean titre (GMT) of 96.04, 95% confident interval (CI): 52.76-174.8. Of the seven TBE unimmunized subjects, four of them had a DENV infection in the past and thus containing anti-DENV antibodies. These were also tested as control for cross-reactivity between flaviviruses. All seven TBE non-vaccinated individuals were negative with both rED3-based ELISA and NT while the four DENV sera were positive using commercial ELISA.

Thus, compared to neutralisation test rED3-based ELISA was shown to have a relatively high sensitivity (11/12, 91.6%) and specificity (7/7, 100%). The commercial ELISA kit showed a high cross-reactivity with all subjects containing anti-DENV antibodies and exhibit a low specificity with 42.8% (3/7) compared to the neutralisation test but has a sensitivity of 100% (12/12) (Table 19). These results support the initial results (3.1.) which indicated rED3 as a suitable antigen for detection of TBEV specific antibodies.

Table 19. Comparison between rED3-based ELISA, commercial IgG ELISA kit (*recom*Well FSME/TBE virus IgG), and neutralisation test

Neutralisation test	**rED3-based ELISA**		**Commercial ELISA kit**		
	Positive	**Negative**	**Positive**	**Negative**	**Total**
Positive	11	1	12	0	12
Negative	0	7	4	3	7
Total	11	8	16	3	19
Sensitivity	**91.6%**		**100%**		
Specificity	**100%**		**42.8%**		

To find out if there is a correlation between number of specific memory B cells and specific plasma antibodies of TBE and Influenza vaccinated individuals a statistic analysis was carried out. It showed that there was no significant correlation between serum levels of anti-TBEV antibodies and the number of rED3 specific IgG ASCs ($p>0.05$ $r = 0.36$). A similar result was also indicated for influenza vaccinated individuals ($p>0.05$, $r = 0.27$) (Figure 23)

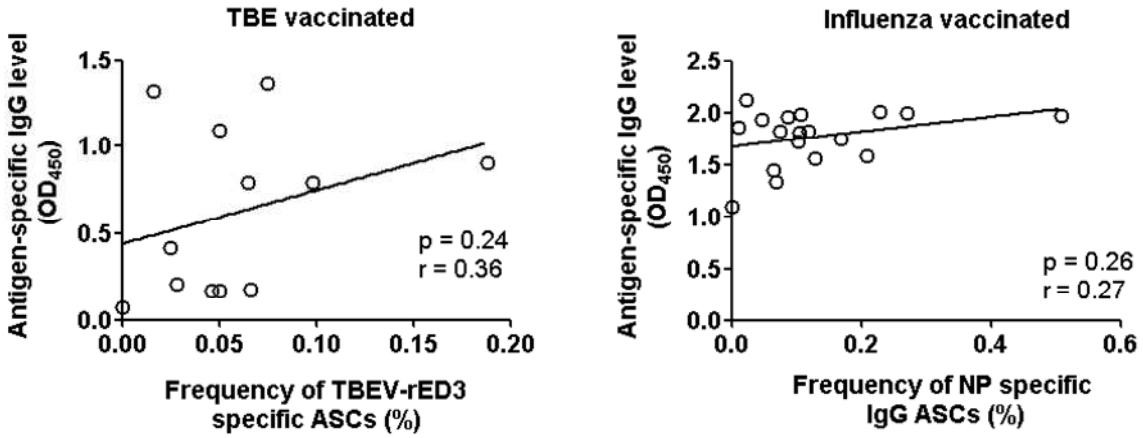


Figure 23. Linear regression and correlation between the frequency of antigen-specific IgG ASCs (x-axis) and antigen-specific IgG antibody level in serum (y-axis)

Neutralizing antibody titres were found in all 12 plasma samples obtained from vaccinated individuals. In order to appraise the significance of ELISA results for evaluating the immune response to TBE vaccination, the correlation was also examined between the levels of antibodies as measured by rED3-based ELISA and levels of neutralizing antibodies determined by Neutralisation test. Taken together, we observed a good significant correlation between IgG antibody detection by TBEV-rED3 based ELISA and neutralizing antibody titres obtained by neutralisation test ($p< 0.05$ and Spearman's coefficient $r = 0.77$) (Figure 24). Therefore, the level of IgG-antibodies determined by rED3-based ELISA is a good marker for predicting the presence of neutralizing antibodies against TBEV.

