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Generation of tools to investigate Chikungunya virus

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Tag der mündlichen Prüfung:

Der Promovend ist Arzt

I dedicate my thesis to my wife and my sons

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1. Introduction

1.1. Chikungunya virus and re-emergence of Chikungunya diseases

Chikungunaya virus (CHIKV) is the prototype of the Alphavirus genus of the family Togaviridae. Togaviridae consists of two genera, Alphavirus and Rubivirus. There are approximately 40 alphaviruses able to infect various vertebrates such as humans, rodents, birds, and horses and in addition invertebrates. Transmission between species and individuals occurs via mosquito vectors, making the alphaviruses a member of arboviruses, or arthropod borne viruses. There are approximately 30 known species of arthropod-borne viruses (Calisher at el., 1988)

CHIKV was first reported in Africa by causing an epidemic in 1952 in the Southern Province of Tanganyika that renamed Tanzania. The name of CHIK was given by a local name for "that which bends up" in reference to the stooped posture as a result of the arthritic symptoms of the disease (Robinson et al., 1955; Lumsden, 1955).

Since the first description of CHIKV numerous outbreaks have been described in India, Southeast Africa and sub-Saharan Africa. The spread of CHIKV from Africa to Asia was recorded by causing severe outbreaks in these areas. In Asia, CHIKV was first isolated in 1958 in Bangkok, Thailand (Hammon, 1960). Subsequently, many Asian countries have reported CHIKV infections such as: the Philippines, Thailand, Singapore, Indonesia, India, Japan, Vietnam, Kampuchea and Myanmar throughout the 1960s and 1970s (Halstead et al., 1963; Thaikruea et al., 1997; Shah et al., 1964; Rao et al., 1965; Thaung et al., 1975; Campos et al., 1969; Macasaet, 1970; Jupp, 1988; Thaikruea, 1997; Macasaet, 1970). The cases of CHIKV-infection in these epidemics were up to 31% in Bangkok, Thailand (Halstead et al., 1963), and ranged from 15% to 25% in Vellore, India (Rao et al., 1965).

Recently, CHIKV has emerged in Southeast Asia and the Pacific region (Mackenzie, 2001; Laras, 2005). In India, the outbreak of CHIKV disease caused 1.3 million cases in 13 States in 2005-2006 after 32 year absence. In the same time, the outbreaks were also reported in the Indian Ocean such as the Comoros, Madagascar, Mayotte, Mauritius, La Reunion and the Sychelles (Lahariya and Pradhan, 2006; Enserink, 2006). On the Comoros Islands more than 500 cases were reported during an outbreak at the beginning

of 2005. Subsequently, the virus was spread to other islands, such as Reunion and Mayotte, Mauritius, Madagascar (Simon, 2007). Especially, in Reunion approximately 266,000 cases which stand for one third of the population were infected with CHIKV and 237 deaths were considered to be caused by CHIKV. During this period about 9000 cases of CHIKV in the Seychelles, 7290 in Mayotte and 6000 in Mauritius have also been reported.

Recently, CHIKV has attracted more attention when the virus caused an outbreak in a temperate region such as Italy where the virus caused approximately two hundred cases (Lines, 2007). Tourism is thought to be the reason for virus introduction to Europe (Chastel, 2005; Depoortere, 2006; Service, 2007). Travelers were infected with CHIKV during visiting the outbreak places and they became a reservoir of virus when they backed home (Bodenmann, 2006; Hochedez., 2007; Krastinova, 2006; Panning, 2006; Pfeffer, 2006). The recent CHIKV outbreaks are presented in figure 1.

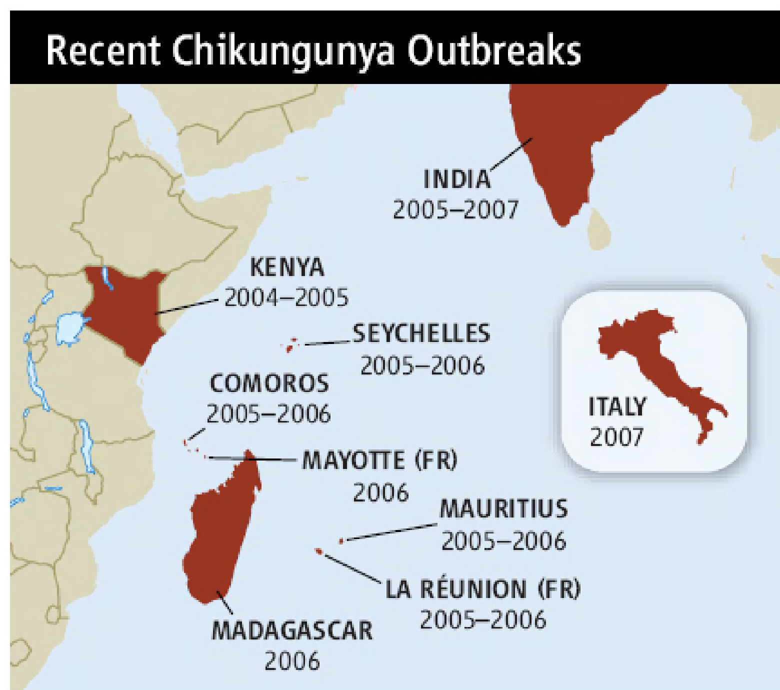


Figure 1: Distribution of recent CHIKV outbreaks (MARTIN ENSERINK-SCIENCE VOL. 318. 21 DECEMBER 2007)

CHIKV is transmitted by mosquitoes in which the virus has a cycle of replication before replication in human. The mosquitoes were considered to be the main vectors for CHIKV *Aedes albopictus* and *Aedes aegypti* (Hammon, 1960; Thaikruea, 1997). The other *Aedes* species have also been reported to transmit the virus (Griffin, 2007; Pialoux, 2007). There is a different geography distribution of these vectors. In Asia, *Ae.aegypti* is the main vector of CHIKV (Jupp, 1988), whereas *Ae. albopictus* (common name Asian tiger mosquito) is shown to be the main vector in islands of the Indian Ocean (Enserink, 2006; Reiter, 2006). *Ae.albopictus* is also thought to be the main vector for transmission of CHIKV in Italy where due to climate change this vector now appears to be epidemic (Enserink, 2007; Knudsen, 1996; Gratz, 2004). Furthermore, reports indicate *Ae.albopictus* to replicate and transmit the old African genotype of CHIKV as well as the recent Indian Ocean strain of CHIKV better than those of *Ae.egypti* (Konishi, 1986; Mangiafico, 1971; Tesh, 1976) and other *Aedes* species (Charrel, 2007).

Figure 2: A female ***Ae. aegypti*** *(right)* and a female ***Ae. albopictus*** (left) feeding on a human (Image Centers for Disease Control Public Health Image Library).

1.2. The genome and replicative cycle of CHIKV

1.2.1. Genome structure and organization

CHIKV is an enveloped particle and has a single-stranded RNA genome of positive polarity. The genome is approximately 12000 nucleotides in length (Schlesinger and

Schlesinger 2001; Strauss and Strauss 1994). Under electron microscopy in green monkey kidney (Vero) cells CHIKV particles reveal a characteristic Alphavirus morphology (Fig.3 and Simizu et al., 1984)

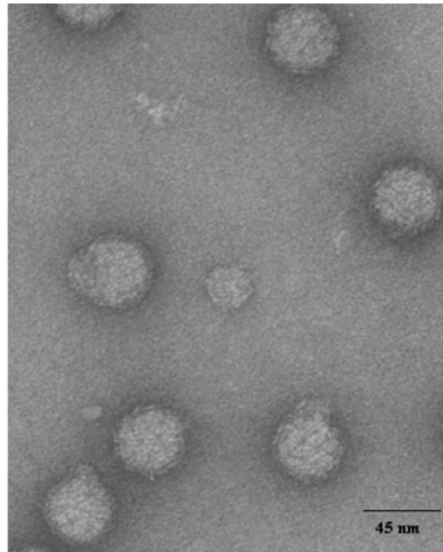
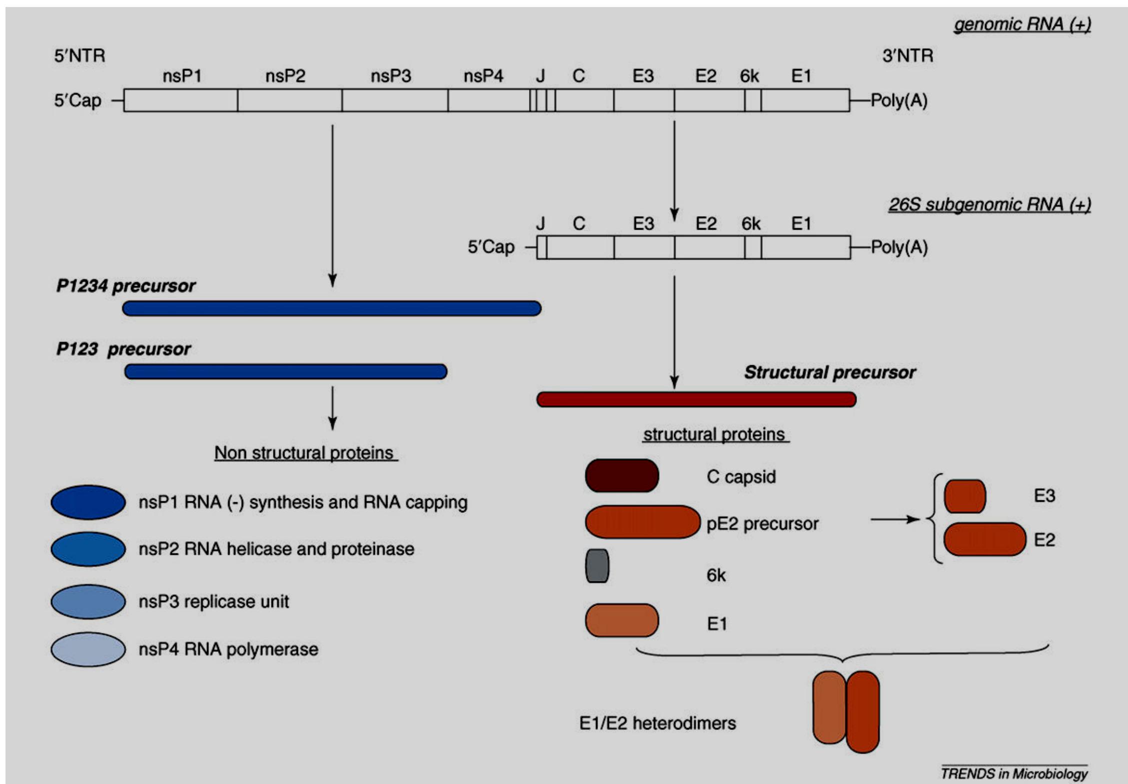


Figure 3: Electron micrograph of purified CHIKV virions. CHIKV virions exhibit typical alphavirus structure. In thin sections the virus shows a roughly spherical shape with a diameter of 42 nm composed of a 25–30 nm core which is surrounded by an envelope (reproduced from Simizu et al., 1984).

The genomic RNA acts as cellular messenger RNA. Therefore, the 5' end is capped with a 7-methylguanosine, while the 3' end is polyadenylated. The genome is divided into two major domains: a nonstructural domain, covering the first two thirds of the genomic RNA, which are directly translated in the non-structural proteins and a structural domain covering the last third of the genome, encoding the structural proteins of the virus.



(Christine et al., 2007)

Figure 4: CHIKV genome and its products

The nonstructural proteins are required for transcription and replication of the viral RNA. In the early of infection, the non-structural proteins are synthesized directly from the two third of genomic RNA as a P1234 polypeptide and in the process of replication this polyprotein is cleaved to form all four nonstructural proteins: Nsp1, Nsp2, Nsp3, and Nsp4. Nsp1 protein is considered to play a role in the synthesis of minus-strand RNA and to be involved in the association of the replication complex to cellular membranes. The main functions of the Nsp2 protein are helicase and proteinase that cleaves the non-structural polyprotein to form the individual non-structural proteins (Kim et al., 2004; Vasiljeva et al., 2001; Hardy and Strauss, 1989; Merits et al., 2001; Strauss et al., 1992). The function of Nsp3 in viral replication remains largely unknown (Strauss at al., 1994). Nsp4 is the viral RNA polymerase and interacts with the N-terminal region and others non-structural proteins and unknown host cellular factors.

The structural proteins, Capsid, E3, E2, 6K and E1, are synthesized from a sub-genomic mRNA as a single polypeptide, which is cleaved both co-translationally and post-

translationally to form structural proteins (Strauss & Strauss, 1986, 1988; Faragher et al., 1988). These structural proteins have important functions in the replication of virus and particularly in the interaction with the host. This interaction was presented by the production of antibodies which played important roles in the recovery from infection (Griffin, 1977). Antibodies can neutralize virus infectivity and decline virus by the complement systems (Jahrling, 1983).

The junction region is located in between the nonstructural and structural domains and functions to promote transcription of an intracellular subgenomic 26S RNA. In addition to the junction region, there are two other untranslated regions: one at the 5'-end, which is required for plus-strand RNA synthesis (Ou et al., 1983; Strauss & Strauss, 1994) and the other which is located at the 3' end between the stop codon of the E1 gene and the poly (A) tail. This region is mainly involved in translation of viral proteins rather than in replication of the genomic RNA (Leathers et al., 1993; Kuh et al., 1991).

1.2.2. The replication-cycle of CHIKV

CHIKV replicates in the vertebrate as well as in the insect host. Principally, the process of virus replication is similar, but the budding of virions is different. In invertebrate cells, virus is released by exocytosis rather than at the plasma membrane (Miller, 1992; Stollar, 1975). In addition, the lifelong infections of virus are supported by noncytopathic infection in invertebrate cells, whereas virus is treated by the immune system after infection in vertebrates. Some human patients suffer from long-lasting symptoms. This could be explained by discovering CHIKV tropism for muscular satellite cells that are considered as reservoirs for virus or virus-encoded components for longer than expected periods (Ozden et al., 2007).

In order to penetrate into vertebrate cells, the interaction between the envelope proteins of CHIKV and receptors of host cells is required. The cellular receptors for CHIKV is still unknown, however, in other Alphavirus the laminin receptor, glycosaminoglycans and DC-SIGN (CD209) molecules are involved in viral uptake. By endocytosis of clathrin-coated vesicles the virus is transported into the cell. Due to reduction of the pH in the vesicle the activation of the E1 protein from the E1-E2 complex is promoted viral and endosomal membrane fuses, resulting in the release of the nucleocapsid into the

cytoplasm. In the cytoplasm, the replication of CHIKV is performed. Firstly, the translation of P1234 precursor polyprotein and RNA replication are carried out. The P1234 polyproteins are translated directly from the viral genome and the process of RNA replication is started by the synthesis of a full-length minus-strand RNA, which is used as a template for the synthesis of the viral genome and to transcribe the 26S sub-genomic plus-strand RNA from the internal promoter in the junction region. Both processes are interlinked. After cleavage from the P1234 polyprotein, Nsp4 associates with P123 and unknown host partners to regulate the synthesis of minus-strand RNA. The switch from genome replication to transcription of sub-genomic 26S positive-strand RNA is also regulated by non-structural proteins which were cleaved from the P123 polyproteins.

The structural protein precursor is translated from the 26S subgenomic RNA, which serves as mRNA. The mature structural protein precursor (C-E3-E2-6k-E1) is cotranslationally cleaved. The capsid protein is produced by autocatalytic cleavage from the N-terminal region of structural polyprotein precursor and encapsidates the viral genomic RNA, resulting in the rapid assembly of nucleocapsid cores in the cytoplasm. In parallel, after being cleaved from the envelope polyprotein precursor, E2 and E1 are transferred to the plasma membrane. Finally, the packaging of virus is performed in cytoplasma by the process of assembly of nucleocapsid cores with glycoproteins and virus is released by budding through the cellular membrane to form an enveloped virion.

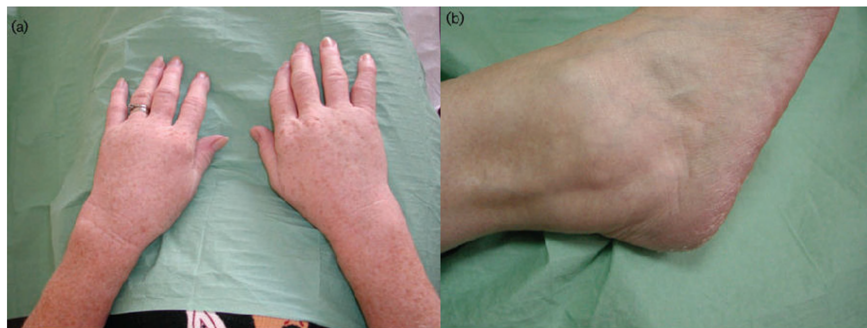
1.3. CHIKV clinical manifestation

Normally CHIKV is not transmitted between humans. CHIKV disease in humans is caused by the bite of an infected mosquito or by transmission of CHIKV infected blood. During feeding blood meal, the mosquito deposits virus-infected saliva extravascularly (Turell, 1995). In typical infected patients, viraemia is highest for the first four days, then declines and is cleared by an immunosystem on day five. The human disease is characterised by a sudden onset of fever up to 39-40^{0}C (Deller and Russell, 1967), which is followed by rash and joint pains (McGill, 1995; Adebajo, 1996; Jadhav, 1965). The manifestation of joint pains is typical symmetrical, poly-articular, and migratory

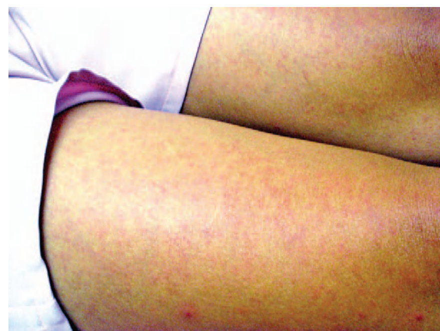
(Kennedy, 1980; Deller and Russell, 1968; Fourie and Morrison, 1979). Joints may be swollen and present redness, and limitation of movement. Fluid in the synovial bursa is uncommon (Brighton, 1983).

Other typical symptoms of CHIKV disease are skin manifestations, presenting on the face, trunk and limbs. The rash can occur when temperature declines and along with itch.

Most CHIKV infected patients have common symptoms such as headache, retro-orbital pain, photophobia, lumbar back pain, chills, weakness, malaise, nausea, vomiting and myalgia, but the presence or frequency of the symptoms in the individual patient are highly variable (Brighton, 1981; McGill, 1995; Calisher, 1999; Munasinghe, 1966). During the recent outbreak in Reunion and in the first outbreak in India, neurological complications such as meningoencephalitis occurred in a small proportion of patients (Quatresous, 2006; Chatterjee, 1965). Mother to child transmission of CHIKV has been also reported (Robillard, 2006). While most symptoms disappear completely in weeks or month, in some cases the patients suffered from chronic joint disease for several years, which may result in joint destructed after 15 years in rare cases (Brighton et al., 1983; McGill, 1995; Calisher, 1999; Brighton and Simson, 1984).



(a) (b)



(c)

Figure 5: Chikungunya-associated rheumatism second stage: **(a)** severe relapse with polyarthritis and multiple tenosynovitis of wrists and fingers, **(b)** hypertrophic tenosynovitis at the ankle (Photos by Dr F.Simon, France). **(c)** This rash appeared on day 2 of a febrile illness in a patient who presented with myalgia and arthralgia after visiting Mauritius (Photos by Richard Foster, South Africa).

Unfortunately, the symptoms of CHIKV infection are similar to those caused by many other infectious agents in the endemic areas. One particular difficulty in identifying CHIKV infection is its overlapping distribution with dengue fever viruses. It has been postulated that many cases of dengue fever virus infection are misdiagnosed and that the incidence of CHIKV infection is much higher than reported (Myers and Carey, 1967; Carey, 1971).

1.4. The development of antiviral drugs and tools of viral diagnosis

In cell culture, some compounds, including ribavirin, sulfated polysaccharides (iotacarrageenan, fucoidan and dextran sulfate), 6-azauridine, glycyrrhizin and interferon-α have been demonstrated their ability to inhibit replication of CHIKV (Briolant et al., 2004).

The diagnosis of CHIKV infection is made by the combination of clinical diagnosis of a patient presenting with typical symptoms and laboratory. For molecular diagnosis Reverse Transcription – PCR (RT-PCR) is commonly performed to detect a part of the E2 gene (Pfeffer, 2002) or a part of nsp1 and E1 genes (Hasebe, 2002). Recently, one-step TaqMan Real-Time-PCR considered as a specific and sensitive assay has been developed to reliably diagnose CHIKV in clinical samples and cell culture supernatant (Pastorino, 2005).

1.5. The aim of thesis

To combat the CHIKV outbreaks, which exploded worldwide and caused thousands of reported cases in new geographical areas, the development of new diagnostic tools is highly desirable. Although many new techniques have been developed and established recently, inconveniences still remain. Virus culture, followed by detection of viral antigens is sensitive, but must be performed under strict BSL3 bio-safety conditions. This is a laborious and time-consuming technique, which can not be used in routine diagnostic. Therefore, this method is used only for virus identification at the beginning of an epidemic. In order to develop diagnostic tools on a serological level, bacterial expression vectors for the non-structural and structural proteins of CHIKV were constructed. The proteins were purified and characterized and used for Western blot and the development of ELISA assays. The tests were evaluated with pre-screened positive sera and, in addition, with sera from healthy human blood donors from Germany. We aimed to use the established analysis to screen samples, from countries with a high CHIKV incidence. Vietnam is the easternmost country in the Indochina peninsula in Southeast Asia. The climate of Vietnam varies greatly from one region of the country to another. The south of Vietnam is a mere 8 degrees north of the Equator, whereas the most northern point is almost on the tropic of Cancer. Most regions in Vietnam have a subtropical climate and are hot, at least in the summer, and wet most of the year. With the climate condition this is perfect environment for development of *Ae.agypti,* due of the CHIKV vectors. Dengue virus infections in victims have often been reported, but there are only a few old reports on CHIKV (Vu et al., 1967; Anonym, 1969). Thus, the newly developed test will be used to screen Vietnamese serum samples and to gain an inside in the situation of CHIKV seroprevalence in Vietnam.

2. Materials

2.1. Enzymes and Buffers

CIAP *(calf intestine alkaline phosphatase)*	MBI Fermentas
SAP *(Shrimp Alkaline Phosphatase)*	MBI Fermentas
Klenow-Polymerase	NEB, MBI-Fermentas
Restriction enzymes	MBI Fermentas
Mung Bean Nuclease	MBI Fermentas
T4 DNA-Ligase	MBI Fermentas
T4 DNA-Polymerase	MBI Fermentas

2.2. Antibiotics

Antibiotics	Stock Solution	Working Solution
Ampicilline	100µg/µl	100µg/ml
Kanamycine	50µg/µl	50µl/ml

2.3. Competent cells

TOP 10 cells	Invitrogen
DH5α	Reference from Invitrogen
BL 21 star	Invitrogen

2.4. Antibodies

Antibodies were diluted for Western blotting and Elisa

First antibodies		***Dilution***	***Identification***
His-Tagged antibody		1: 1000	His-Tagged protein
Second antibodies			
Goat Anti-mouse IgG	Jackson Immuno Research	1:10000	

Goat Anti-Human IgG	Jackson Immuno Research	1:10000
Goat Anti-Human IgG-Alkaline Phosphatase	Sigma	1:1000

2.5. Vectors

Clonging vectors		*Expression vectors*	
pCR II	Invitrogen	PET 200/D	Invitrogen
pCR XL	Invitrogen	PET 101/D	Invitrogen

Virus was isolated from a CHIKV infected patient and the viral genome was sequenced. PCR products were obtained by RT-PCR and inserted into PCRII and PCR XL (Kowalzik et al., 2008).

