Positive stranded RNA viruses: Dissecting the capsid of Hepatitis E and Chikungunya viruses

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to my teacher

Sebastian PC

Abbreviations

The following list does not contain abbreviations that do not require definition according to the instructions for authors of the FEBS Journal available online at the web address http://www.blackwellpublishing.com/products/ journals/suppmat/ ejb/ejbtab4.htm. Such abbreviations are used in the text and the legends of figures and tables without prior definition.

AAV	Adeno-associated virus
aa	Amino acid
BLAST	Basic Local Alignment Search Tool
CIAP	Calf intestine alkaline phosphatase
ChikV	Chikungunya virus
CMV	Cytomegalovirus
CPE	Cytopathic effect
ddH_2O	doubly deionized water
DPT	Days post transfection
DPS	Days post splitting
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
dNTP	deoxyribonucleoside triphosphate
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EGFP	Enhanced green fluorescent protein
FCS	Fetal calf serum
Fw	Forward
Fig	Figure
GFP	Green fluorescent protein
HAV	Hepatits A virus

HEV	Hepatitis E virus
HRP	Horseradish peroxidase
IEM	Immuno electron microscopy
IP	Immuno precipitation
Kb	Kilo base
kDa	Kilo Dalton
LB	Luria-Bertani broth
MOI	Multiplicity of infection
NCBI	National Center for Biotechnology Information
NC	Nucleocapsid
NLS	Nuclear Localization Signal
NES	Nuclear Export Signal
OD	Optical density (absorbance/extinction)
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PI	Post infection
RIPA	Radioimmunoprecipitation assay
rpm	revolutions per minute
Rev	Reverse
RT	1. reverse transcriptase
	2. reverse transcription
	3. room temperature
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
WB	Western Blot

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1. Preface

Positive stranded RNA viruses cause various viral based diseases in human beings. This includes some of the most devastating epidemics over the last few centuries. Viruses that infect mammalian cells must replicate before the host immune system defenses shut down virus production and/or destroy the infected cell. RNA viruses are comparatively simple with regard to their genomic organization. Plus strand RNA viruses often encode very few proteins (mostly less than 10) in which mostly associated with its structure. RNA viruses also face the same challenges as genetically complex viruses in that they must temporarily evade the immune response until viral replication and egress takes place (Roulston *et al.*, 1999).

Capsid proteins form major structural component of virus. They are involved in nucleic acid binding and recognized function involves packaging genetic material of virus into protective structures called nucleocapsids (NC). NC consists of several oligomeric structural subunits made of proteins called protomers. The observable 3-dimensional morphological subunits, which may or may not correspond to individual proteins, are called capsomeres. Capsid proteins are arranged in a manner which provides maximal contact among subunits and structural units. The repetitive interactions among a limited number of proteins results in a regular structure, with a symmetry that is determined by the spatial patterns of the interactions (Urbanowski *et al.*, 2008).

RNA viruses are enveloped like Dengue virus, Hepatitis C virus, Yellow fever virus Influenza virus, Measles virus, Rabies virus, Alphavirus etc. or non-enveloped like Poliovirus, Rhinovirus, Enterovirus, Norwalk virus, Hepatitis E virus etc (Carstens & Ball, 2009; Fauquet & Fargette, 2005). Here, some of the functions associated with viral capsids are further dissected in two viruses, Hepatitis E virus, a non-enveloped virus of *Hepeviridae* and Chikungunya virus, an enveloped virus of *Togaviridae* family.

Chapter 1

2. Hepatits E virus (HEV)

2.1. Introduction

The Hepatitis E virus is an important cause of morbidity and mortality in the regions of the world where fecal contamination of the environment and drinking water are common. The first well-characterized HEV epidemic was reported in Delhi, India, in 1955. The symptoms caused by HEV are typically characterized by a self-limiting acute hepatitis with low mortality (Krawczynski *et al.*, 1999; Mast *et al.*, 1996). However, severe hepatitis has been reported in pregnant women with up to 31.1% mortality (Boccia *et al.*, 2006; Guthmann *et al.*, 2006). HEV is transmitted enterically and can cause large outbreaks with several thousands of people involved (Fig. 1) (Chandra *et al.*, 2008; Jameel, 1999).

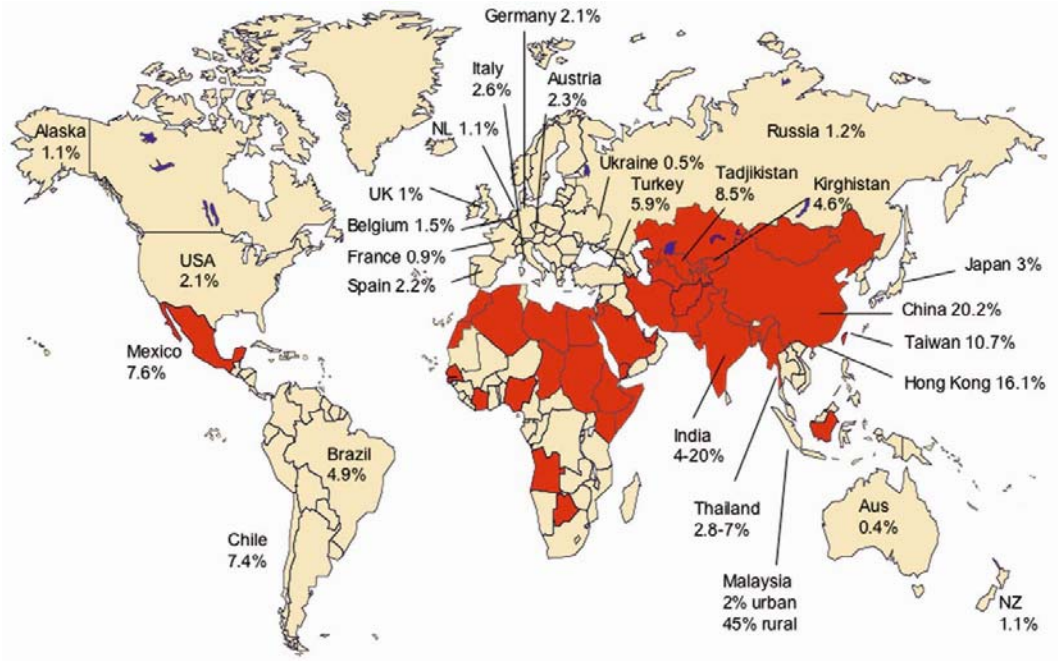


Figure 1: HEV endemic areas and global seroprevalence. Marked in red are areas in which >25% of acute viral hepatitis is due to HEV. Superimposed on this map are seroprevalence rates of HEV from various countries, determined in independent studies (Chandra *et al.*, 2008).

2.1.1. Taxonomy of HEV

Compared to other non-enveloped calcivuruses, HEV is slightly smaller eventhough its sedimentation coefficient in sucrose and the buoyant density are similar. In addition, there exists a superficial resemblance between HEV genome organization and that of viruses belonging to the *caliciviridae*, in which the non-structural polypeptides are located towards the 5' end and the structural polypeptides are located towards the 3' end (Berke *et al.*, 1997). The major change in genome organization is the location of ORF3 protein. In HEV, ORF3 is located between ORF1 and ORF2 (Fig. 2), where as a caliciviral protein corresponding to HEV-ORF3 is located at the 3' end of the viral genome (Jiang *et al.*, 1993). In case of HEV, it shows the presence of a cap structure at the 5' end of its genome (Kabrane-Lazizi *et al.*, 1999) whereas viruses of *caliciviridae* family have protein linked to the 5' end of the genome. With its homologous regions across the genome and with the presence of sub-genomic RNA, it was proposed to be a non-enveloped alpha-like virus (Tam *et al.*, 1991). Sequence analysis of the non-structural proteins RNA dependent RNA polymerase (RdRp) and helicase sequences of HEV and other positive-strand RNA viruses shows that they are phylogenetically related to rubella virus (*Togaviridae* family) and a plant furovirus (Beet necrotic yellow vein virus) (Koonin *et al.*, 1992). However, HEV has recently been classified as the prototype member in the *Hepevirus* genus of the family *Hepeviridae* (Chandra *et al.*, 2008).

2.1.2. Molecular biology of HEV

HEV is a positive-stranded RNA virus of ~7.5 kb containing three overlapping open reading frames (ORFs) (Purdy *et al.*, 1993). ORF1 (nt. 28 to 5107) encodes a 186 kDa polyprotein (Fig. 2) and gets processed (Sehgal *et al.*, 2006). The four conserved domains that code for enzymes in ORF1 includes methyltransferase (MeT), RNA helicase, a cysteine protease and RNA dependent RNA polymerase (RdRp) (Ansari *et al.*, 2000; Koonin *et al.*, 1992). ORF2 (nt. 5147 to 7126) encodes the viral capsid, a 88 kDa glycoprotein (Jameel *et al.*, 1996) that is glycosylated at Asn 310 position (Zafrullah *et al.*, 1999) and is stabilized under acidic pH (Zafrullah *et al.*, 2004). ORF3 encodes a protein of 13.5 kDa (Jameel *et al.*, 1996), which is phosphorylated at Ser 80 (Zafrullah *et al.*, 1997). ORF3 is is shown to interact with

non-glycosylated forms of ORF2 (Tyagi *et al.*, 2002) and is proposed to have a role in modulation of cell signaling by binding to SH3 domains (Korkaya *et al.*, 2001).

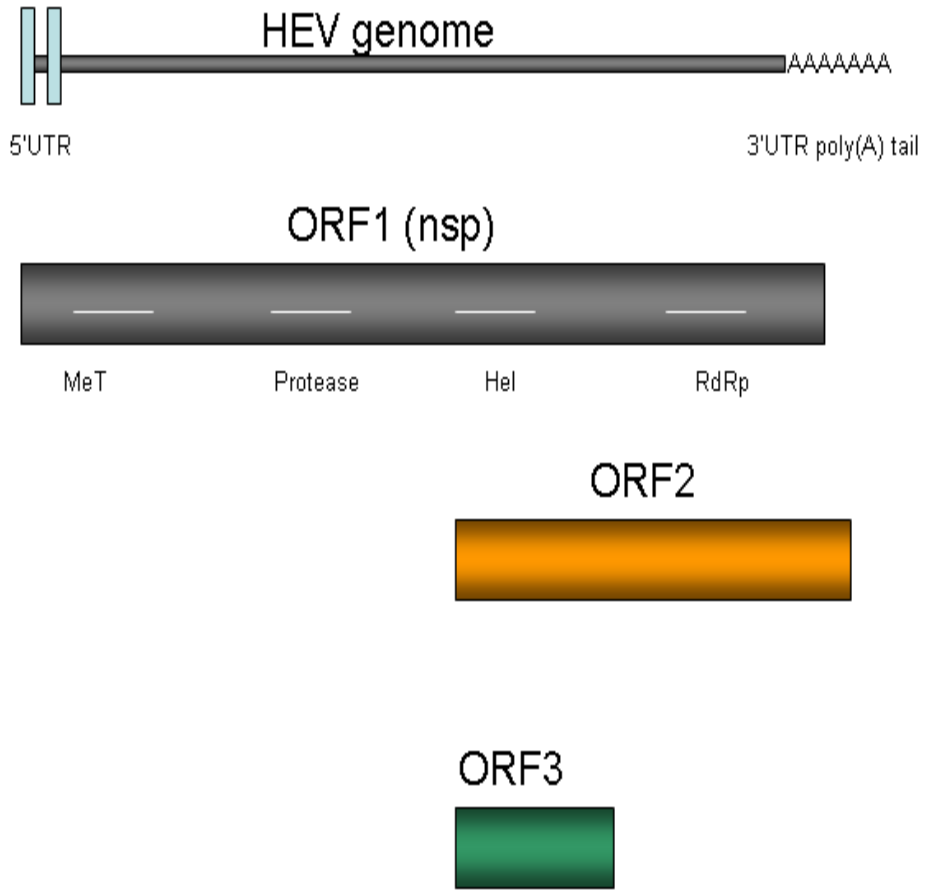


Figure 2: HEV genome and the proteins it encodes.