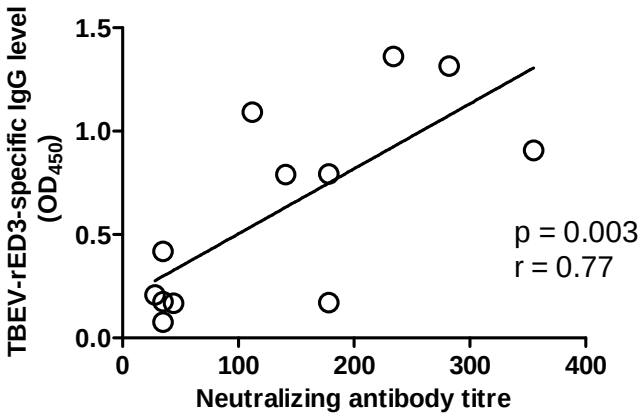


Figure 24. The correlation between neutralizing antibody titres and level of IgG antibodies as OD_{450} values. $p< 0.05$ and Spearman's coefficient $r = 0.77$.

As expected the obtained OD values after almost 17 years post-vaccination (median=0.189 with a 25% percentile of 0.145, a 75% percentile of 0.789) were significantly lower than after 2.5 years post-vaccination (median=0.999 with a 25% percentile of 0.357, a 75% percentile of 1.327) (Figure 25). But no correlation was also found between neutralizing antibody titres and the interval of time since vaccination. No statistically significant influence was also observed (Figure 26).

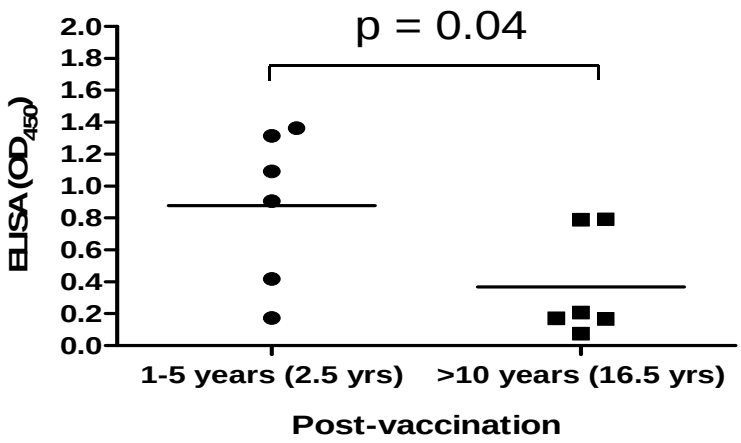


Figure 25. Level of antigen-specific antibodies in TBE vaccinated groups regarded time points of post-vaccination. Median values are represented by lines.

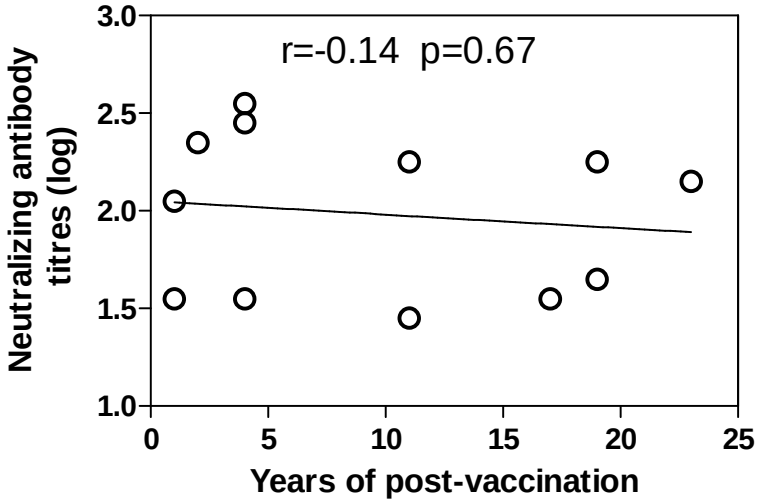


Figure 26. Correlation of TBE neutralizing antibody titres and years between vaccination and sample collection. The Spearman's coefficient r and the p value are indicated.

CHAPTER 4. DISCUSSION

4.1. Expression and purification of recombinant envelope protein domain 3 (rED3) and evaluation of its use for serological TBE diagnosis.

Currently, antibodies to TBE virus are detected and quantified by ELISA employing inactivated whole virus as coating antigen. But due to extensive antigenic cross-reactivity serological diagnosis of flavivirus infections is difficult, especially in areas where more than one virus type circulates. A situation now exists in Europe, where TBEV and WNV are endemic in many countries (Beck et al., 2013). But also the increased mobility of man and increased contact of travellers with flaviviruses complicate a specific serological diagnosis. Several reports demonstrated that cross-reacting antibodies were present in cases of current infection or past vaccination against, other flaviviruses, e.g. West Nile virus, Dengue fever virus, Japanese B encephalitis virus, Yellow Fever virus that are detected by ELISA using whole virus as antigen (Dobler et al., 1996; Holzmann et al., 1996; Litzba et al., 2013; Niedrig et al., 2001). As an alternative antigen, recombinant E protein domain 3 (rED3) is proposed as it contains virus-specific epitopes for specific serological diagnostic of flavivirus infection (Beasley et al., 2004; Holbrook et al., 2004; Marx et al., 2001). Domain 3 represents the receptor-binding domain of the E protein, which is the major flavivirus structural protein and antigen (Gritsun et al., 2003; Heinz, 1986; Marsh and Helenius, 1989; McMinn, 1997). Antibodies directed against D3 are described as highly neutralizing, whereas antibodies to D2 or D1 are cross-reactive (Crill and Chang, 2004; Sukupolvi-Petty et al., 2007).