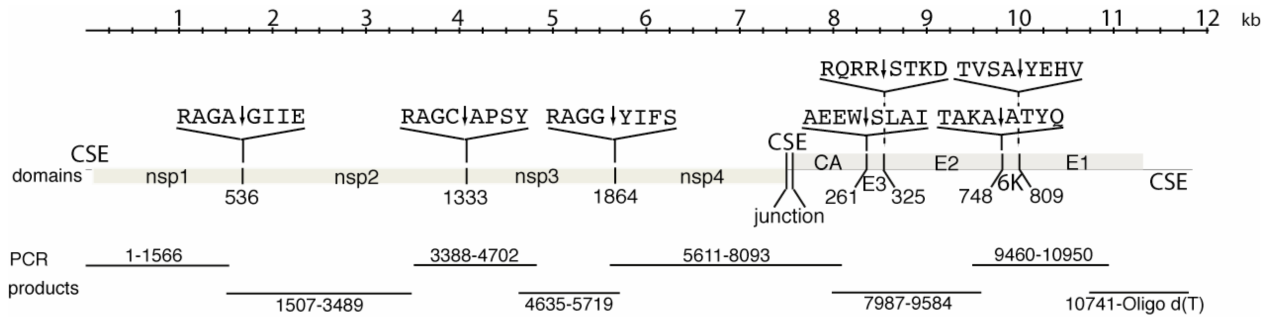


Figure 6: PCR products from CHIKV

2.6. Oligonucleotids

All primers were diluted 1 to 10 with sterile water and kept at -20^0C prior to use.

Primers	*Sequences (5'-3')*	*Usage*
E2-8849s	CACCGCCCGATGTCCAAAGG	CHIKV E2 sense primer
E2-9529as	CTACGTGACCTCGAGCCCTTC	CHIKV E2 antisense primer
E1-10000s	CACCCACGTAACAGTGATCCCG	CHIKV E1 sense primer
E1-10681as	CATTACCGTACCCACAGCCGG	CHIKV E1 antisens primer

C-7567s	CACCATGGAGTTCATCCCAACCC	CHIKV C sense primer
C-8350as	CTACCACTCTTCGGCTCCCTCAG	CHIKV C antisense primer
NSP2-1683s	CACCGGAATAATAGAGACTCCGAG	CHIKV NSP2 sense primer
NSP2-4077as	CTAACATCCTGCTCGGGTGGCC	CHIKV NSP2 antisense primer
pET200-PR s	CACCACACTTTCCGGCGACCCGTG	CHIKV protease sense primer
pET200-PR as	CTAACATCCTGCTCGGGTGACCTG	CHIKV protease antisense primer
pET101-PR s	ATGACACTTTCCGGCGACCCGTGG	CHIKV protease sense primer
pET101-PR as	ACATCCTGCTCGGGTGACCTGTC	CHIKV protease antisense primer

2.7. Kits

Big Dye Terminator v1.1 Cycle Sequencing Kit	Applied Biosystems
ECL ^{TM} Western Blot Detection Kit	Amersham Biosciences
NucleoBond PC500 Reagents	Macherey-Nagel
Qiaquick PCR Purification Kit	Qiagen
Qiaquick Gel extraction Kit	Qiagen

2.8. Others

Blot Filterpaper	Schleicher & Schuell
Hybond-ECL Nitrocellulosemembran	Amersham Biosciences
Plastic Material	Costar, Eppendorf, Falcon, Greiner, Nunc, Roth
X-ray film Retina XBD	Fotochemische Werke GmbH
Filters (0,22 µm, 0,45 µm)	Schleicher & Schuell
INTAS UV system	
Sonicator-Sonifier 250	Branson
FPLC^{TM} system	Amersham Biosciences
Ni-NTA supperflow cartridges	Invitrogen

In addition, the normal laboratory equipments with centrifuges, pipettes, vortex, power supplies etc. were used.

2.9. Media, solutions and buffers

2.9.1. Bacteria cultures

5x LB-Medium	LB Broth Base Lennox L (Invitrogen #12780-029)	100g
	NaCl	25g
	α-D Glucose	5g
	H_2O	Up to 1000ml
	Autoclave	

LB-Agar	LB Broth Base Lennox L	40g
	Agar	40g
	NaCl	10g
	H_2O	Up to 1000ml
	Autoclave	

After autoclaving the LB-Agar was cooled down and Ampicillin 100µg/ml and Kanamycine 50 µg/ml were added. Then LB-Agar was poured in plates and kept at 4^0C until use.

2.9.2. Buffers and solutions for analysis and cloning DNA

50x TAE	Tris	2 M
	Acetic acid	5.71%
	EDTA pH 8.0	50 mM

Preparative competent cell buffer 1	Kaliumacetat	30 mM
	$RbCl_2$	100 mM
	$CaCl_2$	10 mM
	$MnCl_2$	50 mM
	Glycerin	15%

Adjusted to pH 5.8 with acetic acid 10% and filtered by 0.2µl filter, kept at 4^0C.

Preparative competent cell buffer 2	MOPS	10 mM
	$RbCl_2$	100 mM
	$CaCl_2$	75 mM
	Glycerin	15%

Adjusted to pH 6.5 with KOH 1M and filtered by 0.2µl filter, kept at 4^0C.

Miniprep solution 1	Tris-HCl pH 8.0	25mM
	Glucose	50mM
	EDTA pH 8.0	10 mM
	Autoclave	
Miniprep solution 2	NaOH	0.2 N
	SDS	1%
Miniprep solution 3	Natrium Acetat	3M
	Adjust pH 2.0 with Acetic acid	
	Autoclave	
TE/RNase	TE	0.1x
	RNase A	100µg/ml
Tris/$MgCl_2$	Tris-HCl pH 9.0	200 mM
	$MgCl_2$	5 mM
	Autoclave	

2.9.3. Buffers and solutions for protein analysis

2x SDS-PAGE Sample Buffer	1. Combine the following reagents:	
	0.5 M Tris-HCl, pH 6.8	2.5 ml
	Glycerol (100%)	2.0 ml
	β-mercaptoethanol	0.4 ml
	Bromophenol Blue	0.02 g

	SDS	0.4 g
	2. Bring the volume to 10 ml with sterile water.	
	3. Aliquot and freeze at -20°C until use	

1x SDS-PAGE	1. Combine the following	
Sample Buffer	reagents:	1.25 ml
	0.5 M Tris-HCl, pH 6.8	1.0 ml
	Glycerol (100%)	0.2 ml
	β-mercaptoethanol	0.01g
	Bromophenol Blue	0.2g
	SDS	
	2. Bring the volume to 10 ml with sterile water.	
	3. Aliquot and freeze at -20°C until use.	

Coomassie gel stain	Coomassie blue R-250	1.0 g
(1l)	Methanol	450 ml
	H_2O	450 ml
	Acetic acid	100 ml

Coomassie gel		
destain (1l)	Methanol	100 ml
	H_2O	800 ml
	Acetic acid	100 ml

Lysis Buffer for	Potassium phosphate, pH 7.8	50 mM
pilot analysis of	NaCl	400 mM
recombinant	KCl	100 mM
proteins	Glycerol	10%
	Triton X-100	0.5%
	Imidazole	10 mM

Prepare 1 M stock solutions of KH_2PO_4 and K_2HPO_4.

For 100 ml, dissolve the following reagents in 90 ml of deionized water:

KH_2PO_4	0.3 ml
K_2HPO_4	4.7 ml
NaCl	2.3 g
KCl	0.75 g
Glycerol	10 ml
Triton X-100	0.5 ml
imidazole	68 mg

Mix thoroughly and adjust pH to 7.8 with HCl. Bring the volume to 100 ml.
Store at +4°C.

Buffers for purification under denaturing conditions

Lysis buffer

Buffer B (1 liter)	100 mM NaH_2PO_4	13.8 g NaH_2 $PO_4.H_2O$ (MW 137.99 g/mol)
	10 mM Tris-HCl	1.2 g Tris base (MW 121.1 g/mol)
	8 M urea	480.5 g (MW 60.06 g/mol)
	Adjust pH to 8.0 using NaOH.	

Wash buffer

Buffer C (1 liter)	100 mM NaH_2PO_4	13.8 g NaH_2 $PO_4.H_2O$ (MW 137.99 g/mol)
	10 mM Tris-HCl	1.2 g Tris base (MW 121.1 g/mol)
	8 M urea	480.5 g (MW 60.06 g/mol)
	Adjust pH to 6.3 using HCl.	

Elution buffer

Buffer D (1 liter)	100 mM NaH_2PO_4	13.8 g NaH_2 $PO_4.H_2O$ (MW 137.99 g/mol)
	10 mM Tris-Cl	1.2 g Tris base (MW 121.1 g/mol)
	8 M urea	480.5 g (MW 60.06 g/mol)
	Adjust pH to 5.9 using HCl.	

Elution buffer

Buffer E (1 liter)	100 mM NaH_2PO_4	13.8 g NaH_2 $PO_4.H_2O$ (MW 137.99 g/mol)
	10 mM Tris-HCl	1.2 g Tris base (MW 121.1 g/mol)
	8 M urea	480.5 g (MW 60.06 g/mol)
	Adjust pH to 4.5 using HCl.	

Due to the dissociation of urea, the pH of buffers B, C, D, and E should be adjusted immediately prior to use.

Solutions and buffers for coomassie gel and Western blotting

Stacking gel 4x	Tris-HCl	0.5 M
	SDS	0.4%
	Adjust pH 6.8	
Separating gel 4x	Tris	1.5 M
	SDS	0.4%
	Adjust pH 8.0	
Running buffer 5x	Tris	30 g
	Glycin	72 g
	SDS 10%	100ml
	Bring the volume to 1l with d.H_2O	

Western blot buffer	Tris	3.04 g
	Glycin	14.4 g
	Methanol	100ml
	Bring the volume to 1l with d.H_2O	

Reagents and solutions for ELISA

Borate-buffered saline (BBS)	$Na_2B_4O_7.10H_2O$	0.017M
	NaCl	0.12 M
	Adjust to pH 8.5 with NaOH	
Blocking buffer	BBS	0.05%
	Tween 20	1 mM
	EDTA	0.25%
	Bovine Serum Albumin (BSA)	0.05%
	NaN_3	0.01%
	Store at 4^0C	
NPP substrate solution(1x)	*p*-nitrophenyl phosphate (NPP; Sigma)	3 mM
	Na_2CO_3	0.05M
	$MgCl_2$	0.05mM
	Store at 4^0C	

2.10 Standard solutions and Buffers

Tris 1 M pH 8.0	Tris	1M
	pH 8.0	
	Autoclave	
SDS 10%	SDS	10%

PBS(+)	NaCl	137 mM
	KCl	2.7 mM
	$Na_2\ HPO_4 \times 2\ H_2O$	4.3 mM
	KH_2PO_4	1.4 mM
	$CaCl_2 \times 4\ H_2O$	1.5 mM
	$MgCl_2 \times 6\ H_2O$	1 mM
TE pH 8.0	Tris-HCl pH 8.0	10 mM
	EDTA pH 8.0	1 mM
0.5 M EDTA pH 8.0	EDTA	500 mM
	pH 8.0 with NaOH	

2.11. Blood samples

2.11.1. Blood sample control.

Blood samples of 100 healthy blood donors from the Wuerzburg area which were supposed as the negative samples for CHIKV antibodies (Tab.4)

2.11.2. CHIKV Patient's sera

Human sera of 30 patients were obtained from the Berhard Nocht Institut (BNI) known to be positive for CHIKV antibodies (Tab.1).

2.11.3. Vietnamese donor blood samples

158 Vietnamese serum samples were collected from healthy people who live in Hanoi, Northern Vietnam. They did not have CHIKV manifestation at the time of collection (Tab.2 and 3).

3. Methods

3.1. Make competent E. coli cells

1. Innoculate competent *E.coli* cells into 5ml LB 1x without antibiotics and incubate on the shaker at 37^0C overnight.
2. Next day, inoculate 1 ml cell culture overnight into 100ml LB 1x and incubate on the shaker at 37^0C for 2-3 h to get OD_{600}= 0.2-0.3.
3. When samples are ready, pour cell culture into sterile centrifuge falcon 50ml (2 falcons) and put on ice for 10 min. Spin at 2,500g (6,000 rpm) for 10 min. Remove supernatant.
4. Resuspend the cell pellet in 20ml ice-cold competent cell buffer 1.
5. Put sample on ice for 10 min. Spin at 2,500g for 10 min.
6. Pour off supernatant and resuspend in 4ml competent cell buffer 2. Vortex and keep on ice for 15 min.
7. Aliquot 200 µl into 1.5ml tubes and quickly freeze in nitrogen. Store at -80°C. Should last 40 days.

3.2. Isolation of DNA plasmids from *E.coli*

3.2.1 .Mini-prep

1. Grow bacterial colonies overnight in 3ml LB solution with antibiotics (Ampicillin 100µg/ml or Kanamicin 50µg/ml)
2. Take out 1.5 ml bacteria and spin down for 30 sec. Remove supernatant.
3. Add 200 µl Solution 1. Vortex the pellet and keep on ice for 5 min.
4. Add 200 µl Solution 2. Vortex and wait for 5min on ice.
5. Add 250 µl Solution 3. Vortex and keep on ice for 10 min.
6. Spin at 13,000 rpm for 1 min in room temperature.
7. Remove the supernatant and put in new tube.
8. Add 3x volume of 95% cold Ethanol.
9. Invert to mix and spin for 3 min at room temperature or 4^0C.

10. Discard supernatant.
11. Add 500 µl 70% cold Ethanol. Spin 2 min (RT or 4^0 C) and remove supernatant
12. Dry pellet 10 min at RT.
13. Resuspend in 50-100 µl TE.

3.2.2. Maxi-prep

DNA plasmid maxiprep protocol follows standard protocol from Invitrogen which use kit *NucleoBond PC500.*

3.2.3. Measurement concentration of DNA plasmids

The DNA purified by Maxi-prep was measured by spectrophotometer as follow:

1. DNA was diluted into 1:100 in TE buffer or d.H_2O and transferred in cuvettes.
2. Samples were measured by spectrophotometer at OD_{260}
3. The concentration of DNA was calculated by formula: ds DNA (µg/m1) = Dilution factor * OD_{260} * 50µg/ml
4. Dilute the DNA in the final concentration 1µg/µl.
5. Keep the DNA in - 20^0C.

3.3. Running a DNA gel

1. Set up gel container and place in 1 or 2 combs.
2. Pour a 0.8% agarose gel. If mixture is already made, microwave for about 60 sec. (or until melted), then let cool until can place hand comfortably on glass.
3. Set up gel and make sure that there is enough TAE buffer to cover the top of the gel. Place as many dots of gel loading dye as you have samples on a small piece of Parafilm. Pipette samples up and down to mix in the dye and then load the appropriate lane. Fill lanes-add 6µL 1 Kb DNA Ladder to first lane.
4. For PCR products, add 5µl sample. For Restriction Digests, add 20µl or more sample.
5. Run at 90 V until the bands were at expected points.

6. Take a picture of gel using INTAS UV system. Cut out desired DNA band at wave length 320nm.

3.4. Isolation DNA fragments from agarose gel

After finishing running the DNA gel, the desired DNA bands were excised from the agarose gel with a clean, sharp scalpel.
And then follow QIAquick Gel Extraction Kit Protocol.

3.5. Restriction digest

DNA volume should never be more than 25% of total volume and enzyme volume should never be more than 10% of total vol.

1. Add the following in order:

DNA	10µg = 10µl
Enzym buffer 10x	10 µl
Enzymes	10U
dH_2O	Add to 100 µl

2. Pipette up and down a few times (without introducing air bubbles)
3. Incubate at 37°C for at least 2 h or overnight.
4. Run a 0.8 % agarose gel.
5. Take a picture and cut out desired DNA band using a UV light board and a razor. Store band in a 1.5mL tube at 4°C until ready to use.

3.6. Ligation

1. Thaw 10x T4 DNA Ligase Buffer. Set up 1 control (just vector) and X samples (vector + insert). Adjust volumes accordingly.

Vector + insert		Control (vector only)	
Vector	3 µl	Vector	3 µl
Insert	8 µl	Insert	-
10x T4 DNA Ligase Buffer	2 µl	10x T4 DNA Ligase Buffer	2 µl
T4 DNA Ligase	1 µl	T4 DNA Ligase	1 µl
dH_2O	6 µl	dH_2O	14 µl

Final Reaction volume will be 20 µl.

2. Mix in 1.5mL Eppendorf tube in this order: water, buffer, vector, insert, enzymes.
3. Incubate overnight in a 14°C water bath in cold room.

3.7. Sub cloning DNA by PCR with Pfu DNA polymerase

PCR is based on the enzymatic amplification of a fragment of DNA that is flanked by two 'primers', short oligonucleotides that hybridize to the opposite strands of the target sequence and then prime synthesis of the complementary DNA sequence by DNA polymerase. The chain reaction is a three-step process, denaturation, annealing, and extension which are repeated in several cycles.

The reactions are controlled by changing the temperature.

3.7.1. Reaction mixture set up

1. Gently vortex and briefly centrifuge all solutions after thawing.
2. Keep solutions on ice
3. Add to a thin-walled PCR tubes, on ice.
   Reaction mixture

Reagent	Final concentration	
Sterile deionized water	-	37.5µl
10x *Pfu* buffer with $MgSO_4$	1x	5µl
2 mM dNTP mix	0.2 mM of each	1µl

Forward primer	0.1-1 μM	2μl
Reverse primer	0.1-1 μM	2μl
Template DNA	50 pg-1 μg	2μl
Pfu DNA Polymerase	1.25 u/50 μl	0.5μl
Σ		50μl

Gently vortex the sample and briefly centrifuge to collect all drops from walls of tube, place samples in a thermal cycle preheated to 95°C and start PCR.

3.7.2. Thermal cycling conditions:

Step	Temperature, °C	Time, min	Number of cycles
Initial denaturation	95	3	
Denaturation	95	0.5	
Annealing (depend on Tm of primers)	45-55	0.5	30
Extension	70	2	
Final extension	70	5	
Pause	4		

When program was completely finished, the PCR products were analyzed and purified by procedures described above.

3.8. Expression and purification protein methods

A primary consideration for recombinant protein expression and purification is the experimental purpose for which the protein will be utilized. For biochemical and structural studies, it is often important to optimize conditions for the expression of soluble, functionally active protein, whereas for antigen production, the protein can be expressed either in native or denatured form. Freshly transformed bacterial colonies often express recombinant proteins at different levels. Therefore, comparison of the

signals produced after colony blotting to identify highly expressing colonies can help significantly, while establishing expression cultures.

Optimal growth and expression conditions for the protein of interest should be established with small-scale cultures before large-scale protein purification is attempted. In order to judge the toxicity of an expressed protein, cell growth before and especially after induction of expression should be monitored.

3.8.1. Expression protein methods

3.8.1.1. Small-scale expression protein method.

Before proceeding with a large scale preparation, small-scale expression experiment is highly recommended and should be performed. The cells can be lysed in a small volume of sample buffer and analyzed directly by SDS-PAGE. Small expression cultures provide a rapid way to judge the effects of varied growth conditions on expression levels. Expression levels vary between different colonies of freshly transformed cells, and small scale preparations permit the selection of clones featuring optimal expression rates.

The following is a basic protocol for the expression and screening of small cultures.

1. Pick single colonies of transformants and culture them in 3 ml LB medium containing Kanamycine (50µg/ml). Grow the culture overnight at 37^0C with shaking.
2. Use 500µl of the overnight culture to inoculate 10ml of prewarmed LB medium containing Kanamycine (50µg/ml), and grow at 37^0C with shaking for 2 h, until they reach mid-log (OD_{600} = 0.5-0.7).
3. Induce the cultures by adding IPTG to final concentration 1mM. Before induction, take out 1ml culture that serves as a noninduced control (t_0). Harvest the cells by centrifugation for 1min. at 13,000 rpm, and discard supernatants.
4. Grow the cultures for an additional 4 h. At 2 hly intervals, 1ml of the culture is taken and the cells are harvested by centrifugation for 1 min at 13,000 rpm,

discard supernatants. Collect the cell pellets, and keep at -20^0C until all the samples are ready for processing.

5. The cell pellets of the rest of the cultures are resuspended in 2ml lysis buffer. Lyse cells by sonication 6 x 10s with 10 s pauses at 200-300W keep lysates on ice at all time.
6. Centrifuge the lysate for 30 min at 15,000 rpm to collect the cellular debris and the supernatant for analysis.
7. Add SDS-PAGE sample buffer to the samples at time points and the cellular debris with final concentration 1x SDS-PAGE buffer and boil for 5 min at 95^0C.
8. Analyze the samples by SDS-PAGE and Western blotting.

3.8.1.2. Large-scale expression protein method (1 liter)

1. Inoculate 20 ml of LB broth containing 50 µg/ml kanamycine. Grow at 37°C overnight with vigorous shaking.
2. Inoculate a 1 liter culture (LB, 50 µg/ml kanamycin) 1:50 with the noninduced overnight culture. Grow at 37°C with vigorous shaking until an OD_{600} of 0.5-0.7 is reached.
3. Take a 1 ml sample immediately before induction. This sample is the noninduced control; pellet cells and resuspend in 50 µl 2x SDS-PAGE sample buffer. Freeze until use.

1. Induce expression by adding IPTG to a final concentration of 1 mM.
2. Incubate the culture for an additional 4-5 h. Collect a second 1 ml sample. This is the induced control; pellet cells in a microcentrifuge and resuspend in 50 µl 2x PAGE sample buffer. Freeze until use.
3. Harvest the cells by centrifugation at 4700 rpm for 20 min. Freeze the cells in dry ice-ethanol or liquid nitrogen, or store cell pellet overnight at -20°C.

3.8.2. Protein purification method

Expression of recombinant proteins in *E. coli* can lead to the formation of insoluble aggregates; these are known as inclusion bodies. Strong denaturants such as 8 M urea completely solubilize inclusion bodies, and 6xHis-tagged proteins.
Under denaturing conditions, the 6xHis-Tag fusion protein will be fully exposed so that binding to the Ni-NTA matrix will improve, and the efficiency of the purification procedure will be optimized by reducing the potential for nonspecific binding and contaminated proteins.