2.1.3. Structure of HEV

HEV is a small spherical, non-enveloped virus, approximately 27-31 nm in size. Immuno Electron Microscopy (IEM) identified HEV, for the first time, in feces of patients with hepatitis and from experimentally infected animals (Feinstone *et al.*, 1973). The first immune-electron micrograph of HEV isolated from the stool of a patient infected with HEV showed that hepatitis E virions were spherical and non-

enveloped, containing surface spikes as well as cup-like indentations, similar to caliciviruses (Bradley *et al.*, 1987; Feinstone *et al.*, 1973). Virions isolated from stools and bile of infected animals had a size variation ranging from 27 to 32, 34 and some times 38 nm with a mean diameter of 32.3 nm (Bradley *et al.*, 1988) (Fig. 3, 4A). Compared to HAV, the size of HEV particles were distinctly larger i.e. 27-29nm versus 29-31nm, (Ticehurst *et al.*, 1992). Structure of HEV-like particle at a resolution of 3.5-Å shows that the capsid protein contains 3 linear domains that form distinct structural elements: S, the continuous capsid; P1, 3-fold protrusions; and P2, 2-fold spikes (Guu *et al.*, 2009). Capsid protein shows different folding at the protruding and middle domains compared to the members of the families of *Caliciviridae* and *Tombusviridae*, whereas the shell domain shared the common folding. HEV has high resistant towards changes in pH and thus makes it to survive in the gastrointestinal environment causing infection (Zafrullah *et al.*, 2004).

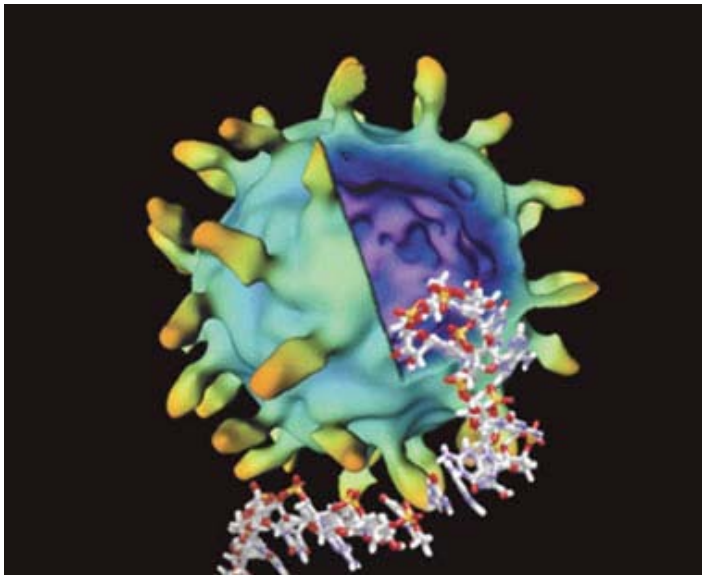


Figure 3: Hepatitis E virus Particle. The three-dimensional structure of a self-assembled, recombinant HEV particle has been solved to 22 A° resolution by cryo-electron microscopy and three-dimensional image reconstruction (Panda *et al.*, 2007).

2.1.4. HEV ORF2

Seacond ORF of HEV (ORF2) constitutes the structural polypeptide of the virus. Analysis of the amino acid sequence (aa) of ORF2 indicated a large hydrophobic domain at the N-terminal followed by region enriched in basic amino acids associated with RNA binding. Hydrophobic region of HEV ORF2 has a typical signal sequence and contain potential cleavage site (PA/PPP) (Tam *et al.*, 1991). This region is involved in virion maturation and also associated with endoplasmic

reticulum transport. ORF2 also has arginine rich region between residues 22 and 322, which makes 10% of aminoacids. These also make isolelectric point of 10.35 for the first half of protein. Due to this highly basic charge, this region is involved in the encapsidation of HEV RNA (Surjit *et al.*, 2004). HEV ORF2 being the main structural protein also constitute the main antigenic protein making it to be used for detection as well as region for designing vaccine.

2.1.5. HEV in swine

Complete sequence of HEV was published in 1991 for a Burmese strain (Tam *et al.*, 1991) this sequence covered greater than 93% nucleotide identity along with other Asian isolates including India, China, Nepal and Pakistan (Aye *et al.*, 1993; Gouvea *et al.*, 1998; Panda *et al.*, 2000; Tsarev *et al.*, 1992; van Cuyck *et al.*, 2003). Compared to Mexican isolate, Burmese isolate is slightly different and forms seacond genotype. Isolates found in in the United States and European countries including Austria, Greece, Italy, Spain and the United Kingdom as well as Argentina, where HEV is not endemic forms the third group HEV genotype (Pina *et al.*, 1998; Schlauder *et al.*, 1998; Schlauder & Mushahwar, 2001; Worm *et al.*, 1998; Zanetti & Dawson, 1994);(Fig. 4).

HEV was suggested to be a zoonosis after it was experimentally transmitted to domestic pigs (Balayan *et al.*, 1990). Sequence alignment of HEV strains US-1 and US-2 shows a high sequence similarity with swine HEV. Both these sequences shared more than 97% amino acid homology with swine HEV covering ORF1 and ORF2. Other than that, all these strains belong to HEV genotype 3. Human US-2 strain was also capable of infecting swine. There is also report in which cross-species infection occurs upon inoculating rhesus monkeys with swine HEV. All these data provides experimental evidence for cross-species infection with swine HEV and shows the possibility of pigs and other animals being a natural reservoir of HEV. Thus some of these HEV infections can be of zoonotic origin. Since the discovery of swine HEV, many other swine HEV isolates has been reported and sequenced. These include Argentina, Australia, Brazil, Canada, China, India, Indonesia, Japan, Korea, Kyrgyzstan, New Zealand, Spain, Sweden, Taiwan, Thailand, the United Kingdom (Lu *et al.*, 2006) and Mongolia, in addition to the United States (Lorenzo *et*

al., 2007). High prevelance of HEV in wild boar population has been reported in Germany (Adlhoch *et al.*, 2009; Kaci *et al.*, 2008; Schielke *et al.*, 2009) Various case studies with HEV have been reported in Germany on patients with close contact to animals which did not had a recent travel history outside Germany (Brost *et al.*) Even though antibodies against HEV have been reported in domesticated pigs (Baechlein *et al.*, 2009) no report has been published on the presence of viral RNA.

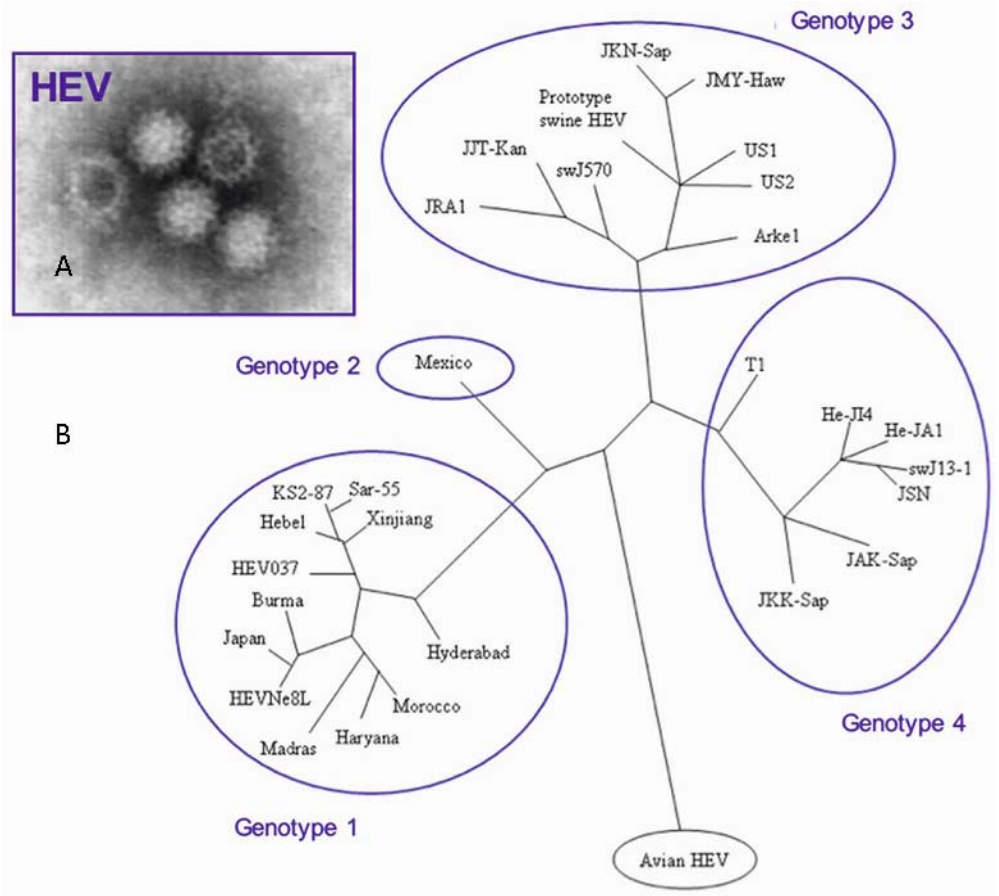


Figure 4: HEV and its genotypes. (A) Electron micrograph showing HEV particles. (B) A phylogenetic tree showing the distribution of human and swine HEV isolates (Chandra *et al.*, 2008).

2.1.6. Cloning and expression of HEV ORF2 in various expression systems, ORF2 as a vaccine candidate

HEV capsid or ORF2, which forms the outer cover of virus, has been proposed as a region for future vaccines (Acharya *et al.*, 2003; Robinson *et al.*, 1998). ORF2 has been expressed *in vitro* through coupled transcription and translation system as well as in heterologous expression systems including *E.coli* (Panda *et al.*, 1995),

mammalian cells using plasmids (Jameel *et al.*, 1996), alphavirus vectors (Torresi *et al.*, 1999; Torresi *et al.*, 1997), baculovirus expression system (Robinson *et al.*, 1998; Sehgal *et al.*, 2003), and in recombinant vaccinia virus (Carl *et al.*, 1994). HEV ORF2 gets translated to a protein of MW 72000 daltons carrying a putative signal sequence and potential sites for glycosylation (Jameel *et al.*, 1996; Tam *et al.*, 1991). Expression of ORF2 in mammalian cells (COS-1 and HepG2) using plasmid-based expression resulted in a glycoprotein of MW 88000 daltons that was shown to be expressed intracellularly, as well as on the cell surface, containing the potential to form non-covalent homodimers (Jameel *et al.*, 1996). Multiple ORF2 species were observed in both plasmid-based expression and Semliki Forest virus expression system (Torresi *et al.*, 1997) with molecular masses estimated as 72,–74, , 79,–84, kDa (gPORF2), 84–88 kDa (ggPORF2), with the larger size of the proteins corresponding to their glycosylation status (Jameel *et al.*, 1996; Zafrullah *et al.*, 1999). Glycosilation status of ORF2 was confirmed by endoglycosidase H and tunicamycin treatments. This is further proved when ORF2 was pulse chased and protein of 82, 84, and 88 were observed. This shows that ORF2 is first translated into a mature high Mw protein and gets processed to mature form by proteases and then gets glycosylated. The glycosylation sites were investigated by mutational analysis (Zafrullah *et al.*, 1999).