In this study TBEV- and WNV-specific antigen ED3 was expressed as MBP fusion proteins with C-terminal histidine tag using the bacterial expression system. Using first amylose affinity chromatography followed by nickel affinity chromatography highly purified rED3 was obtained. Using TBEV-rED3 protein in Western Blot unspecific reaction with serum antibodies of negative serum was detected and a differentiation between WNV infection and TBEV wasn't possible. Probably, flavivirus cross-reactive antibodies bind to linear, conserved epitopes within ED3 that became accessible due to protein denaturation. Otherwise these epitopes are hidden due to the 3D-structure of the protein. This is in contrast to the literature. Holbrook and colleagues found that the rabbit anti-TBEV sera did not recognize rED3 derived from mosquito-borne flaviviruses like WN or YF nor did rabbit anti-YF or anti-WN antisera bound to the TBE-rED3 (Holbrook et al., 2004). The findings might be explained by the used antisera. The rabbit sera were produced by immunization of rabbit with purified rED3 fusion protein, while in this study sera of infected or vaccinated humans was used. Additionally, here the rED3 was used as fusion protein with N-terminal MBP moiety. MBP is

an important bacterial protein from *E. coli* for the maltose/maltodextrin metabolism. *E. coli* is a commensal inhabitant of the human intestinal tract. Consequently, anti-MBP reactive antibodies were detected in all positive and negative sera.

When the purified rED3 protein was used as antigen in ELISA TBE virus-specific antibodies were detected, only. This demonstrated that rED3 retained sufficient antigenicity after purification, which was partly carried out under denaturing conditions. In comparison to the commercial ELISA-kit, that uses whole virus as antigen the rED3-based ELISA has higher specificity. Cross reactivity of WNV and DENV positive sera was detected in 15 of 18 sera by commercial IgG ELISA and thus giving a false positive signal. In TBEV-rED3-based ELISA, these samples were found negative. Thus, that the TBEV-rED3-based ELISA allows a differentiation of infections caused by TBE serogroup and mosquito-borne flaviviruses but not the inactivated whole virus based commercial ELISA. The antigen used might explain this. In the whole virus preparation the complete E-protein is present and thus none neutralizing but highly cross reactive antibodies i.e. D2 specific antibodies can bind and give a positive signal. Using correctly folded ED3 allows only the binding of specific antibodies as it was described in literature that ED3 induces specific neutralizing antibodies (Crill and Chang, 2004; Sukupolvi-Petty et al., 2007). These results were in agreement with previous studies on the use of rED3 as antigen in ELISA. WNV-rED3 for example was found superior to whole virus antigens as it allowed discrimination between WNV and other flavivirus infection, such as St. Louis encephalitis virus (SLEV), Japanese encephalitis virus (JEV) and Murray Valley encephalitis virus (MVEV) (Beasley et al., 2004). Another study described the development of a diagnostic ELISA for JEV infections using JEV-D3 peptide antigen, which showed 98% sensitivity and 96% specificity when compared to a commercial ELISA (Shukla et al., 2009). Recombinant DENV-D3 was also a reliable antigen for ELISA in order to identify the DENV serotypes in primary infection (Wahala et al., 2009). TBEV-rED3 did not allow differentiation of very similar TBE viruses but it was highly specific for the tick-borne flaviviruses and allowed differentiation of TBE serocomplex flaviviruses from mosquito-borne flaviviruses in ELISA (Holbrook et al., 2004).

Noticeably are the obtained lower OD450 values by ELISA on TBEV-rED3. Similar results were found for DENV, where only low amounts of serum antibodies were directed against rED3 (Wahala et al., 2009). This might be explained by the fact that in humans only the minority of produced antibodies are directed against ED3 as shown for WNV-antibodies (Throsby et al., 2006).

In our study, the specificity of rED3 based ELISA obtained 100%, sensitivity was only 91.6% when compared to neutralisation test as the most specific test. One vaccinee was negative by rED3-based ELISA, but positive by commercial IgG ELISA and neutralisation

test. This finding is consistent with previous data on human immune responses, which showed that after vaccination or infection not all individuals generate a significant IgG antibody response against the ED3 epitope (Oliphant et al., 2007). A large fraction of the E-specific antibodies was directed against ED2 epitope, but ED2 specific neutralizing antibodies were less potent as mAbs that bound to ED2 protected only 18–60% of mice when given two days after infection with WNV compared to ED3 specific mAbs, which protected 80–100% of mice (Oliphant et al., 2006). Nevertheless, ED3-responses formed only a very small proportion of the total antibody response (Throsby et al., 2006; Vratskikh et al., 2013; Wahala et al., 2009).