Batch purification of 6xHis-tagged proteins from *E. coli* under denaturing conditions

The purification method carried out followed the protocols offered by Qiagen with modifications.
Firstly, the cell lysates were prepared under denaturing condition as follows:

1. The cell pellets were thawed on ice for 15 min and resuspended in buffer B at 5 ml per gram wet weight.
2. The cell pellets were lysed by sonication 6 x 10s with 10 s pauses at 200-300W, lysates were kept on ice at all time, taking care to avoid foaming.
3. Lysis is complete when the solution becomes translucent.
4. Lysates were centrifuged at 4,500 rpm for 30 min at RT by Multifuge 1s-r (Heraeus) to pellet the cellular debris. Save supernatant (cleared lysate).
5. The cellular debris was resuspended in buffer B. 40µl of the debris solution as well as supernatant were taken out and added with 40µl 2x SDS-PAGE.

Then the supernatant was brought to the next step.

1. Ni-NTA agarose beads were equilibrated by washing with 2 x 1.5ml H_2O and then with 2 x 1.5ml buffer B (washing, mix, spin 3 min at 3500rpm at RT by Biofuge fresco-Heraeus, discard supernatant)
2. Add the Ni-NTA agarose beads to the supernatant and mix gently by rotation 200rpm on a rotary shaker for 60 min at RT.
3. Spin 3 min at 3500rpm in RT by Multifuge 1s-r (Heraeus). Discard unbound materials (keep 40µl for SDS-PAGE)

4. Wash 2 x 4ml with buffer C: mix, spin 3 min at 3500rpm by Multifuge 1s-r (Heraeus), discard supernatant (keep 40µl for SDS-PAGE)
5. Elution with 4 x 500µl buffer D: mix, spin 2 min at 8000rpm by Bioguge fresco (Heraeus), supernatants were collected. Each time keep 40µl for SDS-PAGE.
6. Elution with 4 x 500µl buffer E: mix, spin 2 min at 8000rpm Bioguge fresco (Heraeus), supernatants were collected. Each time keep 40µl for SDS-PAGE.
7. 20µl of the cell lysates were run on SDS-PAGE gel 12% as well as unbound materials and the wash, the elution fractions.

FPLC^{TM} purification of His-tagged proteins under denaturing condition

Ni-NTA superflow cartridges are pre-filled with 1ml or 5ml Ni-NTA superflow and are ready to use for purification of 6xHis-Tag proteins using a liquid chromatography system FPLC.

The cell lysates were prepared as follows:

1. Thaw the cell pellet on ice for 15 min and resuspend with buffer B at 5ml per gram wet weight.
2. Stir cells for 60 min at room temperature and then sonicate them with sonicator until the solution became translucent.
3. Centrifuge lysate at 10,000 rpm for 30 min at RT by SS34 rotor Sorwall $Evolution_{RC}$ (Thermo scientific) to pellet the cellular debris. Take out the cleared cell lysate for next step.
4. Fill system pumps with buffer B and attach cartridge to the pump outlet, taking care not to introduce air into the system.
5. Remove cartridge outlet stopper and attach to the system tubing.
6. Equilibrate the cartridge with 10 column volumes of buffer using a flow rate of 3.3ml/min
7. Using the system pumps load the cleared cell lysate onto the cartridge.
8. Purification process was done by changing the gradient of pH from pH8 to pH4.5.
9. Protein fractions were stored at -80 and analyzed by SDS-PAGE.

3.9. Protein estimation

Proteins were determined by ELISA plate reader (V_{max} Molecular devices) using Bradford assay with a Protein Assay kit (Bio-rad), in which bovine serum albumin (BSA) was used as standard. Briefly, the procedure as follows:

Build up a standard curve

1. Prepare samples
   - Dilution the Bradford reagent at 1:4 with dH_2O
   - BSA was diluted at final concentration 1µg/1µl
   - Standard samples were prepared by adding different BSA concentrations (3µg-30µg) in each eppendorf tube containing 1ml diluted Bradford reagent.
   - Protein samples were also added into eppendorf tubes containing 1ml diluted Bradford reagent with different concentrations.
   - The eppendorf tubes were vortexed and incubated at RT for 5 min
   - 200 µl of samples were pipetted into each well of ELISA plate (96 wells). The samples were prepared in 3 wells at each concentration.

0	0	0	24	24	24	Pro5	Pro5	Pro5	Pro13	Pro13	Pro13
3	3	3	27	27	27	Pro6	Pro6	Pro6	Pro14	Pro14	Pro14
6	6	6	30	30	30	Pro7	Pro7	Pro7	Pro15	Pro15	Pro15
9	9	9	0	0	0	Pro8	Pro8	Pro8	Pro16	Pro16	Pro16
12	12	12	Pro1	Pro1	Pro1	Pro9	Pro9	Pro9	Pro17	Pro17	Pro17
15	15	15	Pro2	Pro2	Pro2	Pro10	Pro10	Pro10	Pro18	Pro18	Pro18
18	18	18	Pro3	Pro3	Pro3	Pro11	Pro11	Pro11	Pro19	Pro19	Pro19
21	21	21	Pro4	Pro4	Pro4	Pro12	Pro12	Pro12	Pro20	Pro20	Pro20

   Red: standard samples

   Black: negative control

   Green: protein samples

2. Plate was read under ELISA plate reader (V_{max} Molecular divices) at 590nm wave length.

3. Standard curve was built up by data of standard samples using Microsoft Exel program.

Calculate the concentration of proteins

The concentration of proteins was measured by the standard curve.

3.10. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

This is a very common method of gel electrophoresis for separating proteins by mass. SDS-PAGE was first employed by U.K Laemmli and is known as Laemmli method. The proteins are dissolved in sodium dodecyl sulfate (SDS), a detergent that breaks up the interactions between proteins, and then electrophorised. The smallest molecules move through the gel faster, while larger molecules take longer and result in bands closer to the top of the gel.

Casting the Gel: The gel used for SDS-PAGE is made out of acrylamide, which forms cross-linked polymers of polyacrylamide. Standard gels are typically composed of two layers, the stacking gel (top layer) and separating or resolving gel (lower layer). The stacking layer contains a low percentage of acylamide and has low pH, while the acrylamide concentration of the separating gel varies according to the samples to be run and has higher pH. The differences in pH and acrylamide concentration at the stacking and separating gel provide better resolution and sharper bands in the separating gel.

Set up some kinds of casting gels.

Reagents	***Separating gel***			***Stacking gel***
Acrylamide percents	*10.5%*	***12%***	*15%*	
4x separating buffer	2.5	2.5	2.5	-
Acrylamide	3.5	4	5	0.75
4x stacking buffer	-	-	-	1.25
H_2O	3.5	3	2	3
10% APS	60	60	60	60
TEMED	6	6	6	6

Electrophoresis: The gel is submerged in the buffer and the proteins denatured by SDS are applied to one end of a layer of the gel. The proteins which are negatively charged migrate across the gel to the positive pole when electricity is applied through the gel. Due to differential migration based on their size, larger proteins are closer to the top of the gel while smaller proteins move to the bottom of the gel. After a given period of time, proteins might have separated roughly according to their sizes. Proteins of known molecular weight (marker proteins) can be run in a separate lane in the gel for calibration.

Staining and analysis: After the electrophoresis run, the gel is stained with Coomassie Brilliant Blue for visualization of the proteins. Coomassie blue staining is based on the binding of the dye, which binds nonspecifically to virtually all proteins, and gives a blue colour. During the destain steps, any dye that is not bound to proteins diffuses out of the gel to remove the background. The proteins are detected as blue bands on a clear background. Within the gel different proteins will be seen as separate bands depending on their sizes. The molecular weight of a protein in the band can be estimated by comparing it with the marker proteins of known molecular weights.

3.11. Western blot assay

The fractions to be analyzed were mixed in SDS-PAGE buffer and heated for 5 min at 95^0C.

Twelve percent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to evaluate visually the level of protein expression, the relative molecular size, and to analyze the homogeneity of purified fractions. Protein bands were visualized after Coomassie blue staining and destained with destaining solution.

The fractions separated by SDS-PAGE also were transferred to a nitrocellulose membrane using semi-dry Western blot assay.

This protocol describes the procedure for transferring protein from a mini polyacrylamide gel onto a nitrocellulose membrane using the semi-dry transfer method.

1. 5 sheets of Whatman the same size as the mini SDS-PAGE gel were cut out.
2. A Nitrocellulose membrane to the size of the gel was prepared.

3. Both sheets of Whatman and the membrane were soaked by blot buffer for 5 min.
4. Assemble sandwich (Bottom up on the surface of a wet semi-dry transfer plate)
    - 3 sheets of Whatman
    - Membrane
    - Polyacrylamide gel
    - 2 sheets of Whatman
5. Use a roller to roll over the sandwich gently to remove trapped air bubbles. Apply few milliliter of blot buffer on the top of the sandwich to avoid drying out during transfer.
6. Power was applied at 200 mA for 1h.

For immune reaction, the nitrocellulose membrane was blocked with 5% nonfat dried milk in PBS at room temperature for 30 min. The membrane was incubated with first antibodies diluted in 5% nonfat dried milk in PBS at 4^{0}C overnight. After two times washing with PBS, the membrane was incubated with second antibodies diluted in PBS at room temperature for 1 h. Blots were finally washed in PBS and bound antibodies were visualized onto Biomax films (Kodak) by chemiluminescence (ECL kit, Amersham).

3.12. Emzyme-Linked Immunosorbent Assay (ELISA)

The ELISA is a fundamental tool of clinical immunology, and is used as an initial screen for detecting and quantitating substances such as: proteins, peptides, antibodies and hormone. Based on the principle of antigen-antibody and antibody-antibody interactions, this test allows for easy visualization of results.

An ELISA is the first and most basic test to determine if an individual is positive for a selected pathogen, such as Chikungunia virus. The test is performed in a 8 cm x 12 cm plastic plate which contains an 8 x 12 matrix of 96 wells, each of which are about 1 cm high and 0.7 cm in diameter.

The procedure as follows:

Coating CHIKV Proteins to microplate

1. The proteins were diluted at final concentration of 220µg/ml in PBSN (Phosphate Buffered Saline Natriumazid). Coat the wells of a microtiter plate with 50 µl the protein dilution by a multichannel pipet and tips.
2. The plate was covered by an adhesive plastic and incubated overnight at 4^0C.
3. Remove the coating solution and rinse the plate three times by filling the wells with deionized water. The solution was removed by flicking the plate over a sink and the remaining solution was removed by patting the plate on a paper towel.

Blocking

1. The remaining protein-binding sites in the coated wells were blocked by adding 50µl blocking buffer.
2. The plate was wrapped and incubated for 30 min. at RT.
3. Rinse the plate twice with deionized water.

Incubation with sera and second antibodies

1. Add 50µl of diluted sera (sera were diluted in blocking buffer at 1:100) to each well.
2. Cover the plate with adhesive plastic and incubate at RT for 2 h.
3. Wash the plate three times with deionized water.
4. Add 50µl of conjugated anti human second antibody, diluted at 1:5000 in blocking buffer.
5. Cover the plate with adhesive plastic and incubate at RT for 2 h.
6. Rinse the plate three times with deionized water.

Add substrate and measure hydrolysis

1. Dispense 75µl of the NPP (*p*-Nitrophenyl Phosphate) substrate solution per well with a multichannel pipet and incubate four 1 h at RT.
2. After sufficient color development (yellow color) read the absorbance of each well with a plate reader under 405nm filter.

3.13. Immunofluorescens assay

Immunofluorescene assay (IFA) is the method in which a fluorescent-labelled antibody is used to detect the presence or determine the location of the corresponding antigen and can be visualized under UV-micoscope.

Cell preparation

Vero cells (ATCC No.CCL81) were splited and grown on sterile glass cover slips. When the density was sufficient, the cells were infected with CHIKV. After infection, the cells were harvested by centrifugation and washed with PBS.

Preparation of slides

1. The cells were resuspended in sufficient PBS and 25 µl were placed into the well area on the slide.
2. The specimen was dried thoroughly at RT and it was fixed by fresh acetone at RT for 10 min. Slides were stored at 4^0C for overnight or freezed at -20^0C for longer storage periods.

Procedure

1. The wells of the slide were incubated with 25 µl sera at appropriate dilution in PBS for 90 min. in a moist chamber at 37^0C.
2. Wash two times with PBS without $MgCl_2$ and $CaCl_2$
3. Rinse in PBS-0.1% Tween 20 for 3 min. two times.
4. Wash two times with PBS without $MgCl_2$ and $CaCl_2$
5. Incubate slides with 25 µl FITC labelled anti-human-IgG conjugate dilution 1:40 plus Evens Blue for 45 min. in a moist chamber at 37^0C.
6. Wash two times with PBS without $MgCl_2$ and $CaCl_{2.}$
7. Rinse in PBS-0.1% Tween 20 for 3 min. two times.
8. Wash two times with PBS without $MgCl_2$ and $CaCl_{2.}$
9. Dry and add drop of mounting fluid to the centre of each well and place coverslip over the mounting fluid and ensure that no air bubbles are trapped.

3.14. Western blot assay with CHIKV infected cells

In this method, the anti CHIKV antibodies were used to detect the CHIKV antigens, which were present in infected cells.

Cell preparation

Vero cells were cultured and when the cell density was sufficent, the cells were infected with high titer virus stocks for 36 h. This time point ensured that viral proteins were produced at high levels and the cells were still viable when harvested. Cells were lysed in RIPA-buffer using the Qiagen shredder as described by the manufacturer.

Western blotting

Cellular proteins were subsequently separated by SDS-PAGE and transferred to nitrocellulose. The membranes were blocked and incubated with sera and second antibodies. After washing, the membranes were developed to identify protein by ECL Western blot kit. All this was performed as described above.

4. Results

The aim of this work was to establish a new detection system for CHIKV infection included antibodies. In order to achieve this goal, viral proteins of the CHIK-Würzburg strain (CHIK-Wü) were cloned and expressed in *E.coli*. Based on the case of a CHIKV infected German woman who returned from Mauritius in spring 2006, we isolated the virus and RT-PCR was performed to clone the whole CHIKV genome (Kowalzik et al., 2008, Fig.7). Then these PCR products were cloned into PCRII-Topo and PCR-Topo-XL vectors according to the manufacturer's instruction (Fig.7). From these sub-clones we constructed expression vectors as shown below:

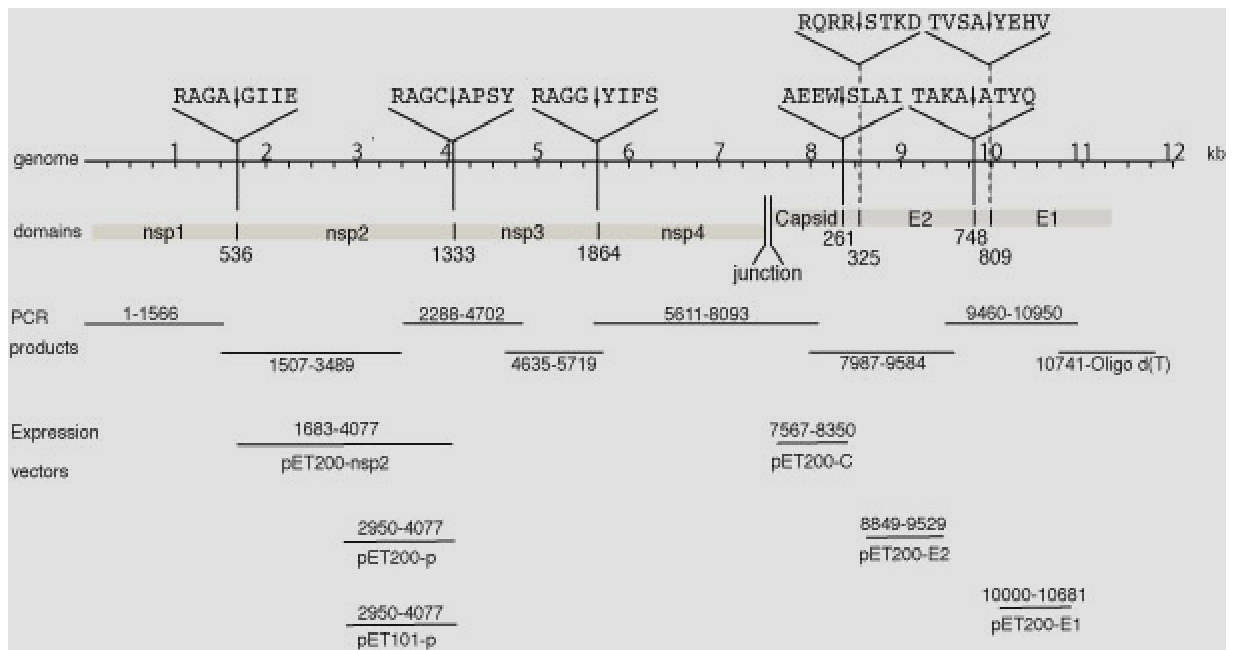


Figure 7: PCR fragments obtained and construction of expression vectors

4.1. Construction of expression vectors

4.1.1. Construction of pET 200 C expression vector.

To express the CHIKV C protein, the C coding region was amplified from CHIK-Wü subclones by PCR. The C of CHIKV is composed of 261 amino acids in length and expressed by 783 bp, which is initiated by a start codon at position 7567-7569. Thus, the C ORF sequence was encoded on two different genomic clones (Fig.7). To construct the pET200/D-TOPO-C expression vector, both PCRII 5611-8093 and PCRII 7987-9584 sub-clones were employed (Fig.7). The PCRII 5611-8093 and the PCRII 7987-9584 vectors were digested with restriction enzymes *ClaI* and *XbaI,* as shown in Figure 8. The 1887 bp fragment of PCRII 5611-8093 (blue) and the rest of PCRII 7987-9584 were isolated using agarose gel electrophoresis and subsequently purified using the QIAquick Gel Extraction Kit according to the manufacturer's instruction. The 1887 bp fragment and the rest of PCRII 7987-9584 were ligated by overnight incubation at 14ºC with T4 DNA ligase. The ligation reaction was subsequently transformed into chemically competent *E. coli* TOP10 cells, plated on LB agar plates containing kanamycin and incubated overnight at 37ºC. The plasmid DNA of the resulting colonies were isolated by the MiniPrep DNA Purification protocol as described above and analyzed by subsequent restriction analysis. The nucleotide sequence was determined by DNA-sequencing. The correct constructs, which contained the Capsid-ORF, were used for further cloning of the expression constructs for the C protein.

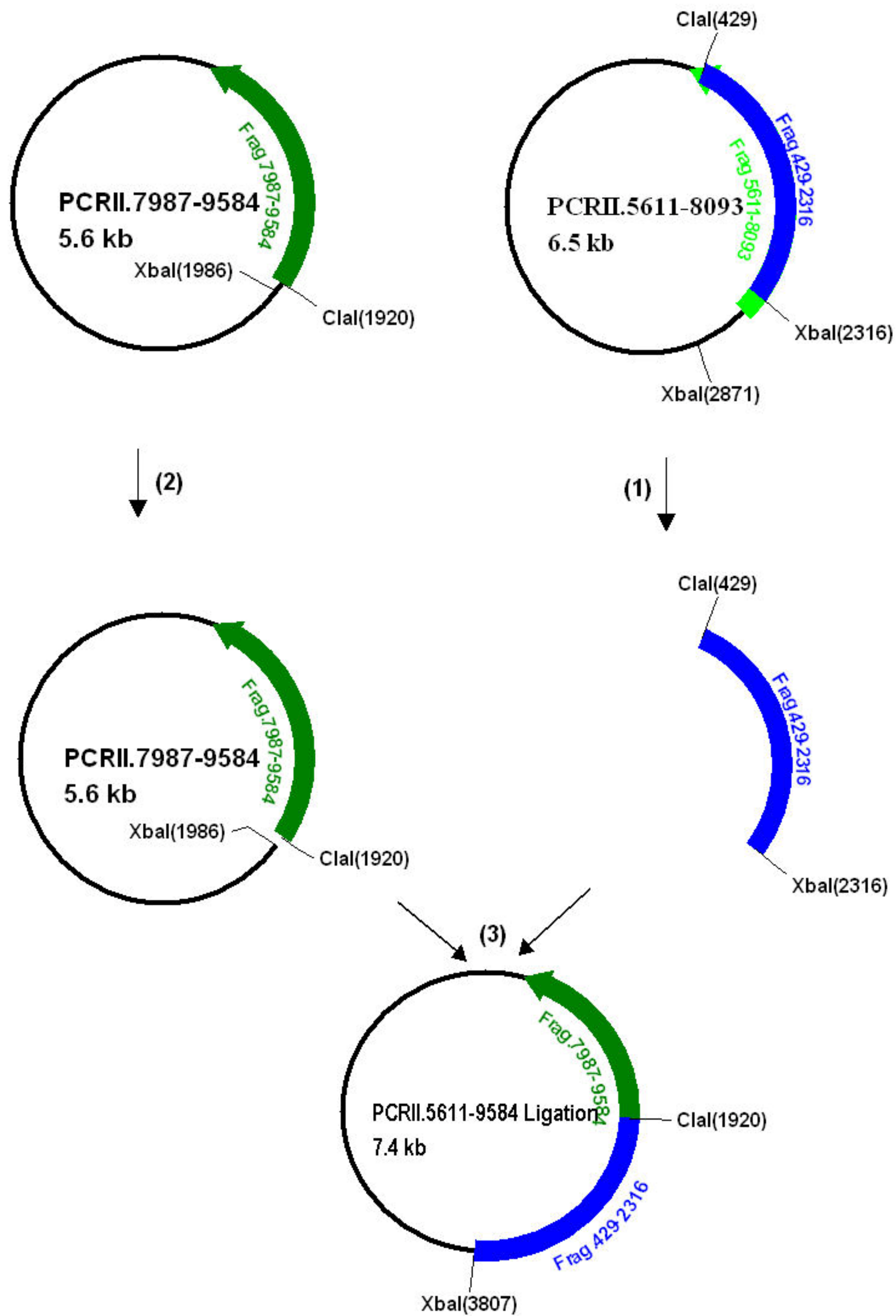


Figure 8: Scheme of the PCRII.5611-9584 cloning strategy. (1): PCRII (5611-8093) was digested with ClaI and XbaI. A fragment (1887 bp - blue color) was used for ligation. The frag.5611-8093 (bright green color) was cloned into PCRII. (2): PCRII

7987-9584 was digested with ClaI and XbaI. The insert (7897-9584 green color) was cloned into PCRII vector. The XbaI-ClaI fragment was removed after digestion. The remaining vector was used for ligation. (3): PCRII 5611-9584 was constructed by ligation of the PCRII 7987-9584 backbone with the fragment 429-2316 derived from PCRII 5611-8093.

For protein expression it was necessary to transfer the coding sequences into a bacterial expression clone. Therefore, the PCRII 5611-9584 was used to amplify the C coding DNA sequence (783 bp) with *Pfu* polymerase and primers Cs and Ca according to the protocol described in the materials and methods. After amplification, the PCR product was analyzed on a 0.8% agarose gel (Fig.9) and purified by using the PureLink quick extraction gel method. The purified PCR product was transformed with the TOPO cloning reaction by using the pET200/D-TOPO vector according to the manufacture's protocol and transformed into *E. coli* TOP10 competent cells. The transformants were plated on LB agar plates containing kanamycin. Colonies were screened by extracting the plasmid DNA using the MiniPrep DNA Purification protocol, as described above and subsequent digesting with *EcoRI*. The DNA was sequenced with primers in both directions. The expression construct was named pET200-TOPO-C.