It is widely accepted that a successful production of HEV vaccine is attainable as HEV has only one serotype. As HEV cell culture system is not robust inactivated or attenuated vaccines are not feasible. High-risk groups to HEV include people living in HEV-endemic areas, travelers visiting these areas and pregnant women. This means that vaccines against HEV will have world wide acceptability. One of the earliest reported study utilizing recombinant protein to induce antibody response to HEV illustrated the importance of ORF2 protein in immunity against HEV (Purdy *et al.*, 1993).

2.1.7. Infectious system of HEV

Various groups have tried to propagate HEV *in vitro* in various cell lines (Divizia *et al.*, 1999; Huang *et al.*, 1992; Kazachkov Yu *et al.*, 1992; Meng *et al.*, 1997; Wei *et al.*, 2000), still an efficient cell culture system has not been reported. Most of the

reports made infectious cDNA clones replicating in non-human primates or pigs. These were also not efficient in HEV propagation. This is mainly because of the virus inability to spread in cell culture system (Emerson *et al.*, 2004; Emerson *et al.*, 2001; Graff *et al.*, 2005; Huang *et al.*, 2005; Panda *et al.*, 2000). One of the promising construct developed using PLC/PRF/5 and A549 cells involve genotype 3 HEV (strain JE03-1760F), obtained from a fecal specimen from a Japanese hepatitis E patient (Yamada *et al.*, 2009); (Fig. 9). This system was further used by us to study the HEV propagation and egress.

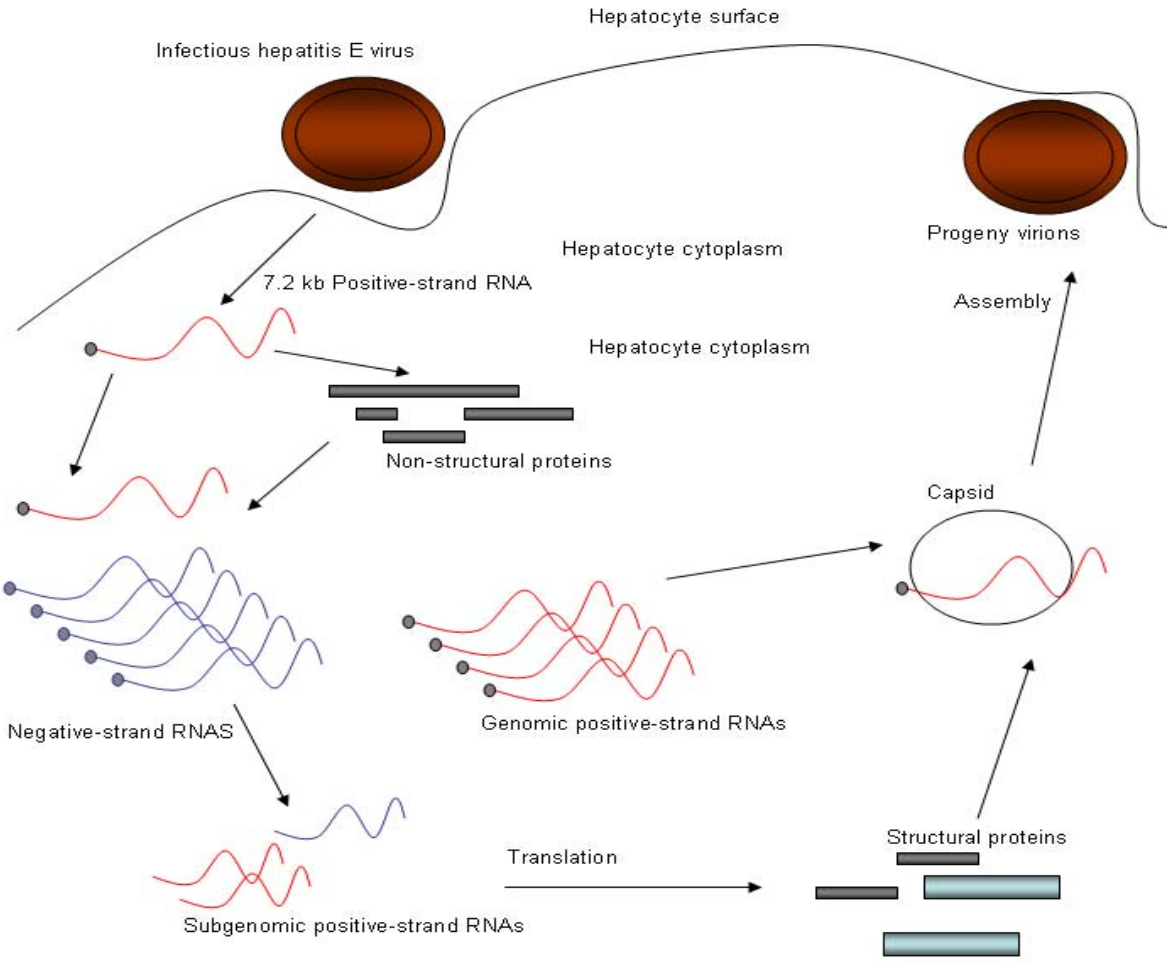


Figure 5: A proposed model for hepatitis E virus replication and egress. Drawn according to (Jameel, 1999).

2.2. Materials and Methods

2.2.1. Materials

2.2.1.1. Devices

Camera	Olympus, Hamburg
Cell incubator	Type B 5060 EC CO_2, Heraeus, Hanau
	Labotect, Göttingen
Centrifuge	Eppendorf, Hamburg
	Biofuge fresco, Heraeus
	AllegraTM 21R, Beckman Coulter, Fullerton USA
Electroblotting chamber	Bio-Rad, München
Electrophoresis chamber	For agarose gels: Wide mini sub cell,
	Bio-Rad, München
	For polyacrylamide gels: mini protean 3
	Bio-Rad, München
Heating block	DRI-BLOCK DB3, Techne, Jahnsdorf
Incubator	Heraeus, Hanau, Arbeitsbank, BDK
	Luft- und Reinraumtechnik
	Sonnenbuhl-Genkingen
Laminar Flow	Arbeitsbank Heraeus-Kendro, Hanau
Minishaker	MS2, IKA, Staufen
Microscopes	Axiovert 25, Carl Zeiss, Göttingen
	Axiovert 40 Carl Zeiss, Göttingen
Microwave oven	Micro-Chef FM 3915 Q, Moulinex
PCR Thermocycler	Personal Cycler, Biometra, Göttingen
	iCycler, Bio-Rad, München
pH-meter	pH 211, Microprocessor pH meter,
	Hanna-instruments, USA
Pipettors	Finn pipettors (5-40 µl, 40-200 µl, 200-
	1000 µl), Labsystems, Finnland
	Eppendorf pipettors (0.5-10 µl, 10-100

	µl, 200-1000 µl), Eppendorf, Hamburg
Power supply	Power Supply Model 3000 X, Bio Rad, München
Shaker	Vortex Genie, Bender & Hobein, München
Sonicator	Bandelin Electronic, Berlin
Spectrophotometers	Smart SpecTM3000, Bio-Rad, München Spectra, Tecan, Crailsheim
Table top centrifuge	Centrifuge 5415 C, Eppendorf, Hamburg
UV transilluminator	Bio-View, Biostep, Jahnsdorf
Water bath	GFL1086, GFL, Wunstorf

2.2.1.2. General material

Cryo tubes, 2 ml	Greiner, Frickenhausen
Culture dishes, 35 mm diameter	Becton Dickinson, Heidelberg
Culture dishes, 90 mm diameter	Nunc, Wiesbaden
Culture flasks, 80 cm^2	Nunc, Wiesbaden
Filter paper (Whatman 3 MM)	Whatman, Göttingen
Filtration units (sterile Millex units)	Millipore, Eschborn
Glass pipettes, 1 ml, 5 ml, 10 ml	Hirschmann, Eberstadt
Glass ware	Schott, Mainz ; Brand, Wertheim
Syringes, 20 ml, sterile	Braun, Melsungen
Nitrocellulose membrane	Bio-Rad, München Amersham, Freiburg
PCR tubes (0.2 ml)	PeqLab, Erlangen
Petri dishes (AD94/H16 mm)	Roth, Karlsruhe
Pipette tips	Braun, Melsungen
Plastic tubes, 14 ml	Greiner, Frickenhausen
Plastic tubes, 50 ml	Nunc, Wiesbaden
Plastic reaction tubes, 1.5 ml	Brand, Wertheim
Pursept®-A disinfectant solution	Merz via Fischer, Frankfurt a. m

Safe Skin Satin Plus powder-free latex gloves	Kimberly Clark, Koblenz-Rheinhafen
Sterile filters (0.2 µm and 0.45 µm)	Renner GmBH, Dannstadt
Sterile single use serological pipets (10 ml)	Falcon via Multimed, Kirchheim Teck
X-ray film	Amersham, Freiburg

2.2.1.3. Chemicals

Ammonium peroxodisulfate (APS)	Fluka, Steinheim
Bradford assay dye reagent	Bio-Rad, München
Bromophenol blue	Fluka, Steinheim
BSA	Sigma-Aldrich, Steinheim
Calcium chloride dehydrate	E. Merck, Darmstadt
Coomassie brilliant blue R 250	Sigma-Aldrich, Steinheim
Dimethylsulfoxide (DMSO)	Sigma-Aldrich, Steinheim
EDTA	Roth, Karlsruhe
Glycerol	Roth, Karlsruhe
Glycine	Roth, Karlsruhe
Mercaptoethanol	Sigma-Aldrich, Steinheim
$MnCl_2$	Sigma-Aldrich, Steinheim
NaCl	Sigma-Aldrich, Steinheim
$NaHCO_3$	Sigma-Aldrich, Steinheim
PageRuler™ prestained protein ladder	MBI Fermentas
Potassium chloride	E. Merck, Darmstadt
SDS	Fluka, Steinheim
Sucrose	Roth, Karlsruhe
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Sigma-Aldrich, Steinheim
Protease inhibitor cocktail EDTA free	Thermo Scientific, USA
RIPA buffer	Thermo Scientific, USA
Tris	Roth, Karlsruhe
Tween® 20	Fluka, Steinheim

2.2.1.4. Kits

Enhanced chemiluminescence (ECL) detection reagent	Amersham, Freiburg
high pure viral RNA kit	Roche, Berlin
QIAquick gel extraction kit	Qiagen, Hilden
QIAquick PCR purification kit	Qiagen, Hilden
RNeasy RNA isolation kit	Qiagen, Hilden
QIAprep Spin Miniprep Kit	Qiagen, Hilden
QIAGEN Plasmid Maxi Kit	Qiagen, Hilden
MEGAscript® T7 Kit	Ambion, Applied biosystems, California, USA
m^7G Capping System	Epicentre Biotechnologies, USA

2.2.1.5. Reagents for molecular biology

Agarose	PeqLab, Erlangen
BSA (100x)	New England Biolabs, Frankfurt a.M.
Dithiothreitol (DTT), 0.1 M	Gibco BRL, Karlsruhe
GeneRuler 1 kb DNA ladder	MBI Fermentas, St. Leon-Rot
GeneRuler 100 bp DNA ladder plus	MBI Fermentas, St. Leon-Rot
Deoxyribonucleoside triphosphate (dNTP; 10 mM each)	PeqLab, Erlangen
Ethidium bromide	Sigma-Aldrich, Steinheim
PCR primers	TIB MOLBIOL GmbH, Berlin Eurofins MWG Operon, Ebersberg
RNAsin ribonuclease inhibitor (40 U/µl)	Promega, Mannheim
T4 DNA Ligation Buffer (10x)	MBI Fermentas, St. Leon-Rot New England Biolabs, Frankfurt a.M.
Turbofect transfection reagent	Fermentas GmbH
TransIT®-mRNA Transfection Kit	Mirus Bio LLC Madison, USA

2.2.1.6. Enzymes for molecular biology

Restriction enzymes	New England Biolabs, Frankfurt a.M.
Calf intestine alkaline phosphatase (1U/µl)	New England Biolabs, Frankfurt a.M.
HotStart Taq DNA polymerase	Qiagen, Hilden
MLV Reverse Transcriptase	Qiagen, Hilden
Pfu turbo High-Fidelity DNA polymerase	Stratagene, Amsterdam
RNAse A	Qiagen, Hilden
T4 DNA ligase	MBI Fermentas, St. Leon-Rot New England Biolabs, Frankfurt a.M.