This study also showed that all TBE vaccinated individuals presented the protective neutralizing antibodies as quantified by neutralisation test. Neutralizing antibodies have been maintained for a long time since last vaccination. This is in line with previous observations that neutralizing antibodies are likely to remain at a sustainable “plateau” titre following three doses of primary immunization without any apparent dependency on elapsed time since last immunization (Rendi-Wagner et al., 2007; Schosser et al., 2009). Additionally, the quantitative determination of specific IgG in TBE post-vaccination sera by rED3-based ELISA exhibits a good correlation with neutralizing antibodies titres. This may be due to the fact that the rED3-based ELISA antigen is employed which represents the primary target for neutralizing antibodies. The presence of specific antibodies in this ELISA is therefore highly predictive for the presence of neutralizing antibodies, and this correlation can probably be used in the future to establish guidelines for recommendations of booster vaccinations (Holzmann et al., 1996; Rendi-Wagner et al., 2004).

Altogether, the results demonstrate clearly that production of a highly antigenic protein using the bacterial expression system is a promising alternative to the conventional production of viral proteins by inactivation of whole virus. The bacterial expression system assures correct protein processing and folding, two important criteria for the preservation of conformational antigenic epitopes. Such recombinant flavivirus specific proteins represent new diagnostic markers and potential candidates for the development of new vaccines.

4.2. Establishment of a memory B cells assay using rED3 as antigen for determination of frequency of rED3 specific antibody-secreting cells (ASCs) in individuals vaccinated against TBE.

Nowadays, common vaccination recommendations often take into account only the antibody titre as a criterion for vaccinations and as an indicator of immunological memory. Long-lived plasma cells (LLPCs) in bone marrow secrete antibodies over a long time to maintain titre but nonetheless levels decline gradually (Ahuja et al., 2008; Manz et al., 1997). But vaccine induced immunological memory is not only based on serum antibody titers. It is also based on memory B cells, which are present for years but don't produce antibodies before re-encounter their specific antigen (Amanna and Slifka, 2010). Thus, long-term effect of vaccination is showing on the frequency of antigen-specific B cells and the level of the antibody titre in the serum (Zinkernagel et al., 1996). It would be desirable for modern vaccine strategies to be based, increasingly, to the memory B cells as a parameter for long-term immunity.

Various methods have previously been described for inducing the differentiation of human memory B cells. These methods involve the culture of peripheral blood mononuclear cells (PBMCs) or purified B cells for 5 to 6 days in the presence of polyclonal stimuli and cytokines, such as ConA with IL-2, IL-4, IL-6 (Slifka and Ahmed, 1996); pokeweed mitogen (PWM) with *Staphylococcus aureus* Cowan I (SAC) (Munoz and Insel, 1987); SAC and interleukin (IL)-2 (Kelly et al., 2006); PWM, unmethylated CpG oligodeoxynucleotides (CpG ODN) and SAC (Crotty et al., 2004); CD40L transfected CDw32L mouse fibroblasts plus IL-2 and IL-10 (Tuaillon et al., 2006); CpG and IL-15 (Bernasconi et al., 2002); PWM, *Staphylococcus aureus* lysate, IL-2, IL-10 and phosphorothioated CpG ODN (Bussmann et al., 2010); TLR7/TLR8 agonist R848 plus IL-2 (Jahnmatz et al., 2013; Pinna et al., 2009; Walsh et al., 2013).

Here, an ELISPOT protocol previously optimized by Jahnmatz et al. for the analysis of vaccine-induced B-cell responses (Jahnmatz et al., 2103) was used in order to determine the numbers of ASCs produced by individuals who got vaccinated against TBE. Memory B cells activated with R848 (Resiquimod) and rhIL-2 indicated to be significantly better than PWM used in combination with various co-activators (Jahnmatz et al., 2013; Pinna et al., 2009; Walsh et al., 2013). In this study, stimulation with R848 and IL-2, TBEV-rED3 specific ASCs could be detected in 11 of 12 TBE vaccinated individuals; even anti-TBE antibodies levels were low in serum. Our finding was in agreement with previous observations indicated that HBs-ASCs were also detected in HBV immunized individuals with serum anti-HBs antibodies under protective level. But HBs-specific memory B cells could rapidly differentiate into plasma cells after re-encounter with HBV antigen and rule out infection of hepatitis B by

secreted antibodies (Margolis, 1993; Tuaillon et al., 2006; West and Calandra, 1996). Only one individual who received two instead of three primary doses of TBE vaccine no TBEV-rED3 specific memory B cells were detected; though, had protective neutralizing antibodies against TBE. This is explained that antigen specific memory B cells in this person may be at low frequency and below the limit of detection of assay. Previously, Buisman and colleagues also demonstrated that ELISPOT using PBMCs were able to identify specific memory B cells, but only when a minimal frequency of these cells was present in blood (Buisman et al., 2009).