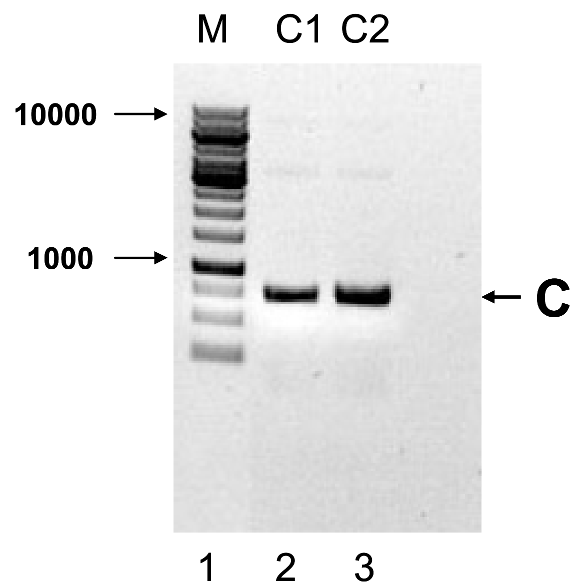


Figure 9: The amplified C DNA fragment (781bp) was analyzed by agarose gel 0.8%. Lane 1: M DNA marker 1kb; Lane 2:C1 and Lane 3: C2 C PCR products.

4.1.2. Construction of pET200 E1 and E2 expression vectors

The CHIKV E1 protein is a post-translational processing product of the structural polyprotein. E1 is encoded by 435 amino acids. It was known from previous studies on alphaviruses that the major immune determinants were located in the E1 and E2 regions. Thus, it was decided to express both proteins in *E.coli* and to use them in Western blot assays.

To construct the pET200 E1 expression vector the PCRII 9460-10950 vector was employed. A part of the E1 sequence 681 bp (nucleotide from 10000 to 10681) of the E1 coding region was amplified using the forward primer E1s and the E1a reverse primers. The primers were designed to ensure that the pET200 vector derived N-terminal 6xHis-tag was fused in-frame to the ORF of the E1 protein. The E1 amplicon (Fig.10) was analyzed on a 0.8% agarose gel and extracted by QIAquick Gel Extraction Kit according to the manufacturer's instruction.

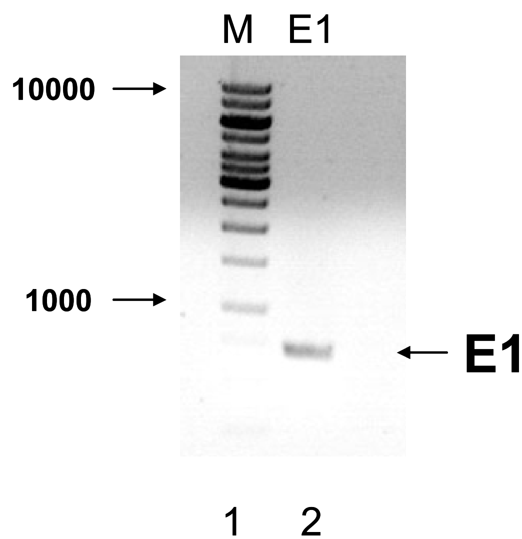


Figure 10: E1 DNA fragment (681bp) was separated by agarose gel 0.8%.
Lane 1: M DNA marker 1kb; Lane 2:E1 PCR product.

The E2 structural protein is cleaved from PE2 during post-translation processing. E2 encompasses AS 261-to-748. In this study we amplified a 681 bp fragment of the E2 coding region from PCRII7987-9584 using the E2s and E2a primers (Fig.7). For the PCR reaction *Pfu* polymerase was used with the protocol as described above (Fig.11). After amplification and purification, both PCR products (E1 and E2) were cloned into the pET200/D-TOPO vector, similarly as described for the cloning of the C PCR product. The expression vectors were named pET200 E1 and pET200 E2 vectors respectively (Fig.12).

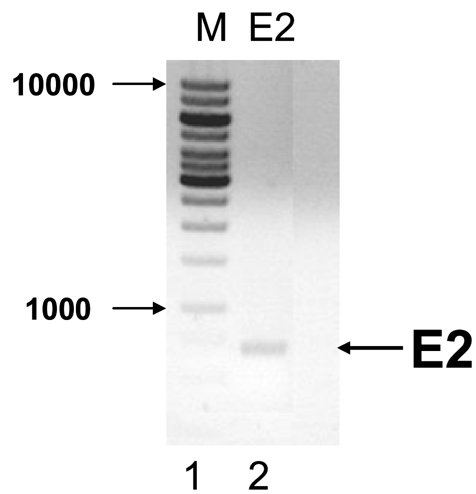


Figure 11: E2 DNA fragment (681bp) was analyzed by 0.8% agarose gel. Lane 1: M DNA marker 1kb; Lane 2:E2 PCR product.

The expression vectors with C, E1 and E2 were cloned into pET200/D-TOPO and can schemetically be depicted as shown in Fig.12.

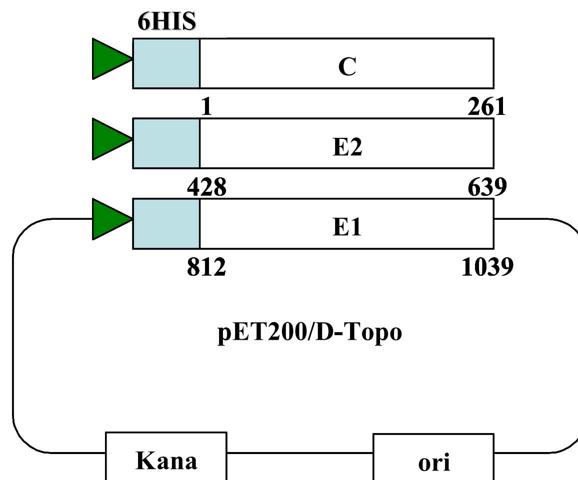


Figure 12: Schemes of pET200/D-TOPO expression vectors with C, E1 and E2 inserts. AA positions are indicated by their position in the CHIKV-Wü nsp-Protein.

4.1.3. Construction of nsp2 and nsp2-protease expression vectors

The non-structural proteins (Nsp) play an important role in the replication of CHIKV. To characterize the NSP2 associated PR activity both, the nsp2 and the nsp2-protease domain (PR), were cloned into pET expression vectors. In order to achieve this, the already described subcloned fragments 3388-4702 and 1507-3489 had to be fused. Therefore, the PCRII 1507-3489 vector was digested with *BglII* and *ScaI* enzymes and the resulting insert of 2919 bp (Fig.13) was excised from a 0.8% agarose gel and purified as described above.

The other vector, PCRII 3388-4702, was also digested with *BglII* to remove a vector fragment of 1038 bp, which was flanked by *BglII* restriction sites (Fig.13). This plasmid was subsequently gelpurified and treated with shrimp alkaline phosphatase to prevent re-circularizing. After purification, both, the rest of the PCRII 3388-4702 (4250 bp) DNA fragment and the 2919 bp DNA fragment, were ligated with T4 DNA ligase to yield a construct, which encompassed nucleotides 1507 to 4702 of the CHIK-Wü sequence. The plasmid was named PCRII 1507-4702. The construct was transformed into TOP10 competent cells as described above. The transformants were screened by restriction analysis and positive clones were verified by DNA sequencing.

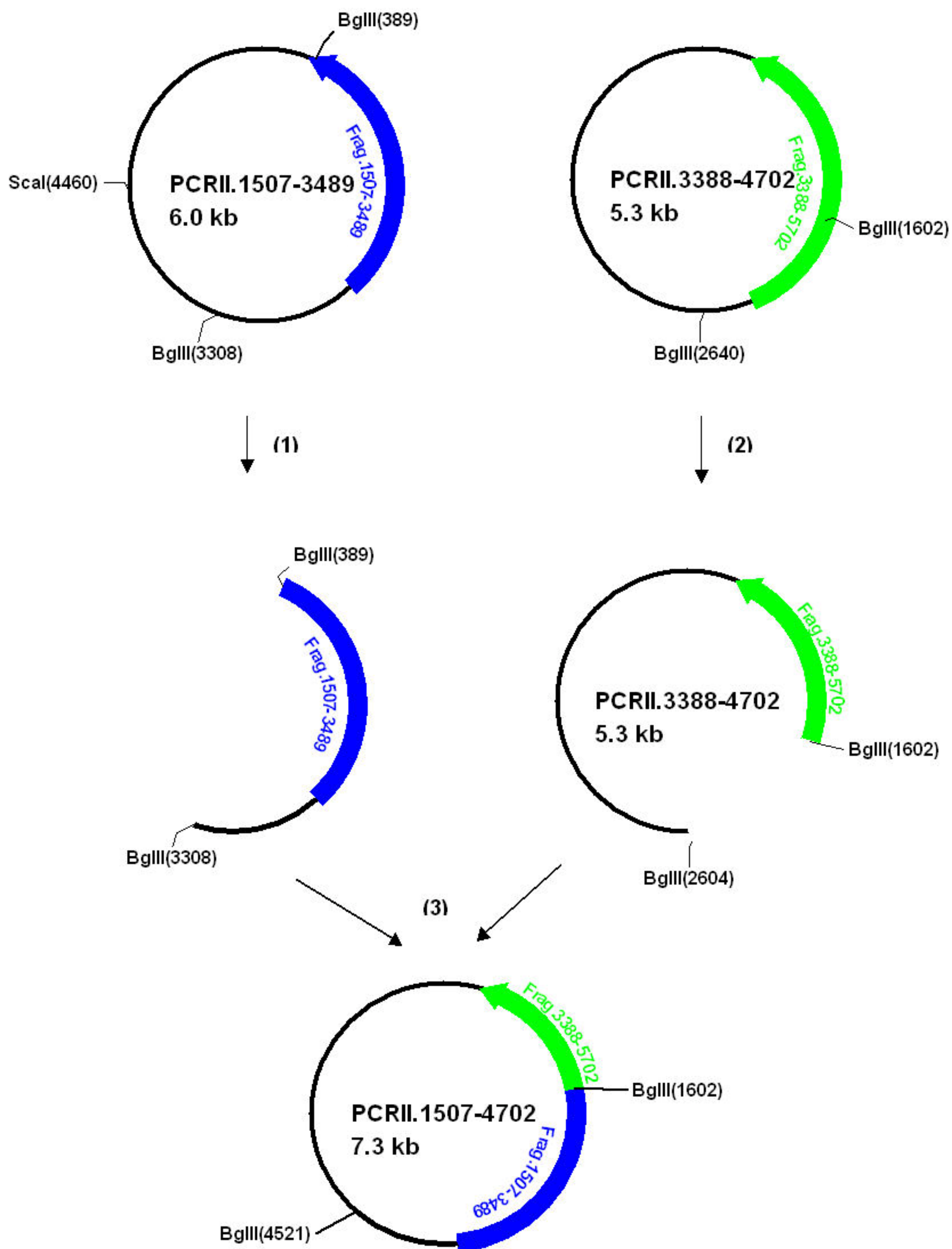


Figure 13: Scheme of the PCRII.1507-4702 cloning strategy. Both, PCRII 1507-3489 and PCRII 3388-4702 plasmids, were digested with BglII and ScaI. The indicated BglII fragments (2919 bp and 4250 bp) were ligated. The resulting clone encompasses nucleotides 1507-4702.

The resulting PCRII 1507-4702 plasmid (Fig.13) was used as a template to amplify the nsp2 fragment by PCR using the primers NSP2s and NSP2a. The amplicon (Fig.14) was purified and cloned into pET200/D-TOPO.

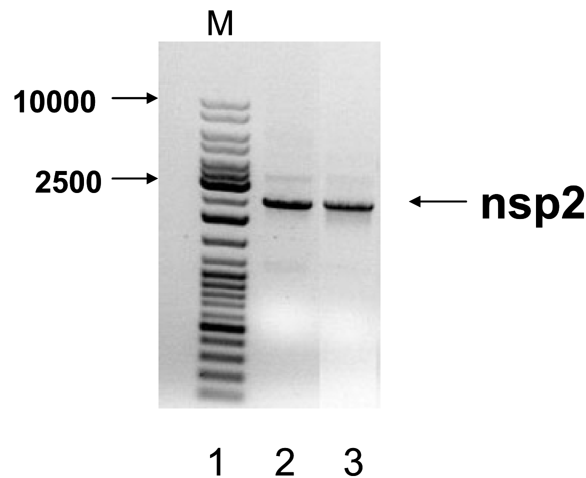


Figure 14: Nsp2 DNA fragment (2394bp) was analysed by 0.8% agarose gel. Lane 1: M DNA marker 1kb; Lane 2 and 3 nsp2 PCR products.

To further characterize the PR-domain, the PR coding sequence was amplified from the PCRII 1507-4702 with primers PRs and NSP2a and cloned into pET-200 as well. In both clones an artificial stop codon at the PR-cleavage site between nsp2 and nsp3 (Kowalzik et al., 2008) was introduced by the NSP2a primer. In addition, a PR clone with a carboxy-terminal HIS-tag was prepared by amplifying the PR-coding region with primers PRs and PSa-w/o-stop. The usage of this anti-sense oligo resulted in a clone, which was in-frame with the vector-encoded carboxy-terminal HIS-tag. The resulting PCR-fragment was gel purified and cloned into pET101/D-TOPO vector as described above. The correct clones were identified by restriction digest and verified by DNA-sequencing.

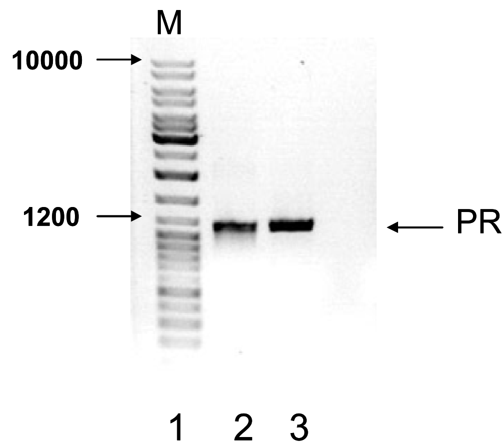


Figure 15: Protease DNA fragments (1024bp) was analyzed by 0.8% agarose gel. Lane 1: M DNA marker 1kb; Lane 2 and 3 protease PCR products.

These plasmids are depicted as follows (Fig.10).

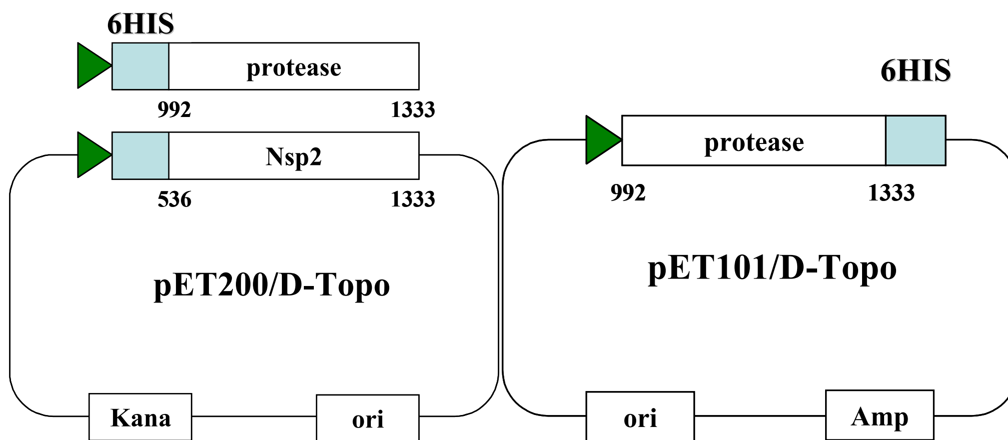


Figure 16: Scheme of nsp2 and nsp2-PR clones in pET200/D-TOPO and pET101/D-TOPO. AA positions are indicated by their position in the CHIKV-Wü nsp-Protein.

4.2. Protein expression and purification of the C, E1 and E2 proteins

4.2.1. The C protein

4.2.1.1. Pilot expression

To express the structural proteins C, E2 and E1 the coding sequences were cloned into pET-vectors as described above. The vectorderived protein expression is directed by the T7 promoter. Therefore, BL21-DE-star competent cells, which express the T7 RNA-polymerase under the control of an IPTG inducible lacUV5 promoter (Dubendorff and Studier, 1991; Studier et al.,1990), were transformed with the expression plasmids. In addition, this bacterial strain carries a mutation in the *RNase E* gene, which improves RNA stability (Grunberg-Manago, 1999; Lopez et al., 1999). To optimize protein stability and solubility, induction times were varied in pilot-experiments. Therefore, several colonies were grown as over-night cultures in small-scale volumes and diluted at the next day 1:20 to grow the bacteria in the exponentional phase, at the time of induction. The protein expression was induced by adding IPTG to 1mM final concentration for 2 and 4 h. After induction, bacteria were collected by centrifugation for 1 min at 13000 rpm and analyzed by coomassie blue stained 12% PAGE and Western blot analyses. For the latter, gels were blotted onto a nitrocellulose membrane. HIS-tagged proteins were detected with a monoclonal anti-HIS antibody at a dilution of 1:1000 and by employing the ECL detection system.
The calculated molecular mass of the fused C protein is approximately 35 kDa. E1 and E2 protein fused with His-tagged protein are slightly smaller with a calculated molecular weight of approximately 30 kDa for E2 and 28 kDa for E1 respectively. The C protein could already be detected after 2h of induction in Commassie stained PAGE as a 35kDa protein (Fig.17: lanes 3, 4, 5, 6). The identity of this band was verified in Western blotting (Fig.18: lanes 2, 3, 4, 5). The proteins were neither detectable prior induction by coomassie stain PAGE nor by immuno-blotting.

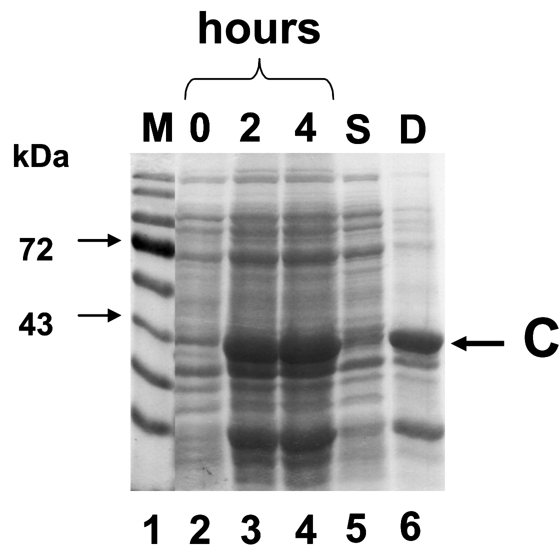


Figure 17: Analysis of the recombinant C protein expression by coomassie blue stained PAGE. The time of IPTG induction is indicated above the lanes. Lane 1: protein marker. Lane 2: prior induction; lane 3-4: samples after 2, 4 h of expression, respectively. Protein solubility was analyzed at 4 h of induction (lanes 5: soluble and lane 6: insoluble).

The optimal induction time for HIS-tagged Capsid protein expression was found to be 4 h. Both coomassie blue stained PAGE and Western blot analysis demonstrated that the amount of the HIS-tagged C protein increased during the induction period from 2 to 4 h. To analyze the protein solubility, bacterial pellets were lysed by sonification for 4 times 30 s in native lysis buffer. The lysates were centrifuged for 5 min at 13000 rpm to pellet the insoluble faction. Most of the HIS-tagged C protein was observed in the cellular debris as an insoluble pellet (Fig. 11 and 12).

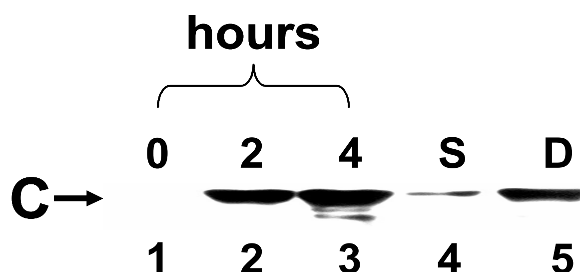
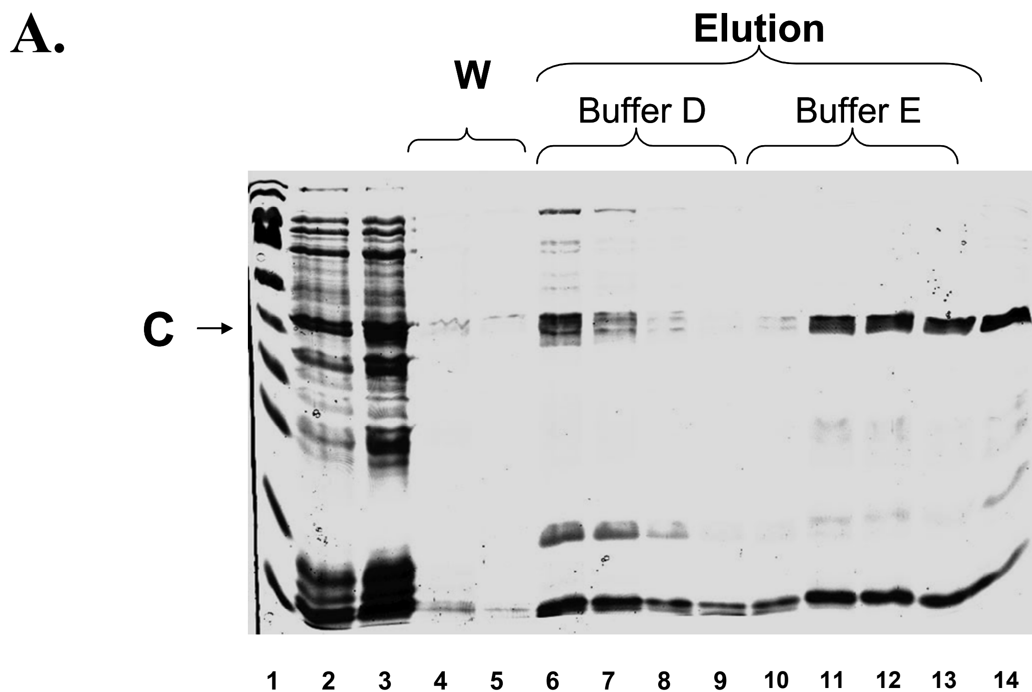


Figure 18: Western blot analysis of the recombinant C protein using anti-His-tag antibody for detection. The concentration of recombinant protein increased from 2 h to 4 h, but was shown to be insoluble under these conditions (lane 5).

These pilot expression experiments demonstrated that the optimal induction time of 4 h resulted in an insoluble protein. Therefore, this protein was expressed and purified under denaturing conditions.