2.2.1.7. Constituents and reagents for bacterial and mammalian cell cultures

Antimycotic/antibiotic	Sigma-Aldrich, Steinheim
Carbenicillin, disodium salt	Roth, Karlsruhe
Dulbecco's modified Eagle's medium (DMEM) powder lacking pyruvate and $NaHCO_3$	GibcoBRL, Karlsruhe
Fetal calf serum (FCS)	Biochrome, Berlin
Kanamycin	Applichem, Darmstadt
LB-agar powder	Fluka, Steinheim
LB-broth powder	Fluka, Steinheim
Penicillin G, potassium salt	Serva, Heidelberg
Superoptimal broth, catabolite repression (SOC) medium	Novagen, Schwalbach/Ts.
Streptomycin sulfate	Serva, Heidelberg
Trypsin	ICN, Eschwege

2.2.1.8. Antibodies

His Probe, (rabbit) polyclonal antibody	Santacruz Biotechnology, USA
Anti-Mouse IgG (Goat) HRP conjugated	Santacruz Biotechnology, USA

Anti-Rabbit IgG (Donkey) HRP conjugated	Santacruz Biotechnology, USA

2.2.1.9. Bacterial strains

Top10 *E.coli* cells	Invitrogen, Karlsruhe
BJ5183 *E.coli* cells	Stratagene, Amsterdam
NovaBlue Singles™ competent cells	Novagen, Schwalbach/Ts.
Bl21 cells	Invitrogen, Karlsruhe

2.2.1.10. Mammalian cell lines

HEK 293 cells	American Type Culture Collection (ATCC)
HuH7 cells	American Type Culture Collection (ATCC)
PLC/PRF/5	American Type Culture Collection (ATCC)

2.2.1.11. Vectors

pcDNA 3.1/v5-His-TOPO	Invitrogen, Karlsruhe
pET-28a+	Merck KGaA, Darmstadt
pSK-HEV-2	Kind gift of S. Emerson and R. Purcell, NIH, Maryland
pAdTrack-CMV	Addgene, Cambridge, USA
pJE03-1760/wt	Kind gift of Hiroaki Okamoto, Yakushiji, Japan

2.2.1.12. Primers list

Name of the primer	**Sequence**
HE040	5’-cccttrtcctgctgagcrttctc-3’ [r = a or g)
HE044	5’-caagghtggcgytckgttgagac-3’ [h=a, t, or c; y =t or c; and k = g or t]
HE110-2	5’-gytckgttgagacctcyggggt-3’, 5’-gytckgttgagaccacgggygt-3’, 5’-gytckgttgagacctctggtgt-

	3’
HE041	5’-ttmacwgtcrgctcgccattggc-3’ [m = a or c, w =a or t]
HEV ORF2 fwd primer	5’-ccatgggcatgcgccctcggcctattttg-3’
HEV ORF2 rev primer	5’-ctcgagtaactcccgagttttacccac-3’
Bgl2f2_fw	5’-agatctatgcgccctcggcctattttg-3’
Sal1f2_rev	5’-gtcgacaactcccgagttttacccaccttc-3’
250 fwd	5’-cccgggtaatacgactcactatagggaggcagacca catatgtggtcg-3
250 rev	5’-ccatggtcagctcattatggataacacgctggatgg-3’

2.2.2. Sample collection

The swine serum samples were collected in Mecklenburg-Vorpommern, Brandenburg, Nordrhein-Westfalen and Niedersachsen with the help of Dr. K. Depner, Friedrich-Loeffler-Institut, Greifswald - Insel Riems.

2.2.3. RNA isolation

Viral RNA from sera was isolated using Roche high pure viral RNA kit according to the manufacture’s instruction. Briefly, the sample was mixed with working solution containing carrier RNA and centrifuged in a table top centrifuge (Eppendorf). After washing, the viral RNA was eluted using an elution buffer and was stored at -80 °C until use.

2.2.4. Detection of HEV by nested RT-PCR

RNA isolated from serum was reverse transcribed with MLV reverse transcriptase (Invitrogen) and an antisense primer (primer HE040; 5’-cccttrtcctgctgagcrttctc-3’ [r = a or g) specific for the HEV ORF2 as described before (Mizuo *et al.*, 2002) and was subjected to nested PCR. A region of the ORF2 sequence was amplified with the primer pair HE044 (sense primer; 5’-caagghtggcgytckgttgagac-3’ [h=a, t, or c; y =t or c; and k = g or t]) and HE040 in the first round and HE110-2 (sense primer; mixture of three sequences, 5’-gytckgttgagacctcygggg t-3’, 5’-gytckgttgagaccacgggygt-3’ and 5’-gytckgttgagacctctggtgt-3’) and HE041 (antisense primer; 5’ttmacwgtcrgctcgccattggc3’ [m = a or c, w =a or t]) in the second round (ORF2 PCR). The PCR amplification was carried out for 35 cycles in the first round (94 °C

for 30 s [an additional 2 min was used in the first cycle], 55 °C for 30 s, 72 °C for 75 s [an additional 7 min was used in the last cycle]) and then for 30 cycles in the second round under the same conditions used for the first round; here it was further extended for 60 s. PCR product size from the first amplification was 506 bp, and that of second round was 458 bp. The amplified products were analyzed on a 1.5% agarose gel, and photographed under UV-transilluminator (Bio-View).

2.2.4.1. Sequence analysis of PCR products

The amplification product from sample 3205-45 was column purified using Qiagen PCR purification kit according to manufacture instructions and sent for sequencing (GATC Biotech) for both forward and reverse directions. The sequences were analyzed for the similarity using Basic Local Alignment Search Tool (BLAST) http://blast.ncbi.nlm.nih.gov/Blast.cgi. Tree view of the sequence was constructed using the tool BLAST Tree view (http://blast.ncbi.nlm.nih.gov/blast/treeview/treeView.cgi).

2.2.5. Expression analysis of HEV ORF2, using mammalian expression system

2.2.5.1. Cloning of HEV ORF2 to pcDNA™3.1D/V5-His-TOPO

HEV ORF2 was amplified using primers, Hev ORF2 fwd primer 5'-ccatgggcatgcgccctcggcctattttg-3', HEV ORF2 rev primer 5'-ctcgagtaactcccgagttttacccac-3' using HEV clone pSK-HEV-2 (GenBank accession no. AAF444002, Kind gift of S. Emerson and R. Purcell, NIH, Maryland) as template and amplified using PFU turbo (Stratagene) polymerase enzyme. The PCR product was A-tailed using taq polymerase (Invitrogen) by incubating it with 0.5 units of taq at 72 °C for 10 min. It was further gel purified and cloned to pcDNA™3.1D/V5-His-TOPO vector (Invitrogen) (Fig. 6). Positive clones were screened by restriction digestion and sequencing at both forward and reverse directions.

2.2.5.2. Transfection and protein extraction

HEK293 cells were used for transfection using turbofect transfection reagent (Fermentas), according to manufacturer's ' instructions. 48 hours post transfection, cells were washed with PBS, and incubated with RIPA buffer and protease inhibitor

cocktail EDTA free (Thermo Scientific), for 10 min and lysed with RIPA buffer. The lysate was centrifuged at 13,000 RPM for 10 min and the supernatant was used for protein estimation.

2.2.5.3. Protein assay (Bradford assay)

Protein concentration was assayed using Bio-Rad Bradford dye reagent concentrate and was performed according to Bradford (1976).The reagent was diluted (1:6 in water) and was mixed with 10 µl of the sample, which makes a final volume of 1 ml, followed by 15 min RT incubation. 96-well plate containing 400 µl reaction mixture was analyzed at a wavelength of 595 nm in a microplate reader. The final concentration was determined after calculating through standard curve using BSA with a range from 10 to 100 µgm.

2.2.5.4. SDS-PAGE

SDS PAGE was carried out as described by(Garfin, 1990; Laemmli *et al.*, 1970) with the modifications by (Garfin, 1990).

Solutions

Acrylamide solution: 29.2% (w/v) acrylamide, 0.8% (w/v) bisacrylamide (Sigma)
SDS: 10% (w/v)
APS solution: 10% (w/v)
TEMED
Running gel buffer: 0.5 M Tris / HCl, pH 8.8
Stacking gel buffer: 1.5 M Tris /HCl, pH 6.8
Electrode buffer: 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS
Five-fold concentrated sample buffer: 0.16 M Tris / HCl, 4% (w/v) SDS, 20% (w/v) glycerol, 0.38 M mercaptoethanol, 0.008 (w/v) bromophenol blue; pH 6.8

Preparation of the gels

The gel size was 8 cm x 10 cm x 1 mm. The stacking gels were prepared with 5% acrylamide at pH 6.8, the running gels with 10-12% acrylamide depending on the

protein size at pH 8.8. The scheme as shown in table 1: was used for casting the gels:

Table 1: Scheme for casting 10% SDS-PAGE gel

Solution	10% running gel	5% stacking gel
30% Acrylamide solution	3.3 ml	0.83 ml
Running gel buffer	2.5 ml	
Stacking gel buffer		0.63ml
Water	4.0 ml	3.4 ml
10% (w/v) SDS	100 µl	50 µl
TEMED	20 µl	5 µl
10% (w/v) APS solution	37.5 µl	200 µl

Preparation of the samples

5X protein sample buffer was added to the samples and filled up with H_2O. The protein buffer mix was then heated to 95 °C for 5 min. After collecting the condensate by brief centrifugation, the samples were loaded to the gel.

Electrophoresis

Electrophoresis was carried out under constant current 20 mA at RT. When the bromophenol blue front had reached the end of the running gel, the process was stopped. The gels were either stained with Coomassie Brilliant Blue R 250 or used for Western blotting.