In this study, frequency of antigen specific memory B cells was between 0.016% and 0.188% for TBE, 0.012% and 0.51% for Influenza per total IgG ASCs. The results were in accordance with previous reports on circulating memory B cell specific to tetanus toxoid (TT) (0.02-0.87%) (Bernasconi et al., 2002; Buisman et al., 2009), smallpox (0.1%) (Crotty et al., 2003), anthrax (0.05-2%) (Crotty et al., 2004), or HBV (0.02-0.58%) (Tuaillon et al., 2006). We have shown that TBE-specific memory B cells could be maintained for more than 20 years of post-vaccination. In this study, there was a significant difference in number of ASCs between vaccinated and non-vaccinated group ($p<0.05$). Interestingly, there was no evidence showing a significant difference between TBE vaccinated groups within 1-5 years (mean of 2.5 years) and >10 years (mean of 16.5 years) of post-vaccination ($p>0.05$). Our results were in agreement with other studies demonstrating that the immunological memory as antigen-specific memory B cells in humans may be maintained for many years (Buisman et al., 2009; Migot et al, 1995; West and Calandra, 1996), even for more than 50 years (Crotty et al., 2003), while antibody titres often decrease after the last exposure to the antigen (Ahmed and Gray, 1996; Simonsen et al., 1984). These findings also proved that memory B cell numbers were stable for years (Crotty et al., 2003; Schittek and Rajewsky, 1990).

No significant correlation was found between the number of circulating antigen-specific memory B-cells and serum level of antigen-specific IgG for both TBE and Influenza. This observation is in agreement with previously published data (Bernasconi et al., 2002; Bocher et al., 1996; Crotty and Ahmed, 2004; Lanzavecchia, 1983; Leyendeckers et al., 1999; Nanan et al., 2001; Rojas et al., 2008; Slifka et al., 1998; Tuaillon et al., 2006). It revealed that memory B cells and plasma cells participate as independently controlled forms of immunological memory (Leyendeckers et al., 1999; Rosado et al., 2011) and LLPCs in bone marrow are an important source in maintaining serological memory (levels of specific serum Ig) (Rojas et al., 2008). Memory B cells on the other hand seem to play a major role in antigen processing and presentation, and act as precursors for the rapid antibody-secreting response after antigen re-exposures as well as promptly differentiating in plasma cells (Bachmann and Koft, 1999; Elkins et at., 1999; Slifka et al., 1998). However, previous reports

indicated a significant correlation between serum antibody titres and smallpox-specific memory B cells as well as the frequency of measles or tetanus toxin-specific memory B cells and the serum antibody titre, and demonstrated that many factors involved in the relationship between memory B cell levels and circulating antibody levels (Crotty et al., 2003; Lanzavecchia et al., 2006).

The mechanisms for the longevity of memory B cells are controversial (Ahmed and Gray, 1996; Mamani-Matsuda et al., 2008; Maruyama et al., 2000; Yoshida et al., 2010). Many authors have demonstrated that memory B cells and LLPCs are exclusively antigen-dependent (Benson et al., 2009; Maruyama et al., 2000). However, it has been proposed that after the generation of memory B cells, they are maintained by periodic re-encounter with antigen presented long-term by follicular dendritic cells (FDCs) (Amanna and Slifka, 2010; Fishman and Perelson, 1995; Gray et al, 1996; MacLennan, 1994). The follicular dendritic cells of germinal centres play an important role in antigen presentation. However, retention of antigen by FDCs only persists for weeks to months (Mandel et al., 1980). For re-encounter of memory B cells with antigen other forms of antigen could be persistent at low levels or memory B cells are stimulated by cross reactive environment or self-antigen (Amanna et al., 2006; Gray et al., 1996; Zinkernagel et al., 1996).

Schittek and Rajewsky (1990) showed in a mouse model that memory B cells could survive in a non-proliferative state for several weeks or they only do decline at rate that may be too slow to be measured by current methods (reviewed in Amanna et al., 2006). Another hypothesis assumes that the maintenance of human memory B cells is a bystander polyclonal activation. As memory B cells were stimulated in vitro with Toll-like receptors (TLRs) or polyclonal activators they will undergo several rounds of proliferation and differentiation into plasma cells, but not naïve B cell (Bernasconi et al., 2003; Crompton et al., 2009; Kasturi et al., 2011). More recently, Komegae et al. indicated that TLRs and myeloid differentiation primary response gene-88 (MyD88) signaling play an important role in longevity of memory B cells and LLPCs (Komegae et al., 2013).