4.2.1.2. Purification of the recombinant C protein

For the desired diagnostic test lager amounts of purified recombinant C protein was required. Therefore, the recombinant C protein was expressed under up-scaled conditions established in the pilot experiments. 50 ml of a starter-cultures were incubated O/N at 37° C. At the next day the culture was diluted in fresh LB-Kana^{+}-medium and grown up to an OD_{600} of 0.6. Protein expression was induced by adding IPTG to a final concentration of 1 mM. The bacteria were harvested after 4 h of induction and lysed using 40 ml denatured lysis buffer B and sonificating on ice. The lysis reaction was incubated for 30 min at RT under constant steering, followed by centrifugation for 30 min at 13000 rpm in the SS34 rotor to remove the insoluble cellular debris. The His-tagged C was subsequently purified by affinity chromatography using 0.7 ml Ni-NTA-Agarose beads. The matrix was washed two-times with 4 ml buffer C and bound proteins were eluted four times with 0.5 ml buffer D and buffer E. All fractions were analysed by Coomassie stained SDS-PAGE (Fig.19A) and by Western blot analysis using monoclonal anti-HIS-antibodies (Fig.19B). Both assays showed a prominent protein band of the expected size. The protein started to elute in the first two fractions with buffer D. Notably, the recombinant C protein was not present in the wash fractions (Fig.19B lanes 3 and 4) prior to the elution. The main protein peak was observed in the fraction eluted with buffer E (Fig.19). In addition, the Coosmassie stained gel showed only one other prominent contaminating bacterial protein band in the fractions eluted with buffer E.



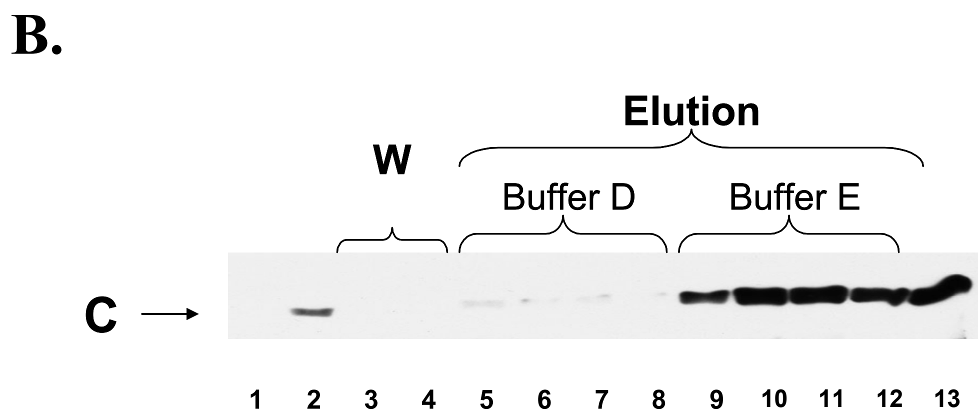
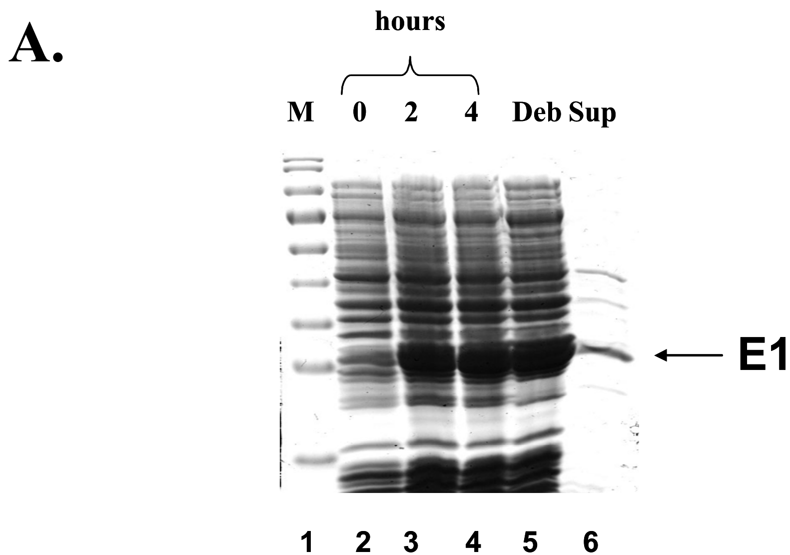


Figure 19: **(A)** Analysis of recombinant C protein purification by coomassi blue stained PAGE. Bacteria were lysed and applied to the gel prior and after induction. Wash fraction (lane 4-5) and elution fraction (buffer D: lanes 6-10; buffer E lanes 11-14) were analysed. The arrow indicates the C protein. **(B)** Western blot analysis of the same fractions using a monoclonal His-tagged antibody diluted 1:10000 in PBS.

4.2.2. Expression and purification of E1 and E2 proteins

4.2.2.1. Pilot expression

In order to identify the optimal expression conditions for E1 and E2 recombinant proteins, both pET200-E1 and pET200-E2 expression vectors were used in pilot expression experiments. The BL21-DE3-Star competent *E.coli* was transformed with expression vectors. Overnight cultures were diluted 1:20 and grown for additional 2 h at 37^{0}C so that the bacteria cultures reached the log-phase. Protein expression was induced by adding IPTG to a final concentration of 1mM. To determine the optimal expression time, the bacteria were harvested after 2 and 4 h of induction. Bacteria were collected by centrifugation at 13000 rpm for 1 min at RT. The cell pellets were lyzed with native lysis buffer and sonificated 4 times for 10 s on ice. The lysates were centrifuged to separate the soluble from insoluble proteins and both fractions were analysed by SDS-PAGE. The molecular weights of the recombinant E1 and E2 proteins were calculated with approximately 28 kDa and 30 kDa, respectively. Both Coomassie-stained SDS-PAGE and Western blot analysis showed that the E1 and E2 proteins were expressed upon induction (Fig.20 and 21). Without IPTG no recombinant protein was expressed (Fig.20A and 21A lane 2; Fig.20B and 21B lane 1). After induction with IPTG for 2 and 4 h a specific band at 28 kDa (Fig.20) or 30 kDa (Fig.21) was observed. The Western blot analysis for both E1 and E2 revealed the specificity of these purified proteins. The determination of the solubility showed that both proteins were found in the insoluble fraction. Hence, we decided to express both proteins under denaturing conditions.



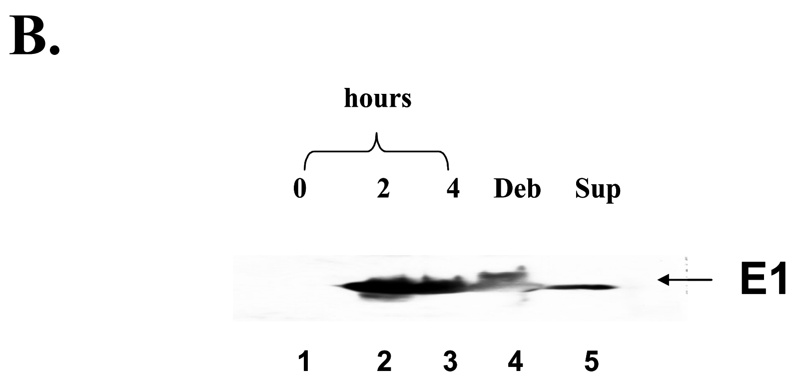


Figure 20: **(A)** Coomassie blue stained PAGE of E1 test expression. Lane 2 shows sample taken prior to induction, whereas lanes 3-4 show samples after 2 and 4h of expression, respectively. Protein solubility at 4h of induction was low (lanes 5 and 6). Marker: lane 1**. (B)** Western blot analysis of the recombinant E1 using anti-His-tagged antibody for detection at a dilution of 1: 1000.

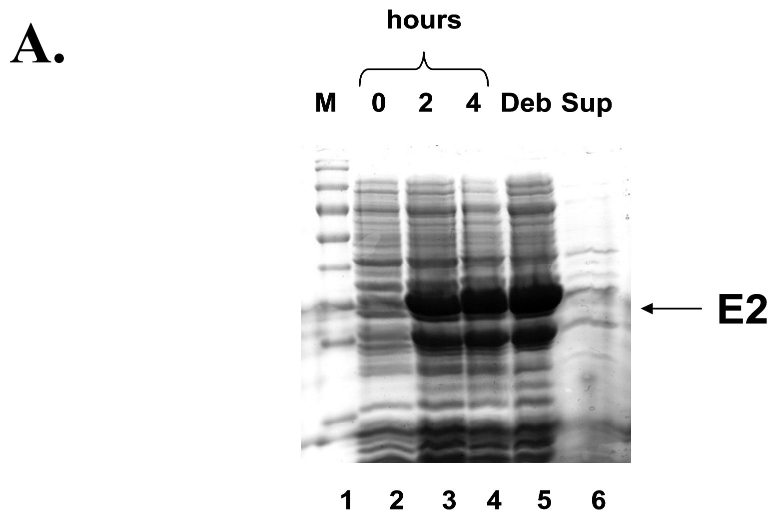


Figure 21: **(A)** Coomassie blue stained PAGE of a pilot expression of the recombinant E2 protein. Lane 2 shows a sample taken prior to induction, whereas lanes 3-4 show samples after 2 and 4 h of expression, respectively. Protein solubility at 4 h of induction was low (lanes 5 and 6). **(B)** Western blot analysis of the recombinant E2 using anti-His-tagged antibody for detection.

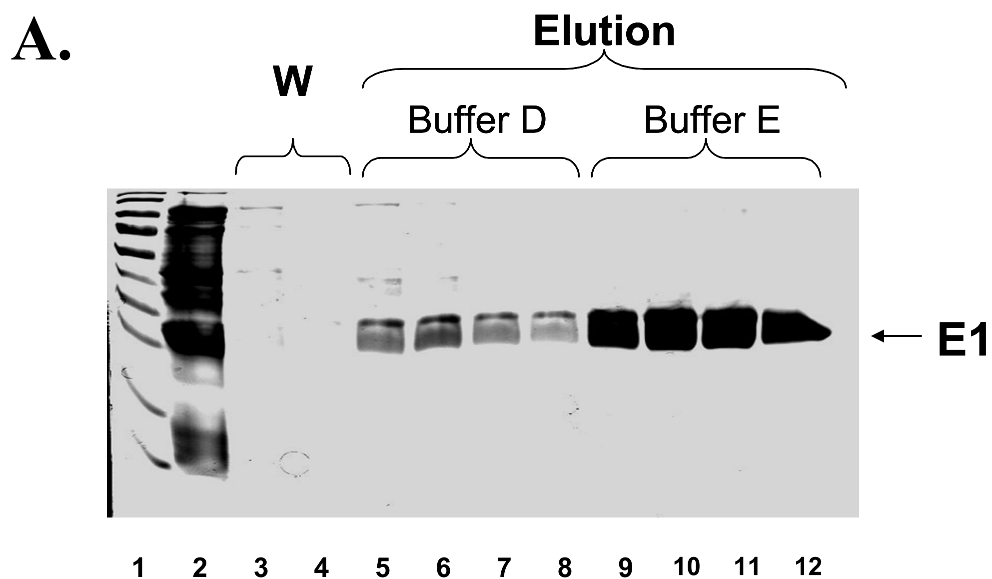
4.2.2.2. Purification of the recombinant E1 and E2 proteins

Having established the conditions for E-protein expression, we attempted to purify both proteins from the *E.coli* lysates. The expression and the purification were essentially

performed as described before. In brief: Both expression vectors, pET200-E1 and pET200-E2, were transformed into competent BL21-DE3-Star cells. The expression was induced by adding IPTG to a final concentration of 1mM for 4 h at 37^0C. The cultures were harvested by centrifugation and the pellets were lysed by resuspension of the bacterial pellet with denatured buffer B and sonificating on ice. The insoluble debris was removed by centrifugation at 14000 rpm at 4^0 C using the SS34 rotor.

To purify the HIS-tagged proteins affinity chromatography was used. The soluble fraction was incubated with Ni-NTA-agarose beads for 1h at RT. The unbound proteins were removed by several washes with ten-time column volume with buffer C. The proteins were eluted four times with buffer D and E. The amount and the purity of the obtained proteins were analysed by Coomassie stained SDS-PAGE (Fig.22A and 23A). The identity of the purified proteins was confirmed by Western blotting with the HIS-antibodies. Most of the E1 and E2 proteins were eluted with buffer E (Fig.22B and 23B). Both proteins were pure and fractions 6 to 12 and 6 to 10 belonged to E1 and E2, respectively, contained no visible contaminations in the Coomassie stained gel (Fig.22A and 23A). The immuno-blotting verified the protein identity (Fig.22B and 23B). The concentration of all eluted fractions was determined by the Bradford assay.

A.



B.

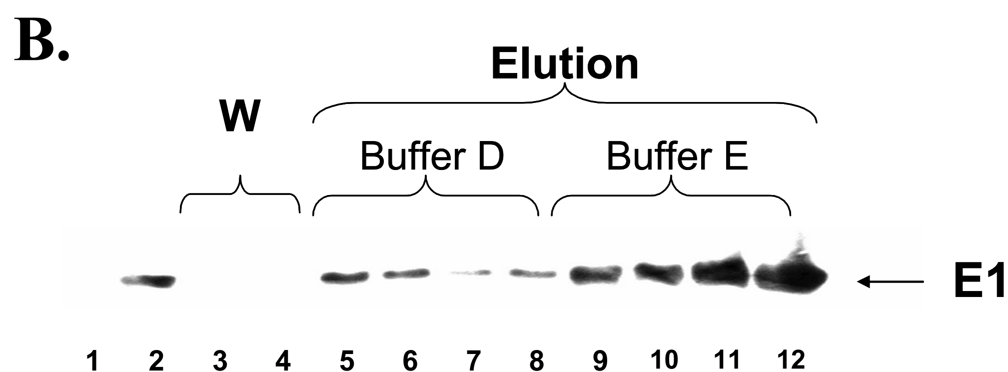
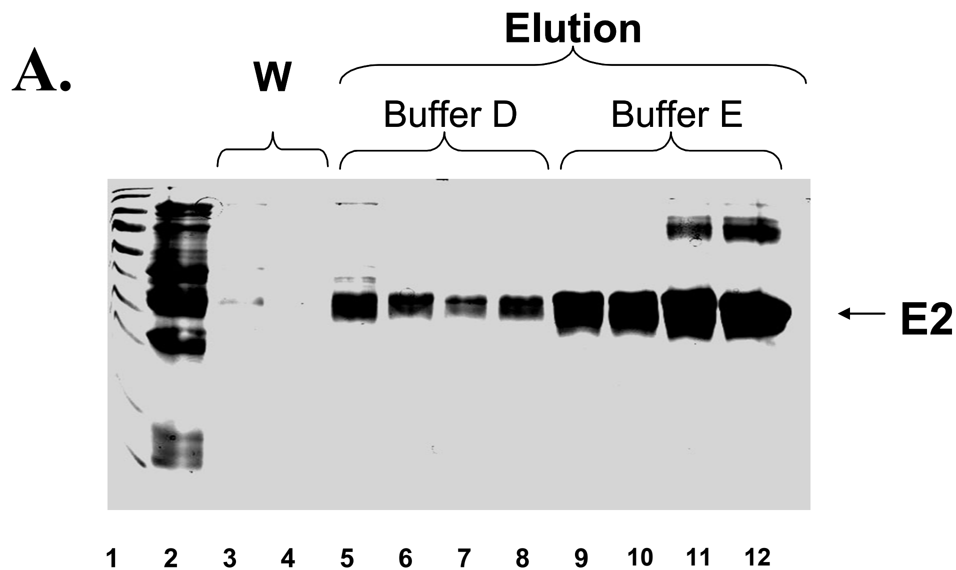


Figure 22: (A) Analysis of recombinant E1 protein purification by 12% SDS-PAGE. Whole cell lysates after induction (lane 2), wash fractions (lanes 3-4), elution fractions (buffer D: lanes 5-8, buffer E:lanes 9-12). (B) Western blot analysis using monoclonal anti-His-tag antibody at a dilution of 1:10000 in PBS. Analysis of total bacterial proteins after induction (lane 2); wash fractions (lanes 3-4), elution fractions (buffer D: lanes 5-8, buffer E:lanes 9-12).

A.



B.

Figure 23: **(A)** Analysis of the recombinant E2 protein purification. Whole bacterial lysates after induction (lane 2). Wash fractions (lanes 3-4), elution fractions (buffer D: lanes 5-8, buffer E: lanes 9-12). **(B)** Western blot analysis using monoclonal anti-His-tagged antibody at a dilution of 1:10000 in PBS. Analysis of total bacterial protein after induction (lane 2); wash fractions (lanes 3-4), elution fractions (buffer D: lanes 5-8, buffer E: lanes 9-12).

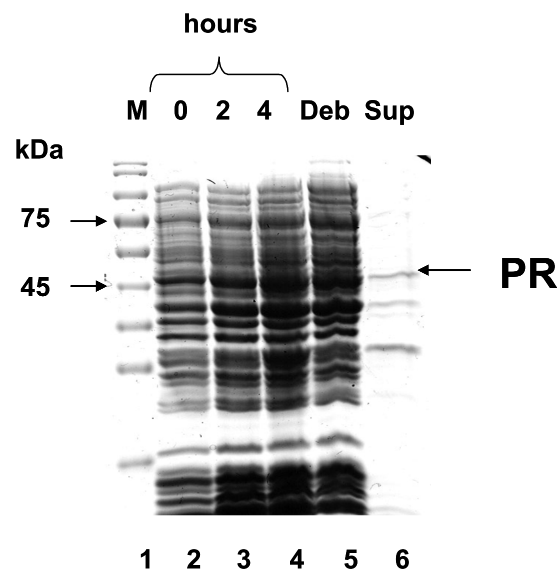
4.2.3. The Protease protein

4.2.3.1. Pilot protease expression

In the first set of experiments, the Nsp2 protein was expressed in *E. coli*, for we expected to see the self processing of the PR and to use this as marker for PR activity. However, in several attempts with a wide variety of expression conditions we failed to obtain significant amounts of the recombinant His-tagged protein. Even the expression in different *E.coli* strains did not result in a detectable protein amounts.

Therefore, we decided to express the CHIKV nsp-protease alone. Competent *E.coli* were transformed with the expression plasmids for the PR fused amino- (pET200D-PR) and caboxy-terminaly to the His-Tag (pET101/D-PR). The cultures were split at the next day and subsequently grown to an OD_{600} = 0.6. Then protein expression was induced by adding IPTG to a final concentration of 1mM. Bacteria were harvested by centrifugation at different time points (0, 2 and 4 h) and analyzed for protein expression by SDS-PAGE and Western blotting (Fig.24 and 25).

A.



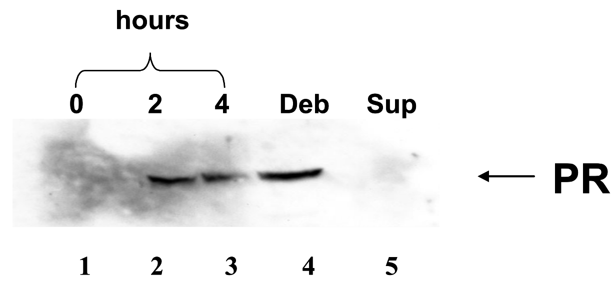
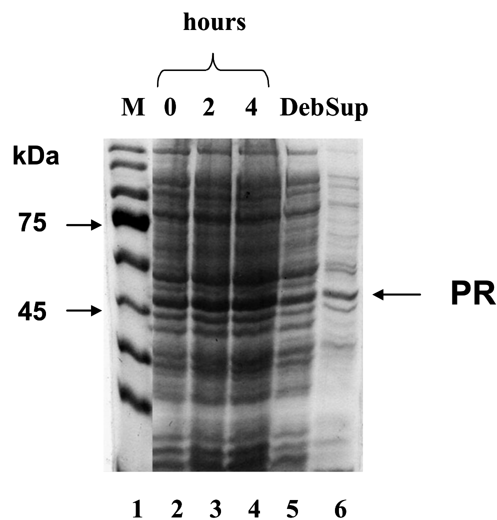


Figure 24: **(A)** Analysis of nsp2-PR expression by pET101-PR transformed *E.coli* and protein solubility by coomassie blue stained PAGE. Sample prior induction (lane 2), samples collected after 2 and 4 h of expression, respectively (lanes 3 and 4), insoluble and soluble proteins at 4 h of induction (lanes 5 and 6). **(B)** Western blot analysis with anti-His-Tagged antibodies. Sample prior induction (lane 1), samples collected after 2 and 4 h of expression, respectively (lane 2-3), insoluble and soluble proteins at 4 h of induction (lane 5 and 6).



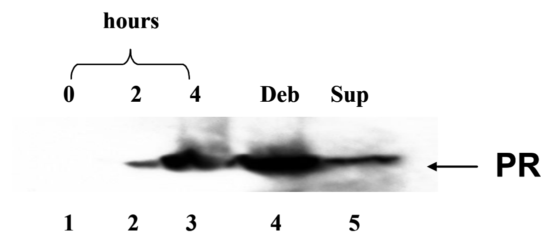


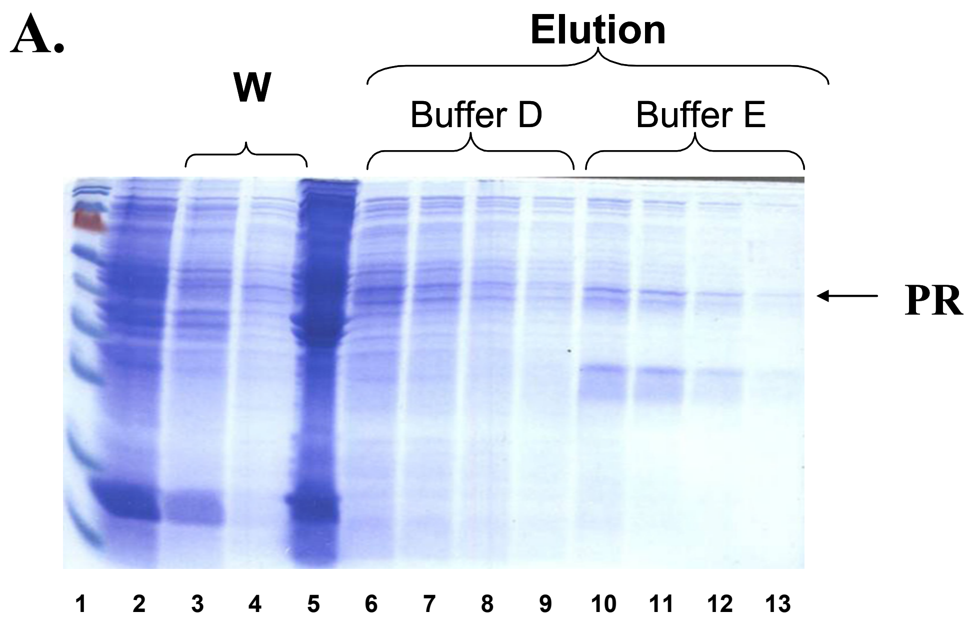
Figure 25: **(A)** Analysis of nsp2-PR expression by pET200-PR transformed *E.coli* and protein solubility by coomassie blue stained PAGE. Sample prior induction (lane 2), samples collected after 2 and 4h of expression, respectively (lanes 3-4), insoluble and soluble proteins at 4 h of induction (lanes 5 and 6). **(B)** Western blot analysis with anti-His-Tagged antibodies. Sample prior induction (lane 1), samples collected after 2 and 4 h of expression, respectively (lane 2-3), insoluble and soluble proteins at 4 h of induction (lane 4 and 5).