2.2.5.5. Coomassie blue staining of polyacrylamide gels

Solutions

Staining solution: 50% methanol, 40% H_2O, 10% glacial acetic acid and 1% (w/v) Coomassie Brilliant Blue R 250 (in water). Destaining solution: Methanol: H_2O: glacial acetic acid (3:6:1).

Experimental procedure

Polyacrylamide gels were incubated with staining solution at RT for 10 min, afterwards; gels were briefly rinsed with water and transferred to destaining solution. Once the blue background had disappeared and single protein bands became

visible, the destaining solution was discarded and the gel was washed with ddH_2O. Protein bands were documented using digital scanner.

2.2.5.6. Western blot analysis with chemiluminescence detection

Protein transfer solutions

Transfer buffer: 25 mM Tris / HCl, 192 mM glycine; pH 9.0

Ponceau S solution: 0.2% (w/v) Ponceau S in 3% (w/v) trichloroacetic acid

Experimental procedure

Proteins (30 µg) were separated through discontinuous SDS PAGE. Gels excised out were rinsed once with transfer buffer. The protein bands were then transferred from the gel to a nitrocellulose membrane using a modification of the protocol described by Burnette (1981) as detailed below.

Nitrocellulose was packed as a “sandwich” containing 3 pieces of filter paper with gel and nitrocellulose membrane in the middle. This was further used for transfer using XCell Blot module (invitrogen). Transfer was carried out at 120 mA at 4 °C for 1 h.

Detection of protein bands with the enhanced chemiluminescence (ECL) reagent

Solutions

Washing buffer: 20 mM Tris / HCl, 150 mM NaCl, 0.02% (v/v) Tween 20; pH 7.4 (PBST)

Blocking solution: 5% milk powder in PBST

Substrate solution: 250 µl ECL solution I + 6 µl ECL solution II (Amersham)

Experimental procedure

The membrane was incubated overnight with 10 ml blocking solution at 4 °C to block unspecific binding. After blocking the membrane was further incubated with appropriate antibodies. Incubation was carried out from 1h to overnight. The membrane was washed three times for 5 min with 25 ml TBST, followed by incubation with 20 ml of the secondary antibody solution for 1 hour to overnight. The membrane was washed again three times for 5 min with 25 ml TBST, rinsed briefly

with PBS and then the ECL detection solution was added dropwise to cover the membrane. After 2 min, the solution was removed and the membrane placed in a exposure casette. An X-ray film (Amersham) was later exposed to the membrane for different time-ponts in the exposure cassette. The film was developed in an X-ray film developing machine.

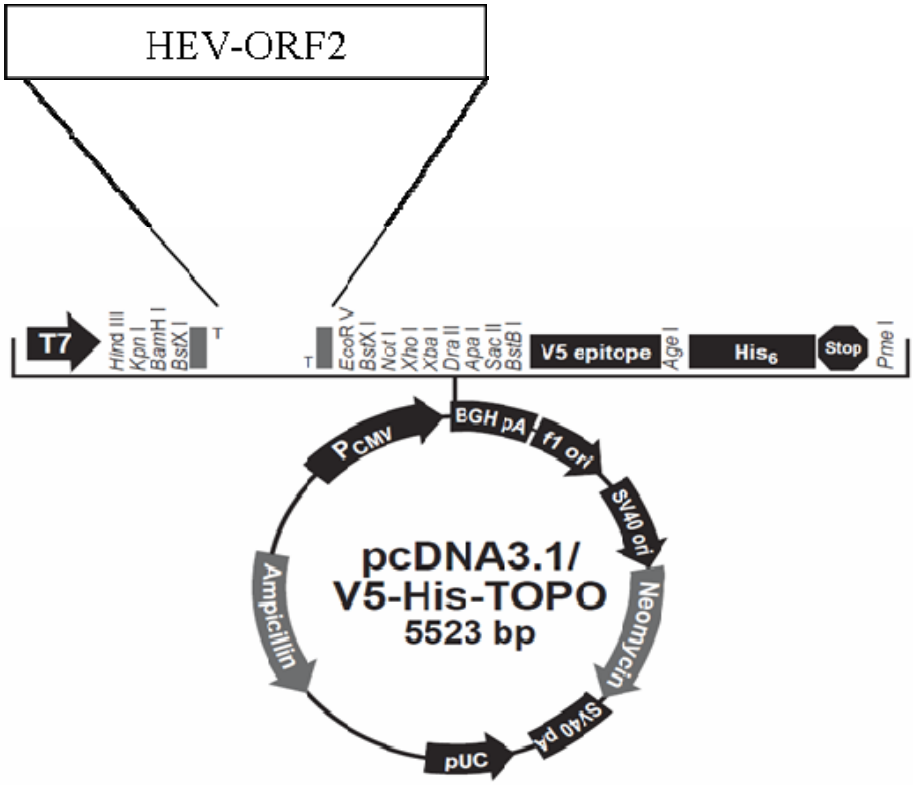


Figure 6: Vector map of clone HEV ORF2 in pcDNA 3.1/V5-His-TOPO

2.2.6. Bacterial expression system

2.2.6.1. Cloning of HEV ORF2 into pET-28a

PcDNA-ORF2 was digested with *BamH*I and *Xho*I to sub-clone into *E. coli* expression vector pet28a (Novagen) (Fig. 7). Positive clones were screened by restriction enzyme digestion.

2.2.6.2. Protein purification

N-terminal His-tagged pet28a-ORF2 was transformed to *Bl21* cells (Invitrogen). The bacterial culture was grown at 37 °C until OD600 had reached 0.6, induced with IPTG at a concentration of 1 mM and incubation continued for additional 6 h. The cells were pelleted and resuspended in 6 ml of buffer containing PBS and lysed by sonication for 3 min. The cell lysate was spun at 5,000 RPM for 20 min and

supernatant was discarded. The pellet which contained HEV ORF2 as inclusion body was resuspended in 5 ml of buffer containing 8 M urea in PBS. Solubilized inclusion body was further centrifuged and imidazole was added to the supernantant at a final concentration of 10 mM. Precharged and prewashed Ni-NTA beads (Invitrogen) of 0.6 ml of bed volume were mixed with the lysate at RT for 1 h. It was then loaded to the column and washed with 50 ml of PBS containing 20 mM imidazole and 8 M urea. Protein bound to the column was eluted using elution buffer containing PBS, 8 M urea, and 300 mM imidazole. Eluents were analyzed by SDS-PAGE and confirmed by Western blot using anti-His or anti-ORF2 antibodies.

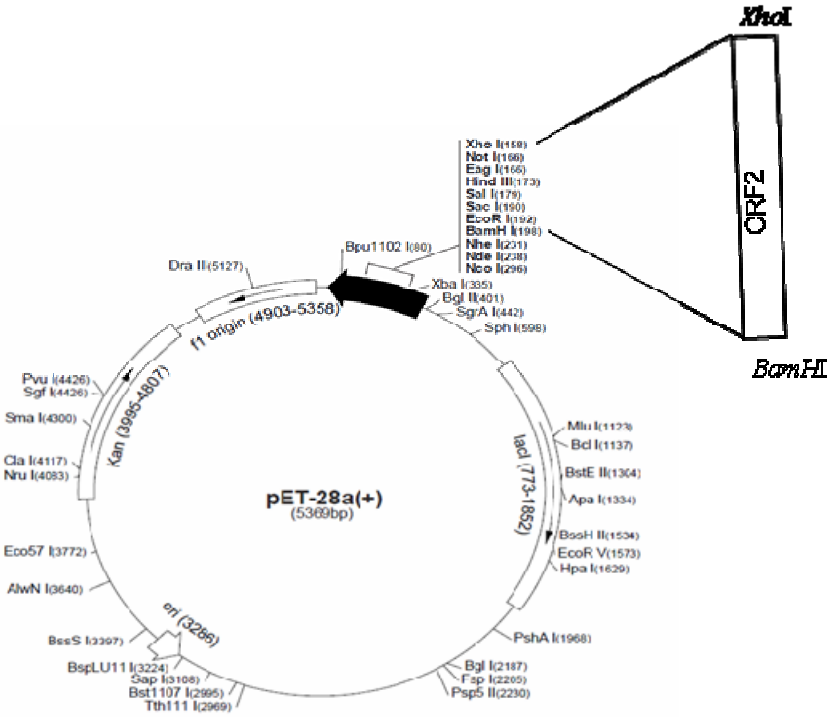


Figure 7: Vector map of pET-28a-ORF2

2.2.7. Adenovirus expression system

Recombinant adenovirus was synthesized using the AdEasy system (Stratagene); (He *et al.*, 1998). Briefly, HEV ORF2 was amplified by PCR from full-length HEV clone pSK-HEV-2 with primers bgl2f2_fw 5'-agatctatgcgccctcggcctattttg-3' and Sal1f2_rev 5'-gtcgacaactcccgagttttacccaccttc-3' and cloned into the vector, pAdTrack-CMV shuttle plasmid. Clones were verified by sequencing in both forward and reverse directions. Shuttle vector pAdTrack-CMV-F2 was linearized with *pme*I and electroporated to *BJ5183 E. coli* cells.

2.2.7.1. Production of adenovirus

*Pac*I-digested recombinant adenoviral DNA (4 ug) was transfected in HEK293 cells in T-25 flask using Turbofect (Fermentas) according to manufacturer's instructions as shown in Fig. 8. Transfected cells were monitored in DMEM containing 10% FCS for cytopathic effects (CPE), which were observed by approximately 6 to 10 days post-transfection. Cells were harvested and exposed to three cycles of freezing in dry ice and thawing at 37 °C water bath to release the virions from the cells. The supernatant was aliquoted in eppendorf tubes and stored at -80 °C until use.