B cells within the different memory populations may have different intrinsic requirements for their survival related to their exposure to antigen (acute, chronic or recurrent infection). Therefore, fundamental differences in memory B cell populations depend on the pathogen that is partly responsible for some vaccines requiring boosters, and others generating lifelong immunity (Tarlinton and Good-Jacobson, 2013).

At the molecular level, the longevity of the memory B cells was regulated by expression of the anti-apoptotic gene Bcl-2. Transgenic mice overproducing Bcl-2 lead to an extended lifetime for memory B cells (Nunez et al., 1991; Smith et al., 1994). Dogan et al.

showed the survival and function of memory B cells on the existence of multiple layers of B cell memory, antigen-dependent and independent and effector functions (Dogan et al., 2009)

Human vaccines induce serum antibody responses that maintain for many years, even for decades as vaccines against smallpox and Yellow fever, and therefore vaccine-specific plasma cells must be present for decades (Amanna et al., 2006). Previously, it was assumed that half-life of short-lived plasma cells were a few days to a few weeks (Shapiro-Shelef and Calame, 2005). Thus, antibody production is maintained by LLPCs with their persistence for years in bone marrow and spleen after vaccination (Good-Jacobson and Tarlinton, 2012; Manz et al., 1997; Slifka et al., 1998). Another possibility is that serum antibodies may be maintained by memory B cells as a positive correlation between serum antibody levels and antigen-specific memory B cells was observed (Crotty et al., 2003; Lanzavecchia et al., 2006). However, many other studies found no correlation between serum antibody titres and antigen-specific memory B cell frequency (Ahuja et al., 2007; Bocher et al., 1996; Crotty and Ahmed, 2004; Lanzavecchia, 1983; Leyendeckers et al., 1999; Nanan et al., 2001; Rojas et al., 2008; Slifka et al., 1998; Tuaillon et al., 2006). Our results are in agreement with these findings that antibody production is independent with the presence of memory B cells.

Antigen-specific memory B cells play an important role for the long-term immunity. This study showed that the number the previous booster vaccinations correlated strongly with the frequency of circulating memory B cells. This suggests that the circulating memory B cells better reflect immunological long-term memory induced by booster immunizations than the amount of the antibody titre. Thus, memory B cells seem to be a far more important parameter for the assessment of long-term immunity.

Our data show that individuals who only got the unique booster increase the specific antibody titre and antigen-specific memory B cell frequency. Looking at the relationship between the time since immunization with last dose and frequency of antigen-specific memory B cells, it is clear that even 10-25 years after vaccination the specific memory B cells are still maintained for protection from disease infection.

In summary, we have found the ELISPOT is a suitable method for detecting TBE antigen-specific memory B cells in individuals who have been previously vaccinated. This study provides data on immunological memory following TBE vaccination; it is useful for the recommendations for a booster dose should be reconsidered.

SUMMARY

Tick-borne encephalitis virus (TBEV) is a serious viral infection that affects the central nervous system. It was estimated that between 1990 and 2009 a total of 169,937 cases of TBE were recorded in Europe. TBEV belongs to genus Flavivirus that comprises over 70 viruses, many of them are important human pathogens. Most members are either transmitted by mosquitoes e.g. West Nile virus (WNV), Dengue virus (DENV) or ticks e.g. TBEV. Due to the extensive antigenic cross-reactivity among flaviviruses serological diagnosis of TBE infections is commonly difficult in areas where more than one virus type circulates. Particularly, a situation now exists in Europe, where TBEV and WNV are endemic in many countries Thus, this thesis focused on the one hand on optimization of serological test systems using recombinant envelope protein domain III (rED3). It represents domain 3 of the major antigen, the surface protein E, which additionally has been shown to induce flavivirus specific neutralizing antibodies. Therefore rED3 was expressed and purified and its application as antigen in ELISA for TBEV diagnosis was verified.

On the other hand a memory B-cell assay was established to analyse antiviral immunity after TBEV-vaccination. Here rED3 was used as antigen to determine the frequency of rED3 specific antibody-secreting cells (ASCs). Vaccination is the most effective method of preventing TBE disease and is currently recommended for all those who live and work or travel to areas of TBE endemicity. An essential requirement of any vaccine is the induction of long-term protective immunity. Several vaccines have defined levels of serum antibody (as measured by ELISA, haemagglutination inhibition test, or neutralisation test) that serve as correlates or surrogates of protective immunity. But this does not take account of vaccine-induced memory B cells. Although, not providing direct protection against infection, they represent an important second line of immune defence that is initiated only if pre-existing antibody levels are too low to prevent infection or if the invading pathogen is able to circumvent the pre-existing antibody response. A thorough understanding of the frequencies of antigen-specific memory B cells and their relationship with the antibodies in serum serological memory is likely to be critical to give information about the long-term efficacy of vaccine as well as its correlates of protection.