Only the Western blot analyses showed a significant protein expression for both constructs, which indicated that the expression levels remained low. The protein amount obtained with the aminoterminaly HIS-Tag PR appeared to be higher compared to the caboxyterminal-tagged PR. Furthermore, the PRs of both constructs were expressed as insoluble proteins and only a very limited amount could be detected in the supernatant (Fig.24 and 25).

4.2.3.2. Protease expression

Based on the established and optimized pilot expression conditions, CHIKV PR was expressed and purified under denatured conditions. After induction by IPTG for expression, bacteria were incubated for 4h at 37^0 C and subsequently collected by centrifugation. The bacteria were lysed as described above for the C, E1 and E2 proteins. The cleared lysates were incubated for 1 h at RT with Ni-NTA beads on a rotator. Beads were washed with 10 times beads volume of buffer B and the bound proteins were eluted with buffer D and E. The concentrations of eluted fractions were determined by the Bradford assay and protein-containing fractions were analysed by both, Coomassie-stained SDS-PAGE and Western blot analysis using a monoclonal anti-His antibody (Fig.26 and 27). The expression of PR in pET200 vector seemed to be better than that in pET101.

A.



B.

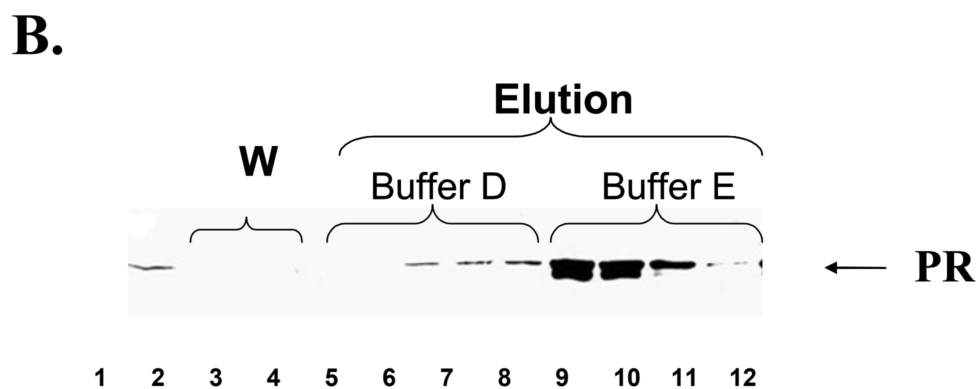


Figure 26: **(A)** Purification of recombinant carboxyterminal His-tagged PR. Whole bacterial lysates after induction (lane 2). Wash fractions (lanes 3-4), elution fractions (buffer D: lanes 6-9, buffer E: 10-13). **(B)** Western blot analysis using monoclonal anti-His-Tagged antibody at a dilution of 1:10000 in PBS. Analysis of total bacterial proteins after induction (lane 2); wash fractions (lanes 3-4), elution fractions (buffer D: lanes 5-8, buffer E: lanes 9-12).

In the Western blot analysis, PR protein expressed by both vectors was detected with monoclonal anti-His-tagged antibodies. These blots showed that the majority of the protease protein eluted with buffer E (Fig 27). The Coomassie stained PAGE indicated that the protease was pure and that significant amounts of PR protein were obtained.

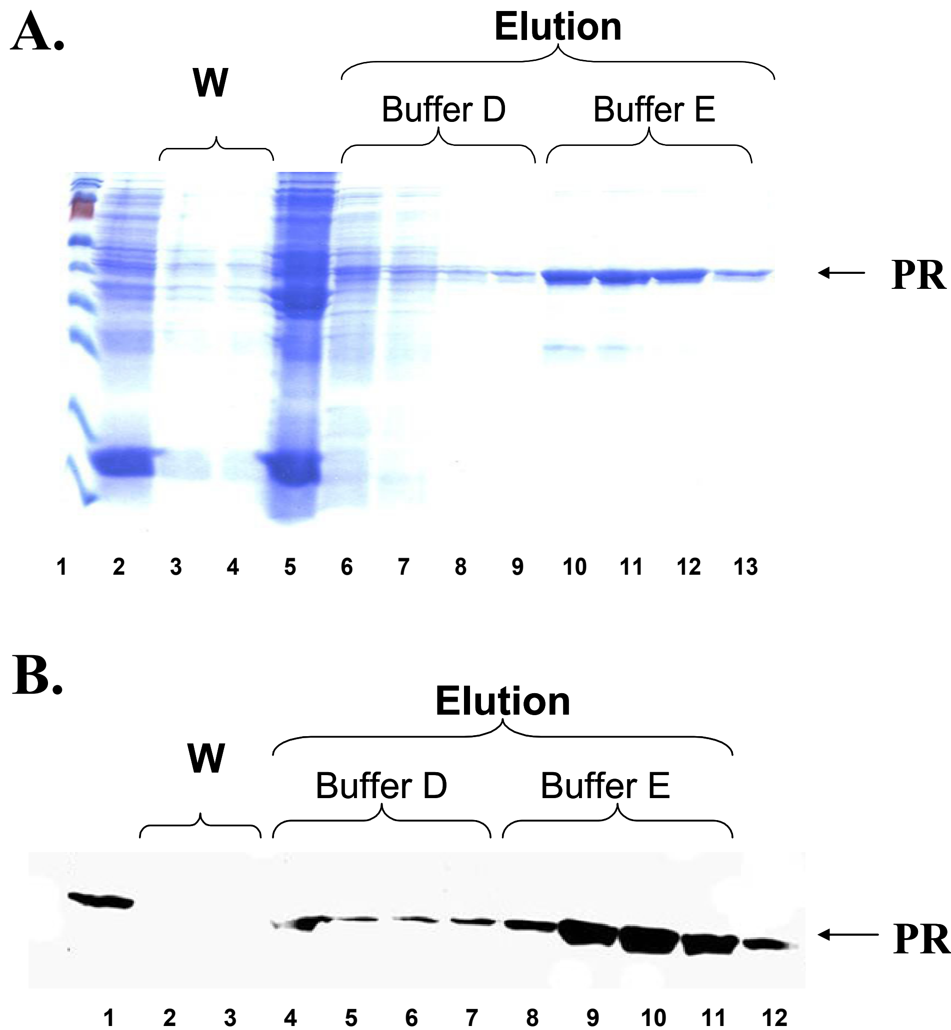


Figure 27: **(A)** Purification of recombinant aminoterminal HIS-tagged PR. Whole bacterial lysates after induction (lane 2). Wash fractions (lanes 3-4), elution fractions (buffer D: lanes 6-9, buffer E: lanes 10-13). **(B)** Western blot analysis using monoclonal anti-His-Tagged antibody at a dilution of 1:10000 in PBS. Total bacterial protein after induction (lane 1); wash fractions (lanes 2-3), elution fractions (buffer D: lanes 4-7, buffer E: lanes 8-11).

To improve purification, the bacterial lysates of both the amino- or carboxy-terminal His-tagged PRs were affinity purified using the AKTA FPLC system. To remove insoluble material the lysates were centrifuged and filtered using 45µm sterile filters cartridges. The flow rate was set to 5 ml/min. The Ni-NTA Superflow columns were pre-equilibrated with 10 column volumes (CV) buffer B. The proteins lysates were loaded via a 50ml "superloop" at a reduced flow-rate of 3.4 ml/min. The unbound proteins were removed by a washing step with 10 CV buffer B. Unspecific bound proteins were eluted with a step gradient of 30% buffer B and 70% buffer E. The specific bound proteins were eluted by linear gradient from 70% to 100 % buffer E with 20 CV. Finally a step of 10 CV buffer E was used to elute the proteins completely. The protein containing fractions were analyzed by SDS-PAGE. Both, the amino- and the carboxy-terminal His-Tagged PRs, eluted at a buffer E concentration of 100 % and their conditions. The amount of the carboxy-terminal PR seamed to be higher concentration and purer than those of the amino-terminal PR (Fig.28).

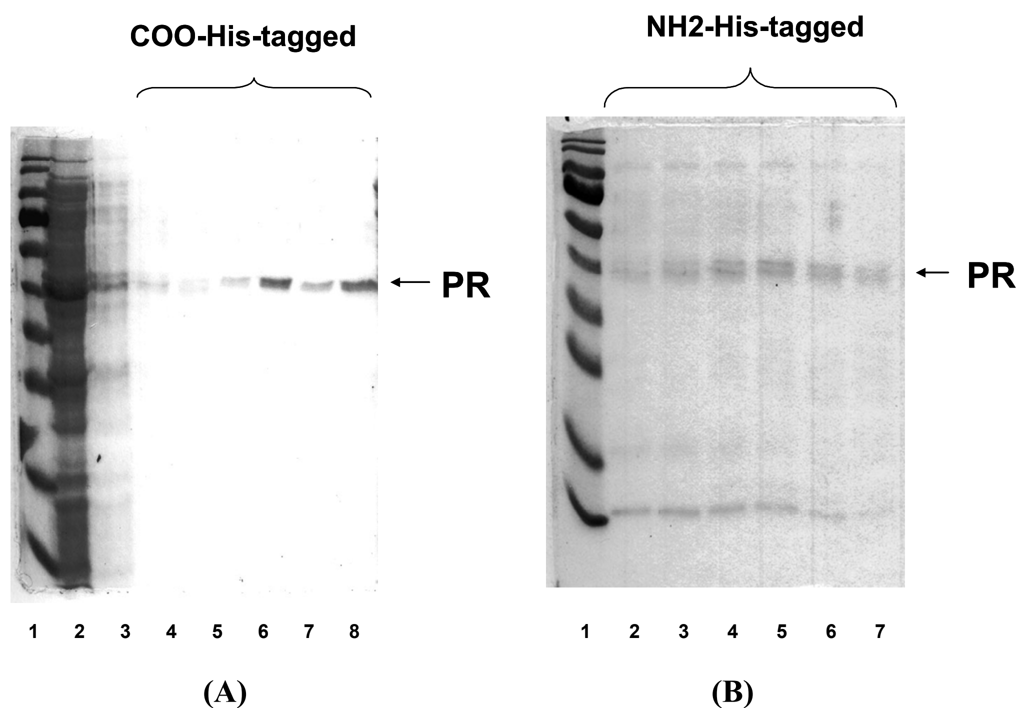


Figure 28: (A) Purification of COO^- -His-tagged proteases by FPLC system. Lane1: Protein marker. Lane 2: the debris cells. Lane 3-8: Elution fractions after purification.
(B) Purification of NH2-His-tagged proteases by FPLC system. Lane1: Protein marker. Lane 2-7: Elution fractions after purification.

4.3. Serologic testing

4.3.1. Establishment of an immunoblot assay with patient derived sera.

Previous reports showed that the structural proteins play important roles in inducing an antibody response of the host to CHIKV. The E2 glycoprotein is responsible for receptor binding. Therefore, most neutralizing antibodies recognize epitopes in E2 rather than in E1 (Strauss and Strauss, 1994). Thus, the recombinant structural E1 and E2 proteins were used to screen for reactive antibodies in CHIKV positive patients sera by immunoblotting.

To test whether sera from patients reacted positive with the purified CHIKV structural proteins, 11µg of the purified proteins were separated by SDS-PAGE and then transferred to nitrocellulose membrane. The proteins were detected by incubation with 30 patient derived sera, which contained CHIKV antibodies and 100 German control sera from healthy German blood donors. The bound antibodies were detected by anti-IgG antibodies, labelled with an HRP using the ECL Western blot kit.

The sera of positive patients and blood donors were treated with Kaolin (1:3) to remove unspecific lipoproteins. An example is shown in Fig.30. Sera were initially screened at a dilution of 1:700 in PBS/milk, which revealed that 13% of the patient derived samples showed IgG antibodies reactive against E1, whereas 43.3% and 50 % of the sera detected C or E2, respectively (Tab.1). None of the control samples reacted positive. Thus, while E2 appeared to be the virus protein recognized by most of the positive sera (50%), our results indicated that only a mixture of at least E2 and C proteins was able to detect the majority of human anti-CHIKV antibody-positive serum samples (Fig.29).

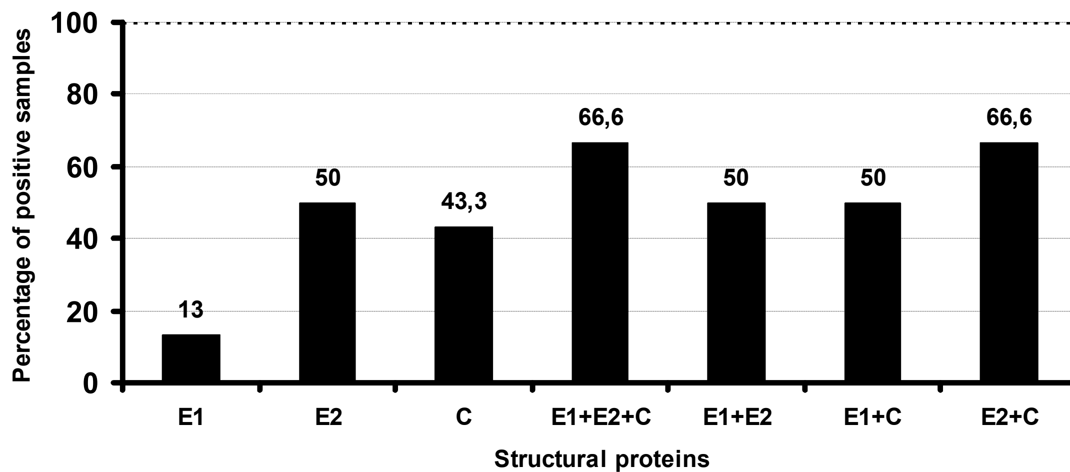


Figure 29: Evaluation of the Western blot assay with predefined IFA positive sera. The numbers indicate the percentage of positive sero-reactivity against recombinant CHIKV structural proteins.

To enhance the sensitivity of the assay we also tested sera at a dilution of 1:100 in PBS/milk. This improved the sensitivity for just two samples, which now reacted positive with E1 or E2 (Tab.1). However, approximately a quarter of the samples (7 sera), which were scored positive in IFA, remained negative in the immunoblot assay (Tab.1). Even serum No.17 that was tested positive by IFA with a titre of 1:2,560 was found repeatedly negative by immunoblotting.

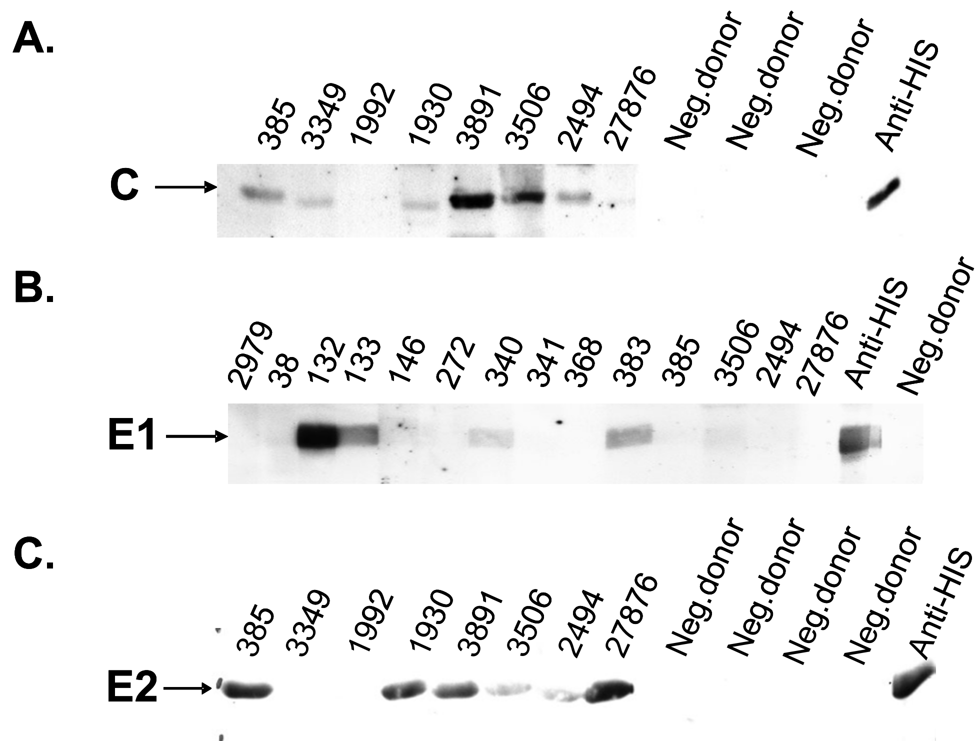


Figure 30: Typical example of an immunoblot analysis of human sera (dilution 1:700) with recombinant structural CHIKV proteins. Anti-His-tagged MAB and donor blood as positive and negative controls. Analyse with recombinant C (A), E1 (B) and E2 (C) antigens. ECL-Western blot was used for detection.

4.3.2. Analysis of anti-CHIK reactivity in serum samples from healthy Vietnamese blood donors

To test the potential of our Western blot analysis and to estimate the percentage of CHIKV infections in the Vietnamese population, 158 sera samples were collected from healthy blood donors living and working in Hanoi, Vietnam. Sera were pre-treated with Kaolin (1:3) as described before and screened with recombinant structural proteins at a dilution of 1:700 in PBS/milk.

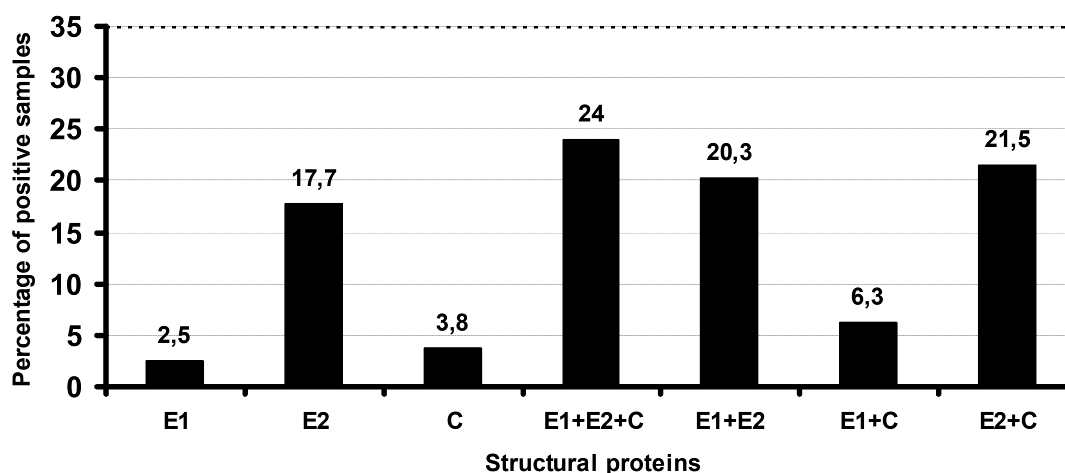


Figure 31: Analysis of the reactivity of Vietnamese sera against CHIKV structural proteins in the Western blot analysis. Sera were diluted 1:700 and ECL-Western blot kit was used for detection.

The results showed that the percentage of samples reacting IgG positive with E1 protein was 4 of 158 (2.5%) (Fig.31). The assay revealed that more sera showed reactivity against the C (3.8%) or E2 protein (17.7%). The analysis with a sera dilution of 1:100 increased the number of positive sera for E1 to 4%, for C to 12% and for E2 to 31%.

4.3.3. Development of an anti-CHICK antibody ELISA

The described recombinant CHIKV structural proteins were used to establish an anti-CHIK antibody ELISA assay. For indirect IgG ELISA, the ELISA plates were coated with diluted CHIKV structural proteins (11 µg/well of E1, E2 or C) in PBS overnight at 4^0C. The reactivity against the recombinant proteins was analysed with all patient derived samples described above. In brief: Sera were first pre-treated with Kaolin (1:3) to remove unspecific proteins and then incubated at a dilution of 1:100 in PBS/milk for 2 h at RT. After 3 times washing with washing buffer, alkaline phosphatase conjugated mouse anti-human IgG antibodies were diluted in PBS and incubated with for 2 h at RT to bind to anti-CHIK IgGs. The alkaline phosphatase was detected by colour reaction using NPP (*p*-nitrophenyl phosphate) substrate. Serum reactivity was considered positive, if the obtained OD_{405} was higher than 0.1± 0.01 the negative control. In a first

experiment the pre-defined IFA positive samples were analysed. 13 of these 28 samples reacted positive with the E1, 7 with C and 14 with the E2 protein (Fig.32).

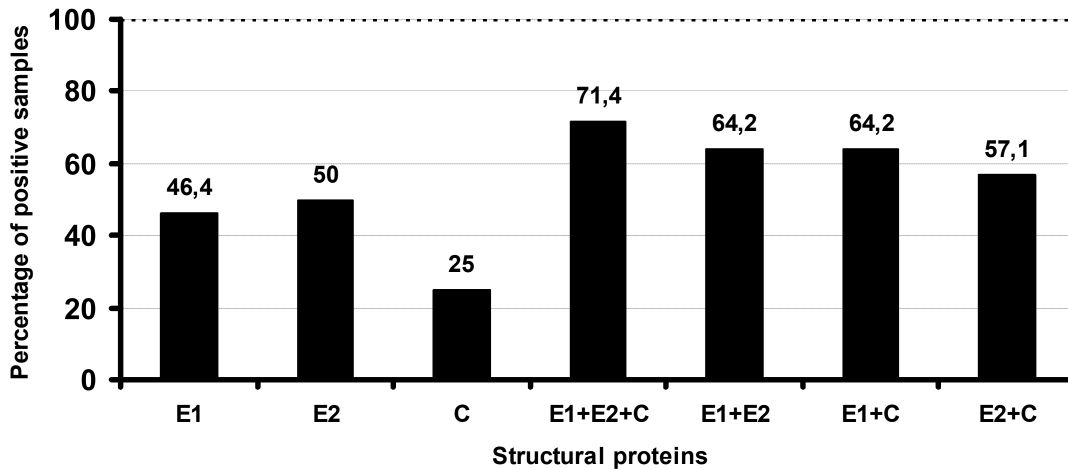


Figure 32: Evaluation of the anti-CHIK antibody ELISA. 23 IFA-positive sera were analysed in the ELISA. Serum reactivity was considered positive, if the obtained OD_{405} was higher than 0.1± 0.01 the negative control.