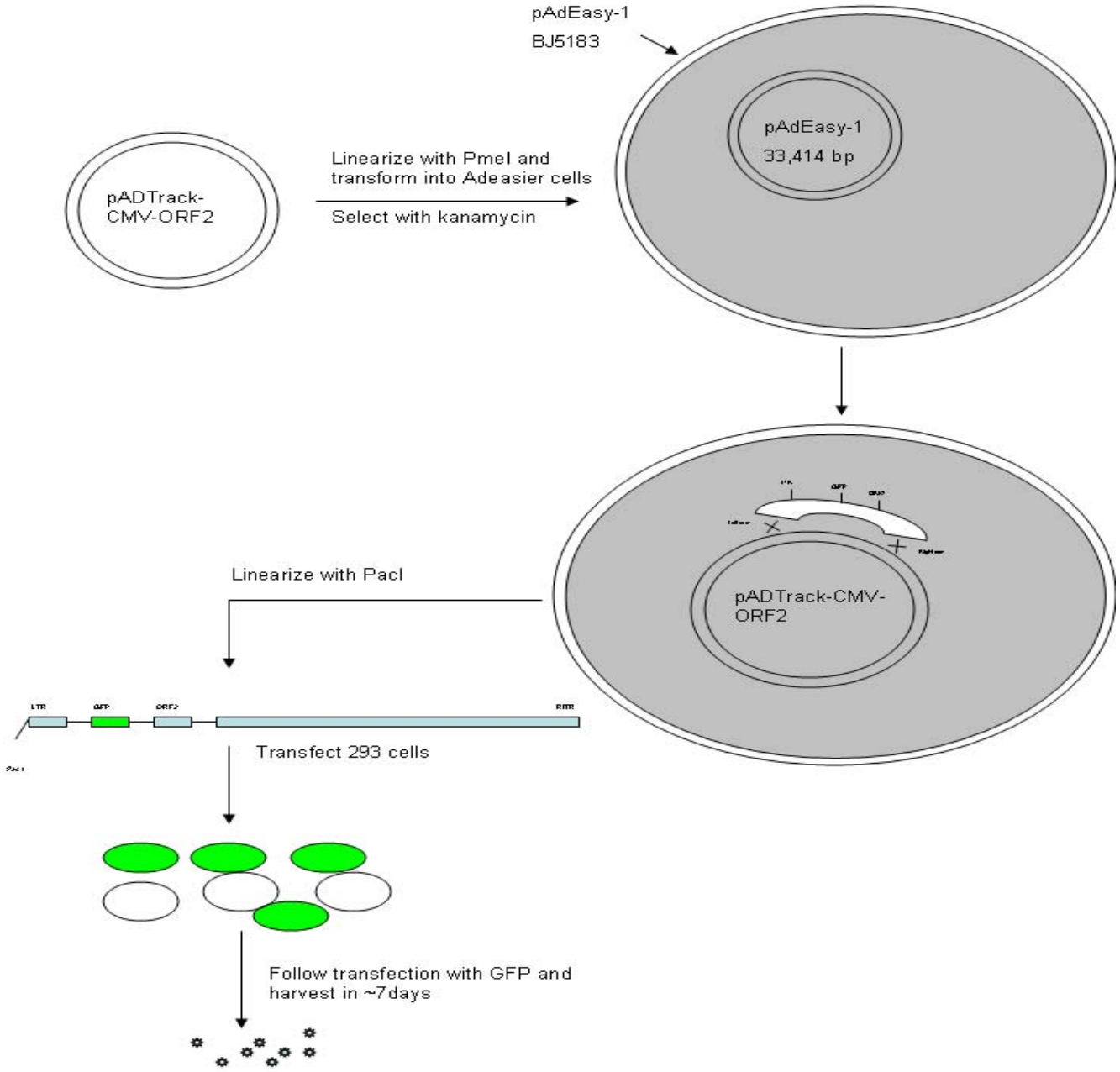


Figure 8: Schematic representation of the AdEasy technology. Drawn according to (He *et al.*, 1998; Luo *et al.*, 2007)

2.2.7.2. Virus generation, infection of HEK-293 cells with recombinant adenovirus

HEK-293 cells were plated in 25-cm^2 tissue culture flasks (Nunc) at 80–90% confluency (approximately 3 × 10^6 cells per flask in 7 ml complete DMEM) 6–15 h before infection. They were then infected by adding viral supernatant from primary transfection. Viral infection was observed using the tracking protein GFP. CPE or cell lysis will be seen at 2- 3 days post-infection. For rest of the experiments cell were infected at an MOI of 10. Cells were harvested once the cells have rounded up and most of the cells detached (3 to 4 days). Infected cells were used for further experiments or stroed at -80 °C until use.

2.2.7.3. Total RNA extraction

Total cellular RNA was isolated using RNeasy RNA isolation kit from Qiagen. The cells (approximately 10^7 cells) were collected by trypsinisation and pelleted by brief centrifugation. The cell pellet was disrupted by adding 600 µl of buffer RLT and repeated pipetting. Then 1 volume of 70% ethanol was added to the lysate, and the solution was mixed well by pipetting. The sample was transferred to the supplied RNeasy spin column and centrifuged (13,000 rpm, RT, 15 s). The flow-through was discarded and the membrane of the column was washed by the addition of 700 µl of buffer RW1 and a brief centrifugation (13,000 rpm, RT, 15 s). The flow-through was discarded and the membrane of the column was washed again with buffer RPE (ethanol added) by centrifugation (13,000 rpm, RT, 1 min). This was followed by brief centrifugation (13,000 rpm, RT, 1 min) to prevent ethanol carry-over and to dry the column. The column was subsequently placed in a 1.5 ml sterile microfuge tube, and the total RNA was eluted with 50 µl of RNase-free water by centrifugation (13,000 rpm, RT, 1 min). The concentration of isolated RNA was measured photometrically

2.2.7.4. Photometric determination of nucleic acid concentration

The concentration of RNA was determined photometrically. For this, the absorbance (OD) was measured at a wavelength of 260 nm in biophotometer (eppendorf). The solvent in which the sample was diluted was used as a reference (blank). An OD_{260} value of 1 corresponded to a concentration of 40 µg/ ml. The sample volume was 3

µl. To estimate the purity of samples, the OD_{260}/OD_{280} ratio was calculated. A protein-free nucleic acid solution was assumed to display a ratio from 1.8 up to 2.0.

2.2.7.5. Reverse transcription of RNA and PCR

Total cellular RNA was isolated using RNeasy RNA isolation kit according to the manufaturers advice. The reaction mixture for reverse transcription of RNA contained 5 mM $MgCl_2$, 10 mM each of dGTP, dATP, dTTP and dCTP. Moloney murine leukemia virus reverse transcriptase (MLV RT), 10 pmol antisense primer Sal1f2_rev 5,-gtcgacaactcccgagttttacccaccttc-3', specific for the HEV ORF2 and 1 µg total RNA in 50 µl of RT-buffer. All the pipetting were done on ice. The mixture was incubated for 1 h at 37 °C, followed by heat inactivation at 95 °C for 5 min. Sample with out the addition of RT was used as control. cDNA was then subjected to PCR with primers bgl2f2_fw 5'-agatctatgcgccctcggcctattttg-3' and Sal1f2_rev 5'-gtcgacaactcccgagttttacccaccttc-3'.

2.2.8. Propagation of HEV, *in vitro* transcription

PJE03-1760F (Fig. 9) plasmid was linearized by digestion with *NheI* and the template was further purified using qiagen QIAquick PCR purification kit. The genomic RNA was transcribed with Megascript kit (Ambion) by *in vitro* transcription with T7 polymerase and 4 ribonucleotide solutions (ATP, CTP, GTP, and UTP). Transcription reaction products were treated with RNase-free DNase (1U) at 37 °C for 20 min, followed by ethanol precipitation and purification of RNA. Purified RNA was dissolved in RNase free water.

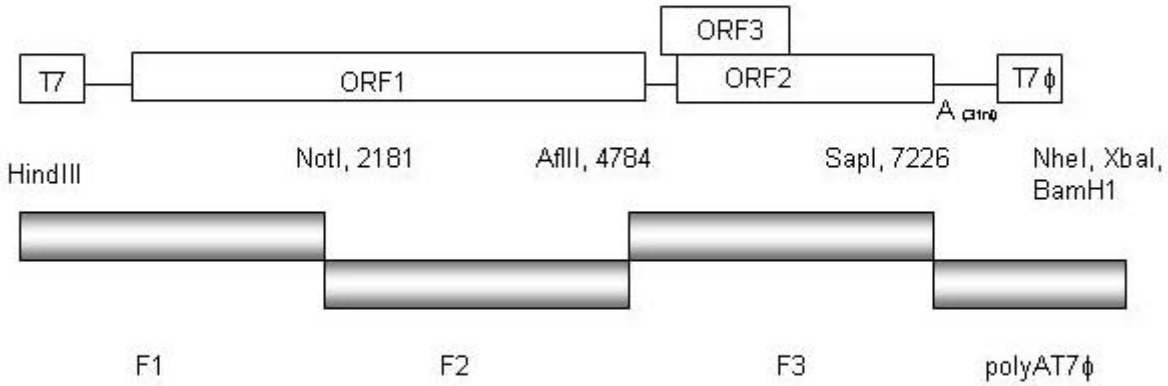


Figure 9: Structure of the infectious HEV construct. Drawn according to (Yamada *et al.*, 2009)

2.2.8.1. Capping

For capping, the ScriptCap m^7G Capping System (Epicentre Biotechnologies) was used according to manufacturer's protocol. Briefly, 45 µgm of RNA was heat denatured by incubating at 65 °C for 10 min and transfered to ice immediately. RNA was then mixed with other components containing 10X ScriptCap Capping Buffer, 10 mM GTP, 2 mM SAM, RNase Inhibitor ScriptCap Capping Enzyme and incubated at 37 °C for 60 min. After capping, the RNA was again purified by ethanol precipitation and redissolved in RNAse free water.

2.2.8.2. Transfection of HEV RNA

PLC/PRF/5 cells, grown in culture medium consisting of Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) fetal calf serum (FCS), 100 U/ml penicillin G, 100 µgm streptomycin/ml and 2.5 µgm/ml amphotericin B was used for transfecting HEV RNA. 3 µgm of the capped RNA was transfected into 60–80% confluent cells, in one well of a six-well plate using the TransIT-mRNA Transfection kit (Mirus Bio); the transfected cells were incubated at 37 °C. Two days posttransfection (dpt.), the culture medium was replaced with 2 ml fresh medium and incubated further. Half of the culture medium (1 ml) was replaced afterwards every alternative day, containing 50% DMEM and 50% medium 199 (Invitrogen), 2% FCS,

30 mM $MgCl_2$, 100 U/ml penicillin G ,100 µgm/ml streptomycin and 2.5 µgm/ml amphotericin B. The collected medium was centrifuged at 800 *g* at 4 °C for 5 min and the supernatant was stored at -80 °C until use. At 19 dpt, the cells were splitted and seeded in 10 cm dishes and maintained in maintenance media or stored in liquid nitrogen.

2.2.9. Simvastatin treatment and viral RNA isolation

To look for the effect of simvasatin on virus egresses, simvastatin (Sigma) was activated as described (Sadeghi *et al.*, 2000). Briefly simvastatin (4 mg) was dissolved in 100 µl of ethanol and to it 150 µl of 0.1 N NaOH was added. The dissolved simvastatin containing solution was subsequently incubated at 50 °C for 2 hours and the pH was brought to 7.0 by HCl. The stock solution was stored at 4 °C. This was added to the HEV containing PLC/PRF/5 cells at a concentration of 20 and 50 µM. Medium was collected after 36 h and stored at -80 °C until use.To monitor virus production in transfected cells, HEV RNA was isolated from culture supernatant using viral RNA extraction kit (Roche) according to manufacturer's instruction. RT-PCR was done with primers 250fwd 5'-cccgggtaatacgactcactatagggaggcagaccacatatgtggtcg-3' and 250rev 5'-ccatggtcagctcattatggataacacgctggatgg-3'.

2.3. Results

2.3.1. Detection of HEV in swine

HEV has been detected in domesticated pigs in several European countries. However, no data is available about German pigs. In order to analyze German pigs for the presence of HEV RNA, I screened swine sera obtained from various German farms.