This thesis focuses on the establishment of a recombinant protein based ELISA and of a memory B cell assay for analysis of specific antiviral immunity after vaccination with the following two objectives: (1) Expression and purification of recombinant envelope protein domain 3 (rED3) and verification of its application as antigen in ELISA for TBEV diagnosis;

(2) Establishment of a memory B cells assay using rED3 for determination of frequency of rED3 specific antibody-secreting cells (ASCs) in individuals vaccinated against TBE.

In this study, TBEV- and WNV-specific antigen ED3 was expressed in *E. coli* as MBP fusion proteins with C-terminal histidine tag using pMAL-c2x vector. By purification with amylose affinity chromatography followed by nickel affinity chromatography, highly purified TBEV-rED3 and WNV-rED3 were obtained. Nevertheless, using TBEV-rED3 protein in Western Blot unspecific reaction with serum antibodies of negative serum was detected and a differentiation between WNV infection and TBEV was impossible, probably due to the MBP moiety.

However, using the purified rED3 protein as antigen in ELISA, TBE virus-specific antibodies were detected specifically. Twenty-three serum samples predefined as TBEV positive were tested positive by rED3-based ELISA and commercial IgG ELISA. Five predefined negative serum samples were tested negative by rED3-based IgG ELISA as well as commercial IgG-ELISA. But cross reactivity of WNV and DENV positive sera was detected in 15 of 18 sera by commercial ELISA. On the other hand, these samples were found negative in TBEV-rED3-based ELISA. Thus, TBEV-rED3-based ELISA allows a differentiation of infections caused by TBE serogroup and mosquito-borne flaviviruses but not the inactivated virus based commercial ELISA. Interestingly, compared to neutralisation test the specificity of rED3-based ELISA obtained 100% with a sensitivity of 91.6%. In contrast, the commercial ELISA obtained 100 % sensitivity but a low specificity with only 42.8%.

In order to determine frequency of antigen-specific antibody secreting cells (ASCs) produced by individuals who received the vaccination against TBE, peripheral blood mononuclear cells (PBMCs) were isolated from blood samples. Subsequently, memory B cells were activated with R848 (Resiquimod) and human recombinant IL-2 (hrIL-2) for 72 hours in 37°C, 5% CO_2, 90% humidity. After 72 hours of incubation, Enzyme-Linked Immunospot (ELISPOT) detected antigen-specific memory B cells. In order to evaluate specificity of TBEV-rED3 in ELISPOT, other antigens including WNV-rED3, Maltose binding protein (MBP) and Influenza Nucleoprotein (NP) were included. Study subjects could be separated into two groups: last vaccination before 5 years or longer than 10 years. TBEV-rED3 specific ASCs could be detected in 11 of 12 TBE vaccinated individuals with different vaccination history and even low serum anti-TBE antibodies levels. TBEV-rED3 specific ASCs were found with frequency of ranging 0.016-0.188 % per total IgG ASCs and lower than frequency of Influenza-NP specific ASCs (between 0.012-0.51%). But TBE-specific memory B cells could be maintained for more than 20 years of post-vaccination. There was a significant difference in number of ASCs between vaccinated and non-vaccinated group ($p<0.05$). Interestingly, there was no significant difference between TBE vaccinated groups between 1-5 years (mean of 2.5

years) and >10 years (mean of 16.5 years) since vaccination ($p>0.05$). These finding proved that memory B cells have been stable for years and are maintained up to 25 years since last vaccination.

A statistic analysis showed that there was no significant correlation between serum levels of anti-TBEV antibodies and the frequency of rED3 specific IgG ASCs ($p>0.05$, Spearman's coefficient $r = 0.36$). A similar result was also indicated for influenza-vaccinated individuals ($p>0.05$, Spearman's coefficient $r = 0.27$). These findings revealed that memory B cells and plasma cells maybe play an independently role in maintaining of immunological memory. Anyway, neutralizing antibodies have been found in all vaccines (a Geometric mean titre (GMT) of 96.04, 95% confident interval (CI): 52.76-174.8) and thus were maintained for a long time since last vaccination. Interestingly, the quantitative determination of specific IgG in TBE post-vaccination sera by rED3-based ELISA exhibits a good correlation with neutralizing antibody titres. The presence of specific antibodies in rED3-based ELISA is therefore highly predictive for the presence of neutralizing antibodies, and this correlation can probably be used in the future to establish guidelines for recommendations of booster vaccinations. Additionally, it became apparent that the number of previous booster vaccinations correlated strongly with the frequency of circulating memory B cells. As expected, individuals who received a booster increased both the specific antibody titre and frequency of antigen-specific memory B cells. This suggests that immunological long-term memory induced by booster immunizations is better reflected by the circulating memory B cells than the amount of the antibody titre. Thus, memory B cells seem to be a more reliable parameter for the assessment of long-term immunity.