The combination of these test showed that 20 of 28 sera showed reactivity against one structural protein. An ELISA with only two recombinant proteins displayed a positive reaction for 57-71 % of the serum samples depending on the used combination (Fig.32). The combination of E1 with E2 or E1 with C showed a significant higher than those of E2 and C. Notably, three samples determined positive with E1 in immunoblot assay (samples 146, 383 and 3506), as well as five samples with E2 and nine samples with C were non-reactive in ELISA assay (Tab.1). Whereas, 8 samples (38, 272, 567, 612, 662, 689, 2979, 27876) non-reactive in immunoblot assay were positive in ELISA assay, indicating reactivity against conformational epitopes. Particularly mentioned should be in this regard sample 689, which had the high value 0.374 in E1-ELISA and 1:320 in IFA, but was negative in immunoblot assay when scanned with E1.

The anti-CHIKV antibody ELISA was also performed with all 158 sera derived from the Vietnamese blood donors. 25 of 158 showed reactivity against E1, 26 of 158 against E2 and 6 of 158 against C recombinant protein. In addition, 6 and 8 sera displayed

borderline reactivity against E1 and E2 proteins, respectively. Again a combination of reactivity against two or more recombinant proteins showed serum reactivity in approx. 20-36 % of the samples (Fig.33).

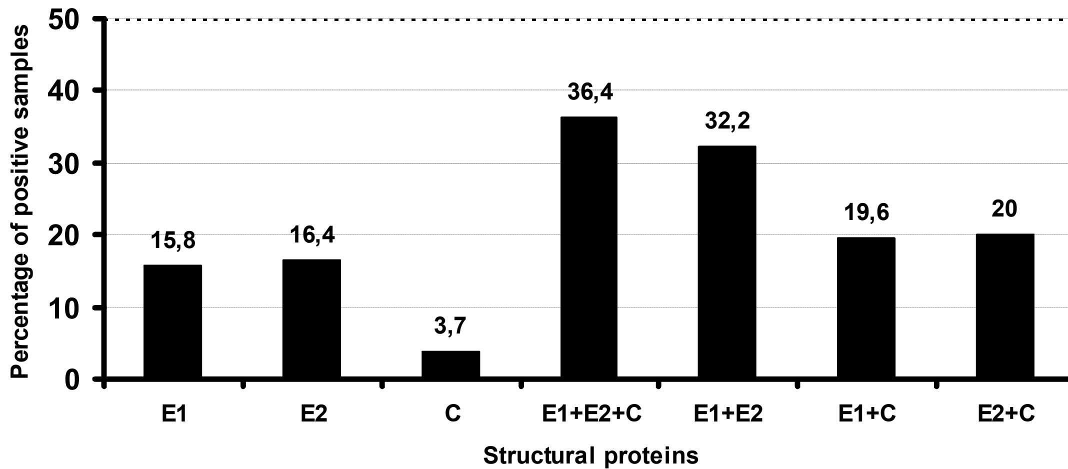


Figure 33: Analysis of the Vietnamese donor sera by ELISA using recombinant CHIKV structural proteins.

4.3.4. Analysis of serum reactivity by immunofluorescence

In order to evaluate our Western blot results Vietnamese and control serum samples were evaluated by immunofluorescence assay (IFA). To determine antibody reactivity to CHIKV, Vero cells were infected with CHIKV-Wü. All available sera, from Vietnamese donors, negative controls and 21 anti CHIKV positive sera, previously screened by the BNI were analyzed. Vietnamese and anti CHIKV positive sera were initially diluted in PBS 1:10 or 1:160 respectively. Reactive sera were further titrated. Titers of ≥ 1:160 were considered as positive (Fig.34). This resulted in 14 of 21 (66.6%) positive samples from the BNI group. This result indicated a lower sensitivity of the assay compared to the titres obtained by the BNI. Of 158 Vietnamese sera 23 of 158 (14.6%) showed titres of ≥ 1.10. One of them had an IgG titre of 1:80, two a titre of 1:60, two a titre of 1:40, six a titre of ≤1:20 and twelve a titre of ≤1:10. No gender specific difference in serum-reactivity was observed.

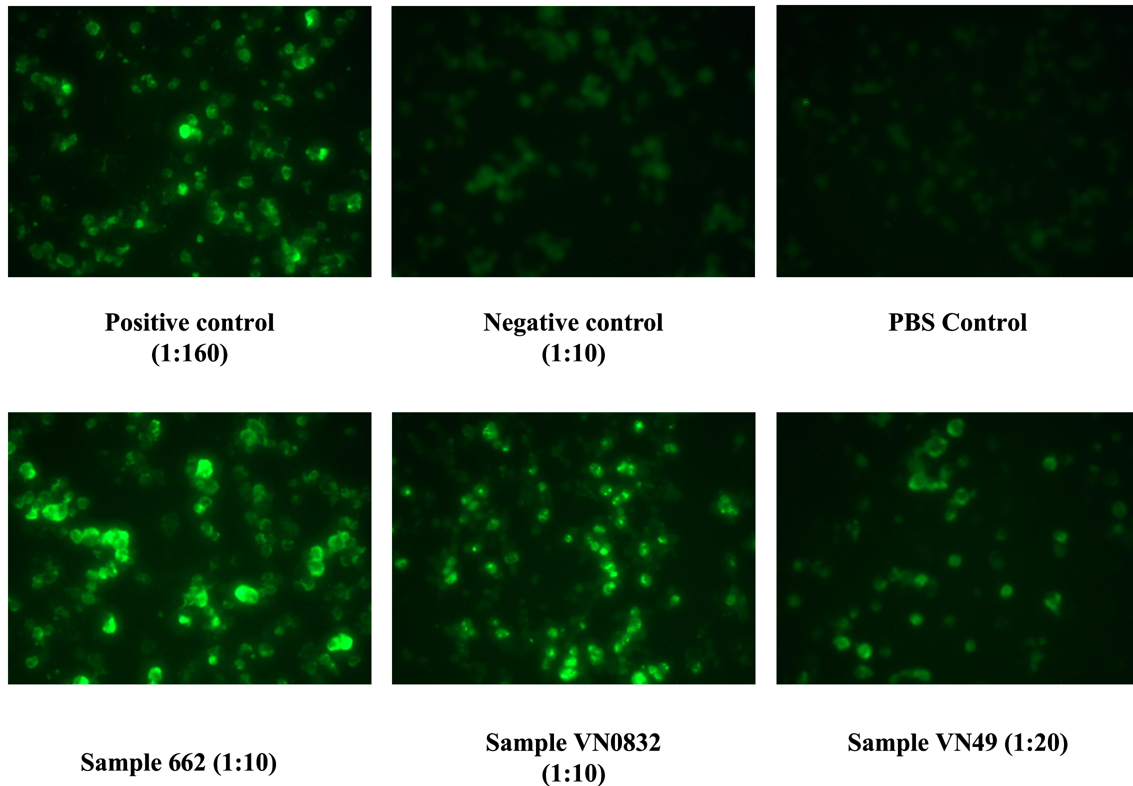


Figure 34: Typical Immunofluorescence assay of CHIKV infected Vero cells.

4.3.5 Analysis of the patient derived antibody responses by western blotting using antigens from CHIKV-infected cells

To evaluate the discrepancies in the results between the different the anti CHIKV antibody analysis in Western blotting, ELISA and IFA, we decided to perform Western blot analysis on infected and uninfected Vero cells. This assay should give an answer whether the found differences were due to the recombinant nature of the protein used above. Vero cells were infected with high titer virus stocks for 36 h. This time point ensured that viral proteins were produced at high levels and the cells were still viable when harvested. Cells were lysed in RIPA-buffer using the Qiagen shredder as described by the manufacturer. Cellular proteins were subsequently separated by SDS-PAGE and transferred to nitrocellulose. After blocking membranes were incubated with positive control and with 100 sera from the Vietnamese blood donors at a dilution of 1:100 in PBS/milk. Bound antibodies were detected with an HRP-conjugated anti-

human antibody and visualized with the ECL Western blot kit. The sera detected three major proteins of approx. 47, 95 and 130 kDa size. The 47 kDa protein band, which was detected by eight Vietnamese and all positive control samples, could be assigned to either E1 or E2, which were not separated on the gel. The 130 kDa and the 95 kDa bands correspond in size to the mass of the non-processed form of the structural protein or to a processing intermediate of the E1 and PE2 proteins, respectively. Both proteins were only detected by the Vietnamese samples. While 9 samples detected the unprocessed 130 kDa form, 7 samples reacted with the processing intermediate.

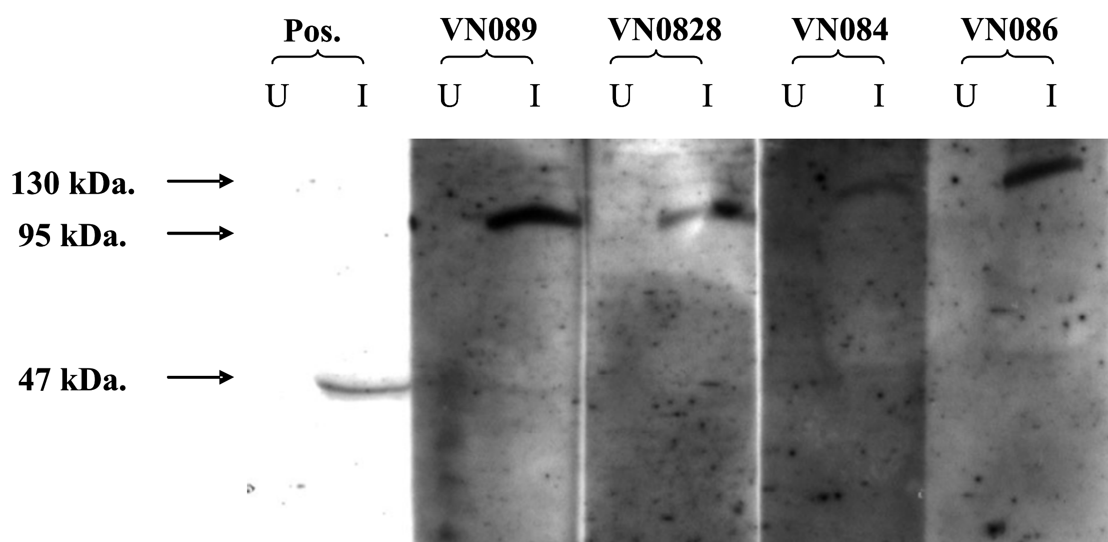


Figure 35: Example of the western blot analysis using uninfected and CHIKV infected Vero cells. The samples were diluted 1:100 and detected with the ECL-detection kit.

5. Discussion

5.1. Development of serological assays for CHIKV diagnoses

In order to diagnose CHIKV infection, many methods have been performed in the laboratory so far. Depending on the phase of infection, different methods were applied to detect CHIKV. In the early stage viremia is present usually between 48 hours and 4 days of illness (Suranjith, 2007). Thus, virus isolation and PCR assays were the main methods in this stage. To isolate CHIKV is a traditional diagnostic tool that is usually possible to perform with human sera within 3-5 days of disease onset. However, the isolation of virus requires the time of cell culture incubation of at least 1-2 days. In addition, virus isolation must be performed in biosafety level 3 facilities. The PCR method is highly sensitive and also suitable for early CHIKV diagnosis (Edwards et al., 2007; Laurent et al., 2007; Santhosh et al., 2007; Hasebe, 2002; Pfeffer, 2002; Yadav, 2000; Pastorino, 2005; Balsubramaniam et al., 2002). Unfortunately, both methods are not available in many public health laboratory centers, particularly in developing countries.

To overcome the limitation of these methods, serological tests for CHIKV diagnosis were developed and analysed in this thesis. Serological tests are the methods of choice to detect CHIKV after 2 weeks of illness when viremia declines. Serological methods are performed to identify antibodies that demonstrate the interaction between the human body and specific infectious agents. Serological tests are easy and fast method to access the CHIKV epidemiology. In CHIKV infection, serological tests could be used to detect both anti-CHIKV IgM in the early phase of infection antibodies and specific IgG after 2 weeks (Pialoux et al., 2007).

To develop the serological assays, the CHIKV structural proteins (E1, E2 and C) were used to screen anti-CHIKV IgG antibody in the sera of CHIKV infected patients, as well as in a German blood donor cohort. The structural proteins E1, E2 and C are the major antigenic components of CHIKV. To test the ability of CHIKV recombinant structural proteins to be detected by anti CHIKV IgG, the CHIKV patient sera obtained from BNI Hamburg which contain anti CHIKV antibodies and German donor blood samples were used. In the immunoblot assay, the results revealed that in the case of

combining the results of the recombinant proteins these proteins could be recognized by the majority of positive samples (up to 77%). As predicted, none of the German donor blood samples reacted with the structural proteins. Particularly, the mixture of E2 and C proteins appeared suitable for anti-CHIKV antibody recognition. In ELISA assay, the recombinant structural proteins were also used as antigens. The results showed that the major of positive sera (71.4%) could recognize the recombinant proteins combining the results of individual assays. The superiority of combining suggests that anti individual recombinant antigens in the serodiagnosis of CHIKV infection has already been mentioned (Muthumani et al. 2008). Among the structural proteins, the E2 recombinant protein reacted best. This result suggests that the ability of E2 protein to elicit antibody is better. In neutralization tests and in mice protection assays it was shown that E2 was also able to elicit antibody responses. These antibodies were efficient in neutralizing virus infectivity and in protecting mice from challenge with new virus (Mathews and Roehrig, 1982; Grosfeld et al., 1989). Conversely, most monoclonal antibodies, which were specific for El epitopes, did not have the ability of virus protection in both neutralization and infectivity protection assays (Razumov, 1991).

However, in comparison with IFA from BNI Hamburg, ELISA and immunoblot methods were less sensitive than BNI's IFA. There were 7 negative samples (567, 759, 798, 1122, 1294, 1302, 1444-Tab.1) in the immunoblotting assay that scored positive in BNI-IFA, particularly, sample 567 with high titer and there were 10 negative samples (368, 554, 555, 798, 1122, 1294, 1302, 1444, 2494 and 3506-Tab.1) in the ELISA assay. Furthermore, the results in both methods, immunoblotting and ELISA, were not congruent.

It has been suggested that various factors could account for the different results observed in this study: (I) The cross-reactivity between different alphaviruses is one of the major disadvantages of serological techniques (Greiser-Wilke et al., 1991; Gould et al., 2006; Suhrbier and La Linn, 2004; Weaver and Barrett, 2004). In Alphaviruses cross-reactive epitopes were found on the nuclecapsid and envelope proteins. These epitopes could account for the observed differences seen in the different detection assays used (Moore et al., 1974; Hunt et al., 1985). On nucleocapsid proteins, conserved epitopes are presented in most alphaviruses (Greiser-Wilke et al., 1989) and in animal models non-neutralizing antibodies were induced by these group-reactive epitopes

(Schmaljohn et al., 1983). For envelope proteins E1 and E2 of alphaviruses, monoclonal antibodies showed relatively broad cross-reactivity in ELISA assay (Roehrign et al., 1982; Wust et al., 1989). Whereas, in neutralization assay, the E2 glycoprotein-specific monoclonal antibodies was found to be restricted to the homologous and some closely related viruses only (Stec et al., 1986; Olmsted, 1986; Roehrig and Mathews, 1985). (II) Another reason of the failure to detect the structural recombinant proteins might be strain differences. Based on the phylogenetic analyses of partial E1 sequences of CHIKV from African and Asian isolates, it has suggested that there exist three distinct CHIKV phylogroups: Asian, Eastern/Central/Southern African (ECSA) and West Africa isolates (Powers et al., 2000). The structural recombinant proteins were expressed and purified from CHIKV-Wü, which has been identified by phylogenetic analysis to be closely related to the strain described by Schuffenecker from the recent Indian Ocean outbreak (Schuffenecker et al., 2006; Kowalzik et al., 2008). The CHIKV positive sera obtained from BNI Hamburg were collected from patients who might be infected with other CHIKV strains. (III) Another explanation for the inconsistent results might be the dependence of the detection system on conformational epitopes which are different between fixed infected cells and recombinant proteins. It has been demonstrated that the reactivity between antigens and antibodies depend on conformation, in particular of the glycoproteins. Epitopes consist of about 10 amino acids, but usually 3-4 amino acids are responsible for the recognition by antibodies. (IV) Furthermore, different antigens used in the various test could be an additional explanation of the results. CHIKV C, E1 and E2 proteins are the main antigens recognized by patient sera. However, other antigens, such as the precursor polyproteins, pE2 or non-structural proteins, which are all present in CHIKV-infected Vero cells (Fig.35) and objects from the recombinant tests.

It should be pointed out that the in-house IFA was less sensitive than the BNI's IFA, possibly reflecting the greater experience with the IFA diagnosis of the BNI team. For example, sample 132 was positive with a titer of 1:20480 at BNI, but it was only 1:320 in the in-house IFA (Tab.1).

5.2. Serological diagnoses in Vietnamese blood donors

The recombinant proteins were also used as antigens for screening the anti-CHIKV antibodies in Vietnamese donor blood samples.

In the past very few papers showed that Vietnam was affected by CHIKV infection. Thus, in order to screen anti-CHIKV antibodies in Vietnamese blood donors, 158 samples were used in immunoblotting. Up to 24% of Vietnamese sera recognized the recombinant proteins. In the parallel with immunoblotting, the ELISA method was performed. The results revealed that the percentage of positive in Vietnamese sera was up to 36 % (56 out of 158 samples). These results suggested that CHIKV might circulate in Vietnam. In Vietnam the climate and temperature conditions are suitable for mosquitoes such as *Ae. aegypti* and *Ae. albopictus*, which are also known to be the main vectors for dengue fever (Gubler, 1988). Vietnam is a country with regular dengue fever outbreaks (Thai et al. 2005). Thus, the Vietnamese population is at high risk to be infected with both CHIKV and dengue fever virus. Both infectious agents are transmitted by the same vectors and can cause clinical symptoms that can easily be mixed up. Recently, a sero survey was carried out in Indian islands. It was shown that the CHIKV seroprevalence was 15.3%, while that of dengue fever was 25.4% (Padbidri et al. 2002). In another study, on Grande Comore Island, the seroprevalence of CHIKV was 63% (Sergon, 2007). In the outbreak in Kenya in 2004, the CHIKV infection rate reacched 72% of the study population (Sergon, 2008) and in Senegal, in the outbreak of the late 1990s, the seroprevalence of CHIKV was 35.3% (Thonnon, 1999). During the late 1970's in Gabon, serological surveys revealed that 20% of the population were infected with CHIKV (Jan et al., 1978; Saluzzo et al., 1982). In the Vietnamese war, the America solders were often doubtfully infected by CHIKV (Deller, 1968). Since the clinical manifestations of dengue fever infection can be very variable (Hoang et al., 2006), our results suggest that infection by CHIKV is frequently misdiagnosed as dengue fever.

However, it must be pointed out that the results of immunobloting, ELISA and IFA with the Vietnamese sera were also inconsistent (Tab.3 and 4). The reason for this is probably the source of our antigens which were derived from a virus identified in the recent Indian Ocean outbreak. Vietnamese sera applied in the assays might not contain

the specific antibodies which could react with the recombinant proteins. Therefore, the isolation of a current Vietnamese CHIKV is of prime interest.

6. Summary

CHIKV is the prototype of Alphaviruses and it causes an acute febrile illness with rash, severely painful arthralgias, and sometimes arthritis. While CHIKV has first been identified in the 1950s in Africa, recent outbreaks of CHIKV in the islands of the Indian Ocean and particular in Italia have re-drawn attention to CHIKV. In the past CHIKV disease was considered self-limiting and non-fatal. However, a number of deaths on Reunion (Anonym, 2006) during the outbreak, which was affected directly or indirectly by CHIKV, have changed this view. To defeat CHIKV outbreaks diagnostic tools and anti CHIKV therapies are urgently needed. In this thesis, we generated tools to investigate CHIKV at the molecular level by serological tests.

CHIKV was isolated from a German woman who was infected during her holidays on the Mauritius Island. To characterize this viral isolate the complete viral genome was amplified by PCR and molecular cloned. In order to analyse antibody responses of infected individuals some of the structural and non-structural genes were subcloned in bacterial expression vectors. The NSP2, proteinase, capsid, E1 and E2 were subsequently expressed in *E.coli* using purified successfully.

In this thesis, the structural proteins were used to develop a screening test for anti-CHIKV antibodies in patient derived serum samples. These tests were evaluated with pre-characterized anti-CHIKV sera (30 samples) obtained from the BNI Hamburg and 100 serum samples from German blood donors used as negative controls. Immunoblotting analysis revealed that up to 77% of precharacterised positive sera could recognize the recombinant proteins and there were no detectable reactivity of CHIKV-negative German donor sera. The recombinant proteins were also recognized by 71.4% of positive sera in the newly established ELISA. In order to go further in analyses of the results, an in house IFA was performed. Positive sera (21 samples) were used. The results showed that all of them reacted positive, but this assay was less sensitive than the IFA from BNI. In comparison with the IFA result from BNI Hamburg, the results were not congruent in all test performed. This could be due to various drawbacks of the tests. A cross reaction in Alphaviruses and the different strains are mentioned as well as the denatured forms of the structural proteins. Besides the main structural proteins (E1,

E2 and C), other proteins such as non-structural proteins, uncleaved precursor proteins could participate in the different outcomes of serological assays.

In order to go further in the CHIKV diagnoses, the CHIKV recombinant proteins were applied to screen the anti-CHIKV antibodies in the Vietnamese population, who are considered to live in the high risk regions. In serological tests, 158 sera of Vietnamese donors were incubated with the recombinant proteins or the fixed CHIKV infected cells. The results showed that 24% of Vietnamese donor sera recognized the recombinant proteins in immunoblot assay, while 36% scored positive in the ELISA assay. In IFA, the sera considered positive were 11.4%. While some discrepancies in serological tests were found, these results showed that the ratio of CHIKV-positive sera seem to be equal to the other regions in the world, which are affected by CHIKV. It is suggested that CHIKV infection in Vietnam has been repeatedly misdiagnosed. This study cohort consisted only of samples originating from Hanoi area of Northern Vietnam, thus, future studies should expand to include samples from other Vietnam areas. To do this the various subtypes of the virus in the different regions should be isolated and the sequences of these viruses should be well characterized.