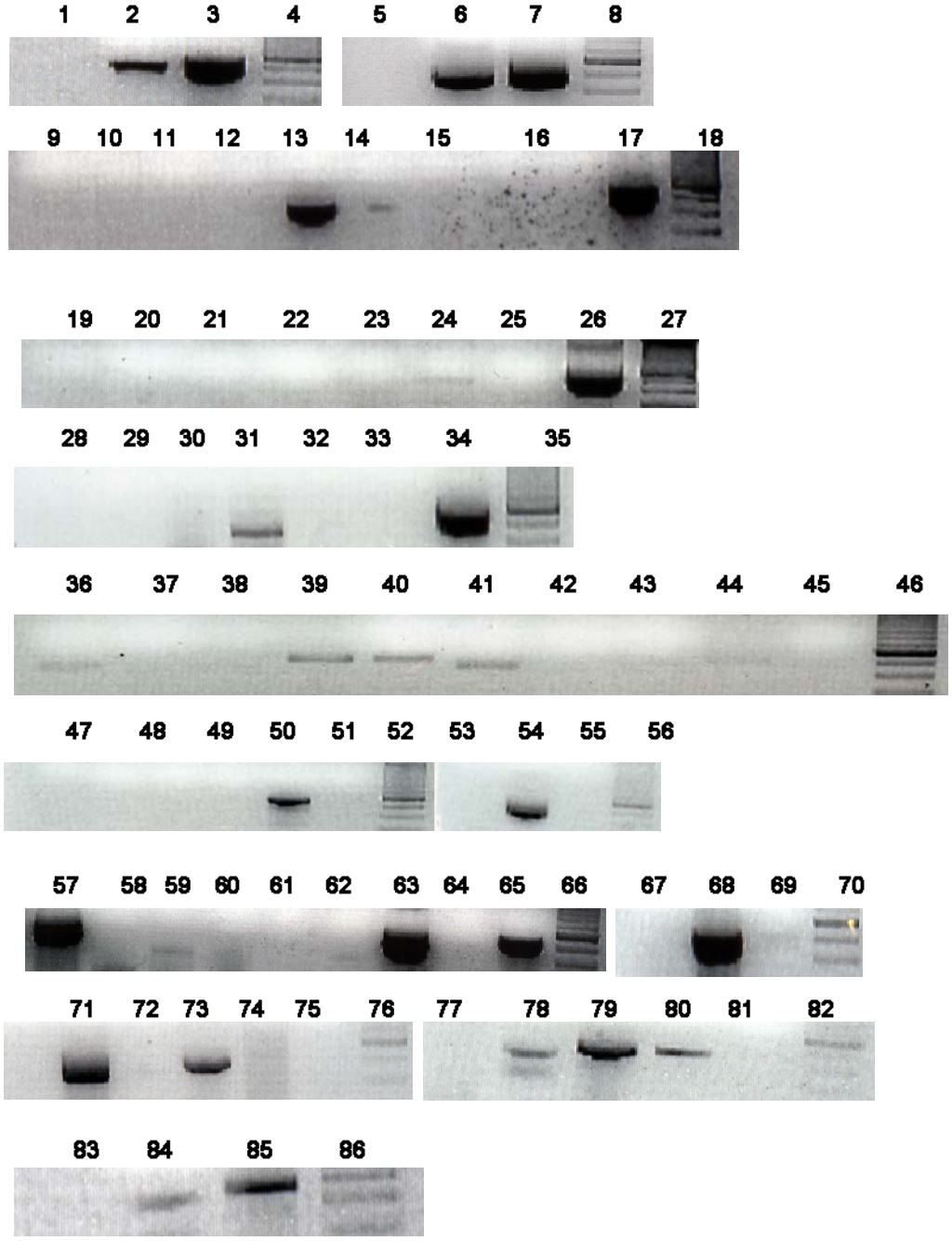


Figure 10: RT-nested PCR of HEV RNA from various swine serum isolates using primers specific to HEV ORF2 region. HEV clone pSK-HEV-2 is used as positive control.

Table 2: Detection of HEV RNA in swine serum using ORF2 specific nested PCR

No	Name of the sample	HEV RNA presence through nested PCR	No	Name of the sample	HEV RNA presence through nested PCR
1	WATER CONTROL	Negative	24	320-5	Positive
2	3205	Positive	25	WATER CONTROL	Negative
3	Positive control	Positive	26	Positive control	Positive
4	DNA marker		27	DNA marker	
5	WATER CONTROL	Negative	28	ESP44	Negative
6	ESP19	Positive	29	ESP35	Negative
7	Positive control	Positive	30	ESP27	Negative
8	DNA marker	Negative	31	ESP19	Positive
9	ESP 61	Negative	32	ESP10	Negative
10	ESP127	Negative	33	WATER CONTROL	Negative
11	669-10	Negative	34	Positive control	Positive
12	ESP-135	Negative	35	DNA marker	
13	669-2	Positive	36	2.1	Positive
14	N1181-7	Positive	37	2.2	Negative
15	730-6	Negative	38	2.3	Negative
16	668-6	Negative	39	40	Positive
17	Positive control	Positive	40	2.1A	Positive
18	DNA marker		41	2.2B	Positive
19	ESP111	Negative	42	2.3C	Negative
20	ESP 102	Negative	43	040	Negative
21	ESP 143	Negative	44	040-B	Negative
22	ESP119	Negative	45	WATER CONTROL	Negative
23	215-7	Negative	46	DNA marker	
			47	ESP	Negative
			48	5750	Negative

No	Name of the sample	HEV RNA presence through nested PCR	No	Name of the sample	HEV RNA presence through nested PCR
49	57529	Negative	71	ESP19-C	Positive
50	Positive control	Positive	72	5750	Negative
51	WATER CONTROL	Negative	73	Positive control	Positive
52	DNA marker		74	3205-M	Negative
53	5750-B	Negative	75	WATER CONTROL	Negative
54	Positive control	Positive	76	DNA marker	
55	WATER CONTROL	Negative	77	W1181-7	Negative
56	DNA marker		78	3205-45	Positive
57	ESP19-B	Positive	79	B4-ESP19	Positive
58	3205-6	Negative	80	Positive control	Positive
59	PS1	Negative	81	WATER CONTROL	Negative
60	PS2	Negative	82	DNA marker	
61	18/12	Negative	83	WATER CONTROL	Negative
62	3205-B	Negative	84	ESP85	Positive
63	1645	Positive	85	Positive control	Positive
64	WATER CONTROL	Negative	86	DNA marker	
65	Positive control	Positive			
66	DNA marker				
67	ESP127-B	Negative			
68	Positive control	Positive			
69	WATER CONTROL	Negative			
70	DNA marker				

Out of 50 samples analyzed, 16 (32%) were found to be positive for the presence of HEV RNA using HEV ORF2 specific nested PCR (Fig. 10 & table 2). This was further confirmed by sequencing one of the PCR products: 3205-45. The sequence obtained was searched for similarities to HEV using BLAST to find regions of local similarity between sequences (Fig. 11). It was found that it is 91% related to its counterpart of swine HEV, accession no AB481226.1 (Swine hepatitis E virus genomic RNA, complete genome, isolate: swJB-E10). The sequence can be thus put in genotype 3

of swine HEV. Though a majority of HEV infections are asymptomatic, sustained transmission may lead to the evolution of virulent strains. HEV genotype 3 is very widely distributed and a newly evolved strain can cause more far-reaching consequences. Swine HEV has been detected in raw pige livers (Yazaki *et al.*, 2003) and the sequence analysis shows recombination of swine and human HEV strains from patients in the USA, Japan and other countries.

HEV was suggested to be a zoonosis after it was experimentally transmitted to domestic pigs (Balayan *et al.*, 1990). Sequence alignment of HEV strains US-1 and US-2 shows a high sequence similarity with swine HEV. Both these sequences shared more than 97% amino acid homology with swine HEV covering ORF1 and ORF2. Other than that, all these strains belong to HEV genotype 3. Human US-2 strain was also capable of infecting swine. There is also report in which cross-species infection occurs upon inoculating rhesus monkeys with swine HEV. All these data provides experimental evidence for cross-species infection with swine HEV and shows the possibility of pigs and other animals being a natural reservoir of HEV. Zoonotic transmission of HEV is a major health issue in developed countries which doesn't have report of faecal-oral HEV transmission. Humans who consume contaminated pork products and are involved in the rearing of pigs are potentially at risk of HEV infection (Matsuda *et al.*, 2003; Tei *et al.*, 2003). Anti-HEV antibodies have been detected in many more animal species, including wild boar and deer and HEV genotype 3 was identified from a boar in Japan (Sonoda *et al.*, 2004).

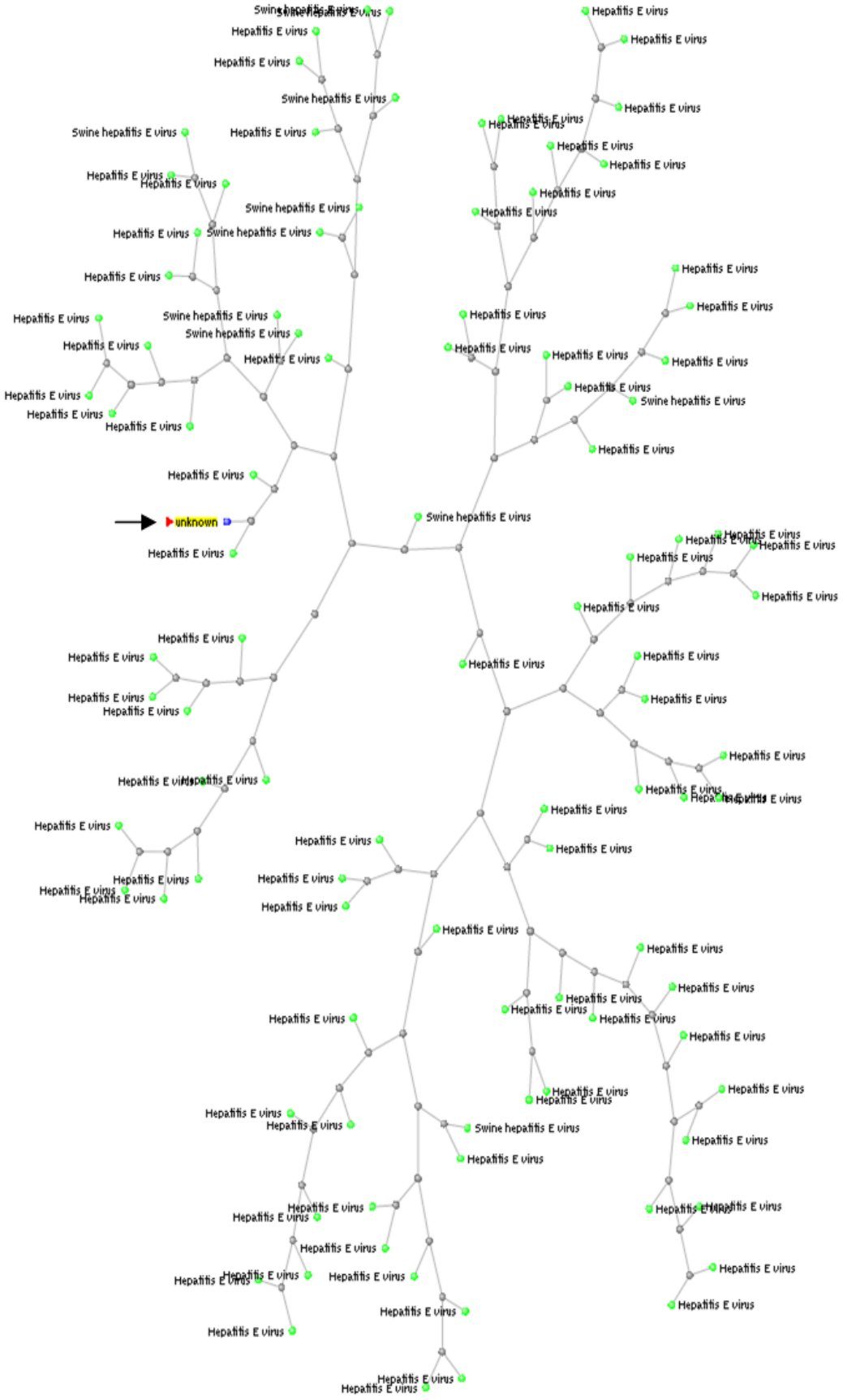


Figure 11: Showing the BLAST Tree view of sequence amplified from ORF2 region of HEV RNA. Unknown sequence with arrow refers to the location of nucleotide sequence of the submitted HEVswine isolate.