Taken together, a highly antigenic rED3 using the bacterial expression system was produced and it is a promising alternative to whole inactivated virus in ELISA. Notably, rED3 was a reliable antigen for detecting antigen-specific memory B cells in individuals who have been previously TBEV vaccinated. This study provides data on immunological memory for TBE vaccination and might be useful for reconsideration of recommendations for booster dose.

In conclusion, boosters of vaccination should be recommended for all individuals who live and work or travel to areas of TBE endemicity. As consequently, vaccine-induced protection is enhanced by both strong humoral and cell-mediated immune responses. If pace of pathogenesis is rapidly growing, pre-existing virus-specific antibodies represent the first line of defence against infection before the memory response is fully activated and implemented. They clearly function best together to efficiently protect against disease.

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Appendix 1: Number of ASCs and frequencies regarding tested antigens of TBE vaccinated and non-vaccinated individuals

	Donor No	Total IG ASCs/10^6 PBMCs	TBEV-rED3 specific ASCs/10^6 PBMCs	% TBEV-rED3 specific ASCs/total IgG	Influenza-NP specific ASCs/10^6 PBMCs	Influenza-NP specific ASCs/total IgG	WNV-rED3 specific ASCs/10^6 PBMCs	Inactivated TBEV specific ASCs/10^6 PBMCs
TBE vaccinated	**< 5 years**							
	1	99 000	50	0.05	70	0.07	0	0
	2	20 000	5	0.025	42	0.21	0	0
	3	29 250	22	0.075	37	0.12	0	0
	4	13 250	25	0.188	10	0.075	0	0
	5	16 500	11	0.066	2	0.012	0	0
	6	31 000	5	0.016	32	0.103	0	0
	>10 years							
	7	15 250	10	0.065	64	0.42	0	0
	8	35 000	10	0.028	60	0.17	0	0
	9	71 000	0	0	34	0.048	0	0
	10	10 750	5	0.046	7	0.065	0	0
	11	19 000	10	0.05	0	0	0	0
	12	15 250	15	0.098	78	0.51	0	0
TBE non-vaccinated	**Without infection of any flavivirus**							
	13	28 500	0	0	66	0.230	0	0
	14	23 000	0	0	20	0.087	0	0
	15	10 500	0	0	11	0.105	0	0
	DENV infected							
	16	18 500	0	0	20	0.108	0	0
	17	21 500	0	0	5	0.023	0	0
	18	22 000	0	0	28	0.13	0	0
	19	16 750	0	0	46	0.27	0	0

Appendix 2: Determination of anti-TBE antibodies in plasma

Group	Donor No	% TBEV-rED3 specific ASCs/total IgG	rED3-based ELISA OD_{450nm} value[a]	Commercial ELISA index value [b]	Neutralisation Test [c]
TBE Vaccinated	**< 5 years**				
	1	0.05	1.092	161.094	112
	2	0.025	0.418	91.073	35
	3	0.075	1.362	187.882	234
	4	0.188	0.906	180.976	355
	5	0.066	0.175	34.618	35
	6	0.016	1.314	190.172	282
	> 10 years				
	7	0.065	0.792	146.290	178
	8	0.028	0.208	59.275	28
	9	0	0.076	25.084	35
	10	0.046	0.171	78.711	177
	11	0.05	0.168	44.555	44
	12	0.098	0.789	198.863	141
TBE non-vaccinated	**Never infected by any flavivirus**				
	13	0	0.062	2.1810	<10
	14	0	0.032	0.934	<10
	15	0	0.037	0.901	<10
	DENV infected				
	16	0	0.016	132.412	<10
	17	0	0.027	47.056	<10
	18	0	0.010	48.741	<10
	19	0	0.017	64.033	<10

a: samples are determined positive if OD_{450} value of sample > 0.146 (cutoff)

b: Samples are positive if ELISA index value >24 U/ml (cutoff)

c: Neutralizing antibodies > 10 were considered protective antibodies

DECLARATION AUTHORSHIP

Herewith, I declare that the work presented in this dissertation has been designed and performed independently, without help from others and without other materials than stated in the text. To the best of my knowledge and belief, thoughts and ideas from other people and colleagues that have been adopted directly or indirectly in this dissertation were specifically indicated and acknowledged in any case. I confirm that others did not either directly or indirectly receive any payment in kind for any work related to the content of this dissertation. This dissertation has never been submitted before for the award of any other degree or during any kind of examination procedure in any other institution.

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Date Tuan Le Van

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