Table 1: Reactivities of CHIKV positive human serum samples with the recombinant CHIKV-Wü1 antigens

No.	Codes	BNI-IFA (IgG)	BNI-IFA (IgM)	N	VR 1:700	WUE-IFA	E1			E2			C		
							Elisa	Western blot 1:100	Western blot 1:700	Elisa	Western blot 1:100	Western blot 1:700	Elisa	Western blot 1:100	Western blot 1:700
1	**38**	1:1280	+				**0.136**	-	-	0.025	-	-	0.088	n.d.	+
2	**132**	1:20480	+		+	**1:320**	**1.194**	n.d.	+	-0.052	n.d.	+	0.049	-	-
3	**133**	1:20480	+				**0.334**	n.d.	+	**0.106**	n.d.	+	**0.1**	n.d.	+
4	**146**	1:1280	+		+	**1:320**	-0.166	-	(+)	0.045	n.d.	+	**0.555**	-	-
5	**272**	1:10240	+			**1:640**	**0.128**	-	-	**0.911**	n.d.	+	0.08	n.d.	+
6	**340**	1:2560	+			**1:320**	**0.141**	n.d.	+	**0.466**	n.d.	+	**0.125**	-	-
7	**341**	1:1280	+			**1:320**	0.015	-	-	**0.844**	n.d.	+	**0.385**	n.d.	+
8	**368**	1:320	+			**1:160**	-0.042	-	-	**0.101**	+	-	0.019	-	-
9	**383**	1:5120	**-**				-0.015	n.d.	+	**0.289**	n.d.	+	-0.005	n.d.	+
10	**385**	1:1280	+			**1:640**	0.015	-	-	**0.288**	n.d.	+	0.032	n.d.	+
11	**554**	1:1280	+			**1:160**	**0.092**	-	-	-0.068	-	-	-0.007	n.d.	+
12	**555**	1:1.024	+					-	-		n.d.	+		n.d.	+
13	**567**	1:5120	+			**1:160**	**0.109**	-	-	**0.347**	-	-	**0.092**	-	-
14	**612**	1:1024	+			**1:164**	**0.107**	-	-	**0.268**	-	-	0.044	n.d.	+
15	**662**	1:10	**-**	1:64		**1:80**	**0.13**	-	-	**0.235**	+	-	0.045	n.d.	+
16	**689**	1:10240	+		+	**1:320**	**0.374**	-	-	0.084	n.d.	+	0.079	-	-

17	**759**	1:80	+			**<1:80**	0.009	-	-	**0.437**	-	-	**0.129**	-	-
18	**798**	1:2560	+	1:32		**1:80**	0.064	-	-	-0.057	-	-	0.025	-	-
19	**844**	1:10	**-**			**<1:10**	0.07	-	-	0.006	-	-	**0.141**	n.d.	+
20	**846**	1:1280	+		+	**1:160**	**0.158**	+	-	**0.521**	n.d.	+	-0.036	n.d.	+
21	**865**	1:160	+			**<1:10**	**0.153**	+	-	**0.214**	-	-	0.014	-	-
22	**1122**	1:80	+				-0.037	-	-	-0.063	-	-	**0.093**	-	-
23	**1292**	1:5120	+		+	**1:320**	0.059	-	-	**0.204**	n.d.	+	**0.128**	-	-
24	**1294**	1:40	+	≤1:8		**<1:40**	0.004	-	-	-0.050	-	-	0.023	-	-
25	**1302**	1:160	+					-	-		-	-		-	-
26	**1444**	1:40	+				-0.013	-	-	-0.029	-	-	-0.03	-	-
27	**2494**	1:5120	**-**	1:256		**1:320**	0.014	-	-	-0.018	n.d.	+	0.032	-	-
28	**2979**	1:160	**-**	1:32		**<1:80**	**0.133**	-	-	0.044	+	-	0.07	-	-
29	**3506**	1:1280	**-**			**1:320**	0.058	(+)	**(+)**	0.041	n.d.	+	0.03	n.d.	+
30	27876					**1:320**	**0.107**	-	-	**0.128**	n.d.	+	**0.104**	-	(+)

n.d: (not done)

Serum 27876: the serum of the index patient from whom Chik-Wü1 was isolated six month after the start of illness

(+): barely detectable band in immunoblotting.

N: Neutralization assay

VR: CHIKV infected vero cell Western blotting

Table 2: Tue-Vietnamese sera against CHIKV structural proteins

Nr.	Code	Gender	Age	WÜ IFA	E1		E2		C	
					Elisa	WB(1:100)	Elisa	WB(1:100)	Elisa	WB(1:100)
1	VN1	M	36	**-**	0,033	-	0,065	-	0,037	-
2	VN2	F	29	**-**	**0,088**	-	**0,148**	-	0,044	-
3	VN3	F	20	**-**	**0,103**	-	**0,139**	-	0,082	-
4	VN4	F	34	**-**	**0,14**	+	0,079	-	0,045	-
5	VN7	M	25	**-**	0,056	-	0,074	-	0,05	-
6	VN8	F	46	**-**	**0,1**	-	0,077	-	0,047	-
7	VN9	M	37	**-**	**0,117**	-	**0,126**	-	0,036	-
8	VN10	M	41	**-**	**0,1**	-	**0,092**	-	0,039	-
9	VN13	M	22	**-**	**0,092**	-	0,087	-	**0,108**	-
10	VN14	M	32	**-**	0,073	-	**0,101**	-	0,06	-
11	VN15	M	25	**-**	0,031	-	0,047	-	0,045	-
12	VN16	F	56	**-**	-0,005	-	-0,002	-	-0,008	-
13	VN17	F	46	**-**	0,032	-	**0,113**	-	0,024	-
14	VN18	F	54	**1:20**	0,077	-	**0,16**	-	0,075	-
15	VN19	F	34	**-**	**0,093**	-	**0,141**	-	0,059	-
16	VN20	F	30	**-**	**0,106**	-	**0,128**	-	0,078	-
17	VN21	M	32	**-**	**0,09**	-	**0,131**	-	**0,089**	-
18	VN22	M	32	**-**	0,065	-	**0,144**	-	0,044	-

19	VN23	M	59	-	-0,002	-	0,069	+	0,005	-
20	VN24	F	55	**1:20**	0,036	-	0,068	+	0,017	-
21	VN25	F	31	**1:40**	0,074	-	**0,097**	-	0,064	-
22	VN26	F	42	-	0,062	-	0,066	-	0,086	-
23	VN27	M	28	-	0,059	-	0,047	-	0,069	-
24	VN28	F	32	-	0,01	-	0,025	+	0,017	-
25	VN30	M	23	-	0,014	-	0,074	-	0,027	-
26	VN31	F	26	**<1:20**	0,035	-	**0,11**	-	0,053	-
27	VN32	M	65	**1:20**	0,036	-	0,071	+	0,053	-
28	VN33	F	59	**1:60**	0,071	-	**0,128**	+	0,07	-
29	VN34	M	34	-	0,028	-	**0,091**	-	0,036	-
30	VN35	F	40	-	0,06	-	0,086	-	0,017	-
31	VN36	M	29	-	0,046	-	**0,099**	-	0,036	-
32	VN37	M	62	-	0,033	-	0,062	+	0,028	-
33	VN38	F	48	-	0,021	-	0,013	-	0,013	-
34	VN39	M	36	-	0,012	-	0,019	+	0,028	-
35	VN40	F	39	-	0,028	-	0,032	-	0,054	-
36	VN41	M	63	-	-0,008	-	0,006	-	0,029	-
37	VN42	F	39	-	0,069	-	0,005	-	0,057	-
38	VN43	F	36	-	0,072	-	**0,131**	-	0,049	-

39	VN44	M	46	**-**	0,082	-	0,085	+	0,056	-
40	VN45	M	38	**-**	0,037	-	0,054	-	0,029	-
41	VN46	M	39	**-**	0,061	-	0,052	-	0,031	-
42	VN47	F	34	**-**	0,075	-	0,052	-	0,036	-
43	VN48	F	21	**-**	**0,103**	-	0,074	-	0,07	-
44	VN49	F	33	**1:60**	0,07	-	**0,127**	+	0,048	-
45	VN50	M	41	**-**	0,073	-	0,049	-	0,057	-
46	VN51	F	28	**-**	0,079	-	0,04	-	0,046	-
47	VN52	F	62	**-**	0,068	-	0,015	-	0,002	-
48	VN53	F	65	**-**	0,022	-	0,003	-	0,011	-
49	VN54	M	37	**-**	-0,001	-	-0,002	-	0,003	-
50	VN56	M	70	**-**	**0,088**	-	0,035	-	0,074	-
51	VN57	M	20	**-**	0,06	-	0,017	-	0,038	-
52	VN58	F	32	**-**	0,047	-	0,032	-	0,016	-
53	VN59	F	37	**-**	0,05	-	0,034	+	-0,002	-
54	VN60	F	46	**1:20**	0,079	-	0,025	-	0,059	-
55	VN65	M	39	**-**	0,009	-	0,036	-	0,007	-
56	VN66	F	70	**-**	0,061	-	0,054	-	0,027	-
57	VN67	F	43	**<1:20**	0,014	-	0,026	-	0,007	-
58	VN68	M	36	**-**	0,019	-	0,074	-	0,013	-

Table 3: Vietnamese sera against CHIKV structural proteins

No.	Codes	Gen.	Age	N	BNI-IFA		WB Vero			Wue IFA	E1			E2			C		
												WB			WB			WB	
				(1:4)	IgM	IgG	45	95	130	IgG	Elisa	1:100	1:700	Elisa	1:100	1:700	Elisa	1:100	1:700
1	VN081	F	43				-	-	-	-	0,079	-	-	0	-	-	- 0,058	-	-
2	VN082	F	32	+-	+-	+-	+-	-	-	<1:10	**0,119**	-	-	**0,107**	-	-	**0,212**	-	-
3	VN083	M	4	-			-	-	-	-	0,011	-	-	0,041	+	-	0,035	+	+
4	VN084	M	58	-			-	-	+	-	**0,191**	-	-	**0,156**	+	+	0,042	-	-
5	VN085	M	56	-			-	-	-	-	-0,08	+	-	- 0,042	+	+	-0,01	-	-
6	VN086	M	45				-	-	+	-	- 0,044	-	-	0,036	-	-	- 0,069	-	-
7	VN087	M	31	-			-	-	-	-	**0,094**	-	-	0,064	+	+	0,034	-	-
8	VN088	M	26				-	-	-	-	- 0,201	-	-	- 0,194	-	-	- 0,213	-	-
9	VN089	M	27				-	+	-	-	- 0,049	-	-	- 0,077	+-	+-	- 0,126	-	-
10	VN0812	M	21	-			-	-	+	-	**0,206**	-	-	0,1	+	+-	**0,104**	+	+
11	VN0814	M	30				-	-	-	-	- 0,153	-	-	- 0,134	-	-	-0,12	-	-
12	VN0815	M	34				-	-	+	-	- 0,078	-	-	-0,09	+-	+-	- 0,108	-	-
13	VN0816	F	50	-	**US**	**US**	-	-	-	<1:10	- 0,121	-	-	- 0,123	-	-	- 0,166	+-	-
14	VN0817	F	38				-	-	-	-	-	-	-	-	+	+	-0,15	-	-

											0,129			0,096					
15	VN0819	F	59	-			-	-	-	-	-0,157	-	-	-0,115	+	+	-0,177	+	+
16	VN0820	M	46				-	-	+	-	-0,133	-	-	-0,094	-	-	-0,091	-	-
17	VN0821	F	42				-	-	-	-	**0,17**	-	-	**0,137**	-	-	0,041	-	-
18	VN0823	F	38	-			-	-	-	-	0,06	-	-	0,013	+	+	-0,045	+	+
19	VN0825	F	23				-	-	-	-	**0,111**	-	-	0,081	-	-	-0,008	-	-
20	VN0826	F	21	+			-	-	-	-	0,055	-	-	**0,089**	+	+	-0,103	-	-
21	VN0827	M	53				-	-	-	-	**0,112**	-	-	0,066	-	-	-0,018	-	-
22	VN0828	F	44				+	-	-	-	-0,139	-	-	-0,138	+-	-	-0,151	-	-
23	VN0829	F	59				+	-	-	-	-0,105	-	-	-0,118	+	-	-0,122	-	-
24	VN0830	M	30	+			-	-	-	-	-0,083	-	-	-0,031	+	-	-0,082	-	-
25	VN0831	M	43	+	-	-	-	-	+	1:10	**0,123**	-	-	0,081	-	-	0,028	-	-
26	VN0832	M	37		-	-	-	-	+	1:80	-0,185	-	-	-0,183	+-	-	-0,181	-	-
27	VN0833	M	18				-	-	-	-	**0,218**	-	-	**0,144**	-	-	**0,089**	-	-
28	VN0834	F	25				-	-	+	-	**0,136**	-	-	0,087	-	-	-0,001	-	-
29	VN0835	F	30				+-	-	-	-	**0,2**	-	-	**0,111**	-	-	0,048	-	-
30	VN0836	F	26				-	-	+	-	-0,056	-	-	-0,061	+	+	-0,075	+	-

31	VN0837	M	48				-	-	-	-	-0,082	-	-	-0,132	-	-	-0,108	-	-
32	VN0838	M	38				-	-	-	-	-0,037	-	-	-0,04	-	-	-0,126	-	-
33	VN0839	M	57				-	-	-	-	-0,023	-	-	-0,032	+	+	-0,028	-	-
34	VN0840	F	32				-	+	-	-	-0,071	-	-	-0,074	-	-	-0,116	-	-
35	VN0841	M	43				-	-	-	-	-0,074	-	-	-0,088	+	-	-0,104	-	-
36	VN0842	M	27				-	-	-	-	-0,056	-	-	-0,071	-	-	-0,082	-	-
37	VN0843	M	17				-	+	-	-	-0,037	-	-	-0,051	-	-	-0,055	-	-
38	VN0844	F	33				-	-	-	-	-0,08	-	-	-0,087	-	-	-0,067	-	-
39	VN0845	M	19				-	+	-	-	-0,037	-	-	-0,035	-	-	-0,058	-	-
40	VN0847	M	24				-	-	-	-	**0,265**	-	-	**0,17**	-	-	**0,128**	-	-
41	VN0848	F	19	**-**	**+/PCR?**	**-**	-	-	-	<1:10	**0,198**	-	-	**0,109**	-	-	0,029	-	-
42	VN0849	M	49				-	-	-	-	-0,015	-	-	-0,024	-	-	-0,077	-	-
43	VN0851	M	33		**+**	**-**	-	-	-	1:10	-0,002	-	-	-0,056	-	-	-0,084	-	-
44	VN0852	M	24				+	-	-	-	-0,072	-	-	-0,102	-	-	-0,149	-	-
45	VN0854	F	45				+	-	-	-	**0,276**	-	-	**0,217**	+	+	**0,188**	-	-
46	VN0855	M	29				-	-	-	-	-0,165	-	-	-0,134	-	-	-0,044	-	-

47	VN0856	F	34				-	-	-	-	-0,141	-	-	-0,139	-	-	-0,072	-	-
48	VN0857	F	55				-	-	-	-	-0,023	-	-	-0,022	-	-	-0,04	-	-
49	VN0858	M	44				-	-	-	-	-0,091	+	-	-0,004	+	-	-0,018	-	-
50	VN0859	F	49				-	-	-	-	-0,152	-	-	-0,117	-	-	-0,157	-	-
51	VN0860	M	58				-	-	-	-	-0,045	-	-	0,002	-	-	-0,048	-	-
52	VN0861	F	30	-	**US**	**US**	-	+	-	1:10	**0,134**	-	-	**0,21**	-	-	0,085	-	-
53	VN0862	F	47				-	-	-	-	-0,154	-	-	-0,074	-	-	-0,096	-	-
54	VN0863	M	26				-	+	-	-	0,04	-	-	0,023	-	-	-0,029	-	-
55	VN0865	F	53				-	-	-	-	0,033	-	-	0,014	-	-	-0,009	-	-
56	VN0868	F	46				-	-	-	-	-0,032	-	-	-0,064	-	-	-0,089	-	-
57	VN0869	F	31		**-**	**-**	-	+	-	1:40	-0,184	-	-	-0,168	-	-	-0,182	-	-
58	VN0870	M	56				-	-	-	-	-0,062	-	-	-0,09	-	-	-0,118	-	-
59	VN0872	M	52				-	-	-	-	-0,077	-	-	-0,091	+	-	-0,096	-	-
60	VN0873	M	28				-	-	-	-	**0,152**	-	-	0,075	-	-	-0,002	-	-
61	VN0880	F	20				-	-	-	-	**0,15**	+	+	**0,119**	+-	-	0,032	-	-
62	VN0881	F	45				-	-	-	-	-0,197	-	-	-0,2	-	-	-0,198	-	-

63	VN0882	F	16	-	-	-	-	-	-	<1:10	**0,146**	-	-	0,084	+	+	0,01	-	-
64	VN0883	F	13				-	-	-	-	-0,11	+	+	-0,091	+	+	-0,168	-	-
65	VN0884	F	46				-	-	-	-	-0,14	-	-	-0,126	-	-	-0,158	-	-
66	VN0885	F	44				-	-	-	-	-0,095	-	-	-0,094	-	-	-0,15	-	-
67	VN0886	M	44				-	-	-	-	-0,14	-	-	-0,148	-	-	-0,173	-	-
68	VN0887	M	25				-	-	-	-	-0,069	-	-	-0,045	-	-	-0,14	+	+
69	VN0888	M	34				-	-	-	-	-0,079	-	-	-0,081	-	-	-0,119	+	-
70	VN0889	M	58				-	-	-	-	0,057	-	-	0,036	-	-	0,01	-	-
71	VN0890	M	56				-	-	-	-	-0,028	-	-	-0,056	-	-	-0,014	-	-
72	VN0891	M	44				-	-	-	-	-0,086	-	-	-0,077	-	-	-0,126	-	-
73	VN0892	M	32				-	-	-	-	-0,178	-	-	-0,12	-	-	-0,169	-	-
74	VN0893	F	18	+	US	US	-	-	-	<1:10	-0,093	-	-	-0,1	-	-	0,015	-	-
75	VN0894	F	40				-	-	-	-	-0,046	-	-	-0,028	-	-	-0,104	-	-
76	VN0895	M	14				-	-	-	-	-0,049	-	-	-0,025	-	-	-0,065	-	-
77	VN0896	M	36				-	-	-	-	-0,164	-	-	-0,137	-	-	-0,188	-	-
78	VN0897	M	20		+	+-	-	-	-	1:10	-0,183	-	-	-0,142	-	-	-0,189	-	-

79	VN0898	M	65				-	-	-	-	-0,079	-	-	-0,044	-	-	-0,073	-	-
80	VN0899	M	27				-	-	-	-	-0,132	-	-	-0,123	-	-	-0,168	-	-
81	VN08100	F	21				-	-	-	-	-0,112	-	-	-0,11	-	-	-0,185	-	-
82	VN08101	M	24				-	-	-	-	-0,141	-	-	-0,114	-	-	-0,144	-	-
83	VN08102	M	56		++	++	-	-	-	1:10	-0,024	-	-	-0,086	-	-	-0,113	-	-
84	VN08103	M	42				-	-	-	-	-0,061	-	-	-0,065	+	-	-0,085	-	
85	VN08104	M	32		**+/CR**	**+/CR**	-	-	-	1:10	-0,187	-	-	-0,144	-	-	-0,182	-	-
86	VN08105	M	43				-	-	-	-	**0,161**	-	-	**0,093**	+	+	0,009	-	-
87	VN08106	M	32				-	-	-	-	**0,269**	-	-	**0,224**	-	-	**0,126**	-	-
88	VN08109	M	4				-	-	-	-	**0,11**	-	-	**0,154**	-	-	0,033	+	+
89	VN08110	M	58		**+/US**	+	-	-	-	1:10	-0,156	-	-	-0,15	-	-	-0,132	-	-
90	VN08111	M	56				-	-	-	-	-0,183	-	-	-0,145	-	-	-0,166	-	-
91	VN08112	M	45				-	-	-	-	**0,195**	-	-	**0,142**	+	-	0,069	+-	-
92	VN08113	F	31				-	-	-	-	-0,066	-	-	-0,054	-	-	-0,055	-	-
93	VN08114	M	26				-	-	-	-	-0,092	-	-	-0,033	+	-	-0,082	-	-
94	VN08115	M	27				-	-	-	-	-0,095	-	-	-0,053	-	-	-0,121	+-	-

95	VN08116	M	21				+	-	-	-	-0,046	-	-	-0,056	+	-	-0,037	+	-
96	VN08117	M	30				-	-	-	-	0,027	-	-	0,012	-	-	-0,014	-	-
97	VN08118	M	34				-	-	-	-	0,013	-	-	-0,016	+	+	-0,062	-	-
98	VN08119		50				+	-	-	-	-0,196	-	-	-0,15	-	-	-0,174	-	-
99	VN08120		38				-	-	-	-	-0,081	-	-	-0,103	-	-	-0,147	-	-
100	VN08121		59				-	-	-	-	-0,049	-	-	-0,033	-	-	-0,029	+	-

N: Neutralization assay

VR: CHIKV infected vero cell Western blotting

US: unspecificity

CR: Cross reaction

Table 4: German blood donors

No.	Sera code	No.	Sera code	No.	Sera code
1	2007024438	35	2007024705	68	2007025018
2	2007024439	36	2007024706	69	2007025019
3	2007024440	37	2007024707	70	2007025020
4	2007024441	38	2007024708	71	2007025021
5	2007024442	39	2007024709	72	2007025022
6	2007024443	40	2007024800	73	2007025118
7	2007024444	41	2007024801	74	2007025119
8	2007024445	42	2007024802	75	2007025120
9	2007024446	43	2007024803	76	2007025121
10	2007024447	44	2007024804	77	2007025122
11	2007024448	45	2007024835	78	2007025123
12	2007024573	46	2007024836	79	2007025124
13	2007024574	47	2007024837	80	2007025125
14	2007024575	48	2007024838	81	2007025129
15	2007024576	49	2007024839	82	2007025130
16	2007024577	50	2007024920	83	2007025131
17	2007024578	51	2007024921	84	2007025132

18	2007024579	52	2007024922	85	2007025133
19	2007024580	53	2007024923	86	2007025134
20	2007024583	54	2007024924	87	2007025135
21	2007024584	55	2007024925	88	2007025136
22	2007024585	56	2007024926	89	2007025226
23	2007024586	57	2007024927	90	2007025227
24	2007024587	58	2007024928	91	2007025228
25	2007024588	59	2007024929	92	2007025229
26	2007024589	60	2007024930	93	2007025230
27	2007024590	61	2007024931	94	2007025231
28	2007024696	62	2007024932	95	2007025232
29	2007024697	63	2007025011	96	2007025238
30	2007024698	64	2007025012	97	2007025239
31	2007024699	65	2007025013	98	2007025240
32	2007024700	66	2007025014	99	2007025241
33	2007024701	67	2007025015	100	2007025242
34	2007024704				

8. Abbreviations

Σ	In total
BBS	Borate-buffered saline
BNI	Berhard Nocht Institut, Hamburg Germany
bp	Base pairs
C	Capsid protein
C^0	Degree Celsius
cDNA	Complementary DNA
CHIKV	Chikungunya virus
CIAP	Calf intestine alkaline phosphatase
CR	Cross-reactive
d.H_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphates
NTPs A, G, C, T	Adenine, Guanine, Cytosine, Thymidine
E1	E1 envelope protein
E2	E2 envelope protein
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzym-linked immunosorbent assay
IFA	Immunofluoresencens assy
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPTG	Isopropyl-ß-D-thiogalactopyranosid
kb	Kilo – base pairs
LB	Luria-Bertani
M	Molar
mAbs	Monoclonal antibodies
NPP	*p*-nitrophenyl phosphate
nsp	Non structural protease
OD	Optical density
PBS	Phosphate-buffered saline

PCR	Polyemerase chain reaction
PR	Protease enzyme
RNA	Ribonucleic acid
rpm	Round per minute
RT	Reverse Transcriptase enzyme
RT-PCR	Reverse Transcription Polyemerase chain reaction
SAP	Shrimp alkaline phosphatase
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TAE	Tris Acetic acid EDTA
U	Unit
V	Voltage

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