2.3.2. HEV ORF2 expression in various expression systems, ORF2 as a vaccine candidate

2.3.2.1. Mammalian expression system

HEV ORF2 is expected to be translated into a protein of MW 72 kDa carrying a putative signal sequence and potential sites for glycosylation (Jameel *et al.*, 1996; Tam *et al.*, 1991). ORF2 expression in mammalian cells (COS-1 and HepG2) using plasmid-based expression yielded a glycoprotein of MW 88 kDa that was shown to be expressed intracellularly, as well as on the cell surface and formed non-covalent homodimers (Jameel *et al.*, 1996). As it was important to analyze the use of ORF2 as a vaccine candidate, I went for its expression in various expression systems. ORF2 was expressed as a protein of ~80 kDa and ~72 kDa when cloned and expressed using pcDNA mammalian expression plasmid and can be seen using Western blot with anti-his antibodies (Fig. 12). The larger size or multiple forms may be associated with the glycosylation status of the protein.

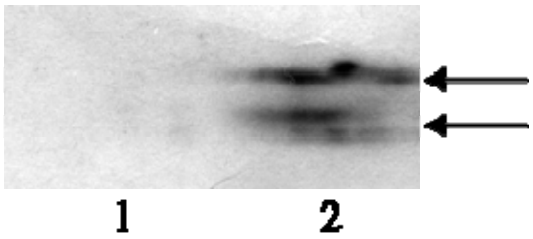


Figure 12: Expression of HEV ORF2 in mammalian cells. Lane 1 transfected with pcDNA, lane 2 transfected with pcDNA-ORF2.

2.3.2.2. Bacterial expression system

The fragment of ~2 kb, encompassing complete ORF2 was cloned into the prokaryotic expression vector pET28a+ as described. After induction with IPTG, abundant amounts of the ORF2 was expressed (Fig. 13A, lane 2), as evidenced by their absence in uninduced cells (Fig. 13A lane 1) and by their reactivity on Western blots to the anti-His tag antibody (Fig. 13B). The protein accumulated as inclusion bodies, a feature exploited for further purification. The protein in washed inclusion bodies, after solubilization with urea, was purified by affinity chromatography on a nickel-agarose resin (Quiagen) (Fig. 13A lane 6-8). The purified protein of ~1mg was used for the production of antibodies (DAVID's Biotech GmbH, Regensburg) in rabbit. The serum collected was able to detect the purified protein using ELISA at a

concentration of 1:80,000 according to the provider. I also checked the specificity of serum to HEV ORF2 through Western blot in various dilutions of serum. It was possible to detect the ORF2 even at a dilution of 1:30,000 (Fig. 15A&B). Detection was done using DAB (Sigma) detection system and ECL (Amersham).

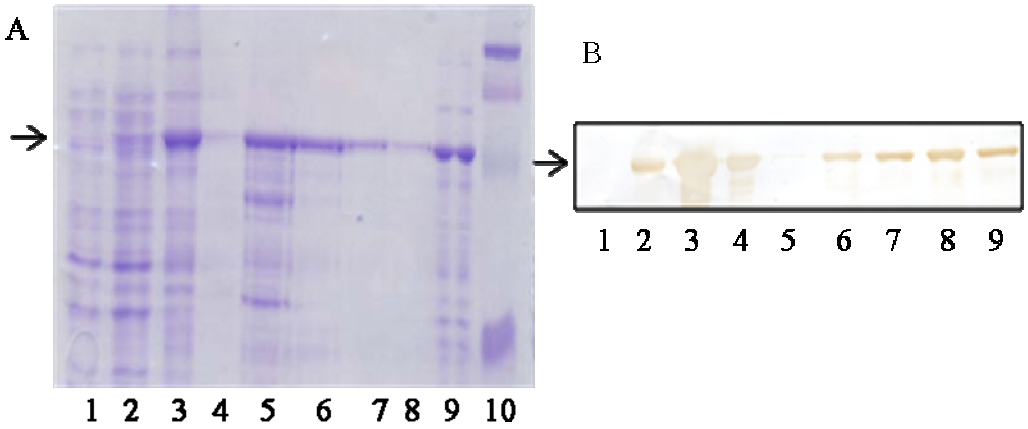


Figure 13: Expression and purification of His tagged HEV ORF2 in *E. coli*. A. The gel was stained with Coomassie blue. Lane 1 un-induced, lane 2 induced preload, lane 3 100 mM imidazole wash, lane 4 50 mM imidazole wash, lane 5 Eluent fraction 1, lane 6 eluent 3, lane 7 eluent 6, lane 8 eluent 10, lane 9 beads, lane 10 protein marker. (B) Western blot analysis for *E. coli* expressed and purified HEV ORF2 protein. lane 1 un-induced , lane 2 induced , lane 3 preload, lane 4 100 mM imidazole wash, lane 5 50 mM imidazole wash, lane 6 Eluent fraction 1, lane 7 eluent 3, lane 8 eluent 6, lane 9 elunet 8.

2.3.2.3. Adenoviral expression system

HEV ORF2 was expressed using adenoviral expression system to study its use as a vaccine candidate. The overall strategy for adenovirus construction is diagrammed in Fig. 8. HEV ORF2 was cloned into pAdTrack-CMV (Fig. 16), and the resultant construct is cleaved with a restriction endonuclease to linearize it and transformed into E*. coli* strain BJ5183 which has the supercoiled adenoviral vector pAdEasy-1. Recombinants were selected with kanamycin and screened by restriction digestion with *Pac*I. Positive clones released a fragment of 4.5 kb (Fig. 14A) or 3.0 kb to expose its inverted terminal repeats. The resulting plasmid, pAd-GFP-F2, were subsequently transformed to Top10 *E.coli* cells (Invitrogen) for large-scale plasmid amplification followed by sequencing using gene specific primers in both forward and reverse directions. The plasmid was re-digested with *Pac*I and transfected into a packaging cell line HEK 293 cells. After ~7 days, viruses were harvested by freeze thaw cycle and were used for infecting HEK 293 cells. Infection was carried out at an MOI of 10 and was monitored by tracking the GFP expression on cells (Fig. 14C and D). For checking the expression, RNA was isolated from the infected cells and used

for RT-PCR using gene specific primers (Fig. 14B). Expression in RNA level was seen in both 24 hpi and 48 hpi (Fig. 14B, lane 2 and 3), but not in the RNA control. To check the expression of ORF2 in protein level the infected cell lysate (48 hpi) was separted through SDS-PAGE, followed by a Western blot using anti-ORF2 antibody raised in rabbit. The HEV ORF2 was detected as a protein of MW ~88 kDa (Fig. 15B: lane 3), slightly above the *E.coli* expressed ORF2 band (Fig. 15B, lane 2).

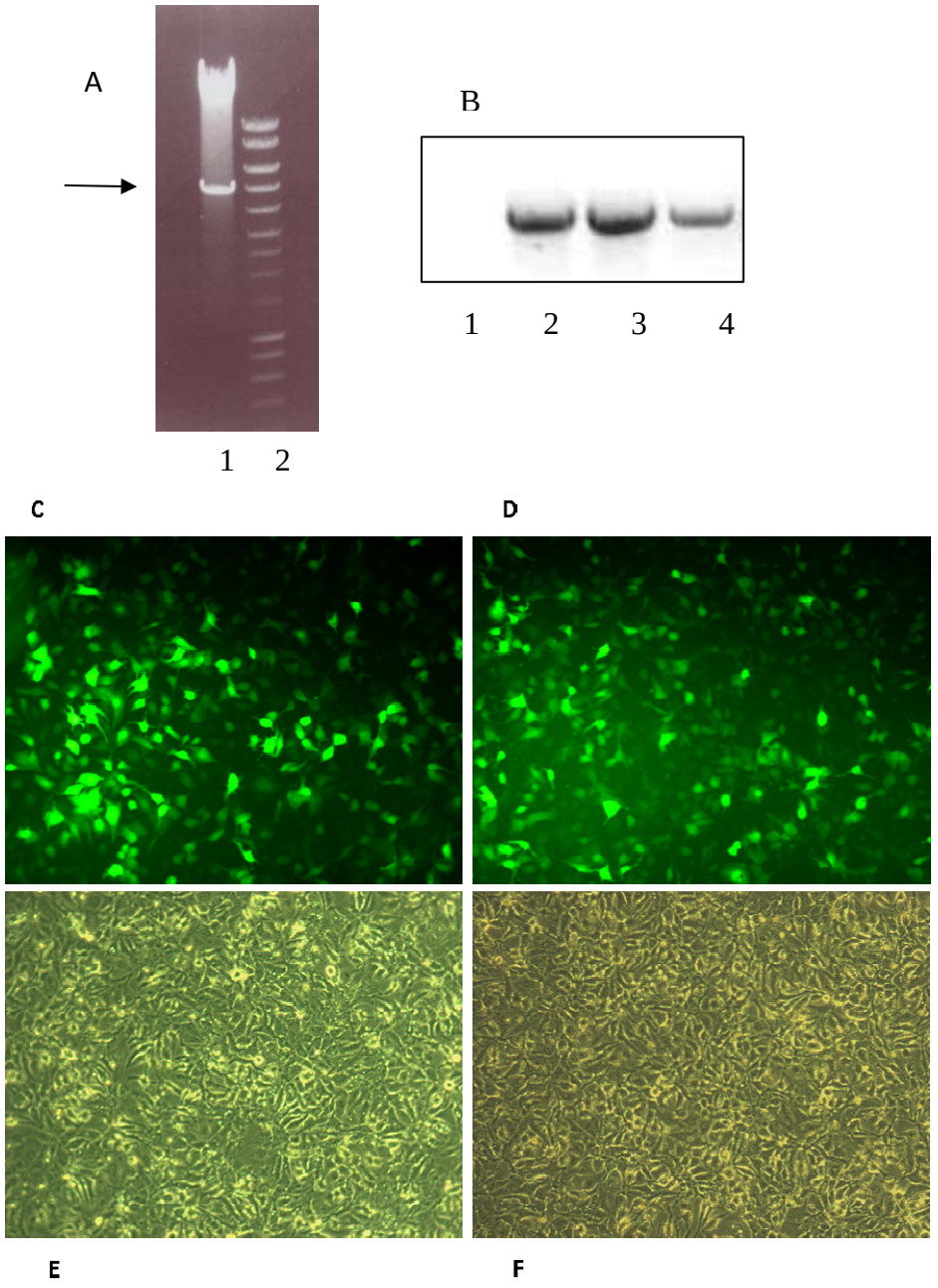


Figure 14: A, lane 1, Agarose gel electrophoresis showing the positive clone of Ad-ORF2 after digesting with *Pac*I. Lane 2, DNA marker. B, RT-PCR after infecting HEK293 cells with Ad viruses. Lane1 control, infected with Ad-GFP. Lane 2 and 3 with Ad-ORF2 at 24 and 48 hrs post infection respectively. Lane 4, positive control. C, GFP expression after infecting HEK 293 cells with Ad-GFP virus. D, infected with Ad-ORF2 virus. E and F, the respective phase contrast images.

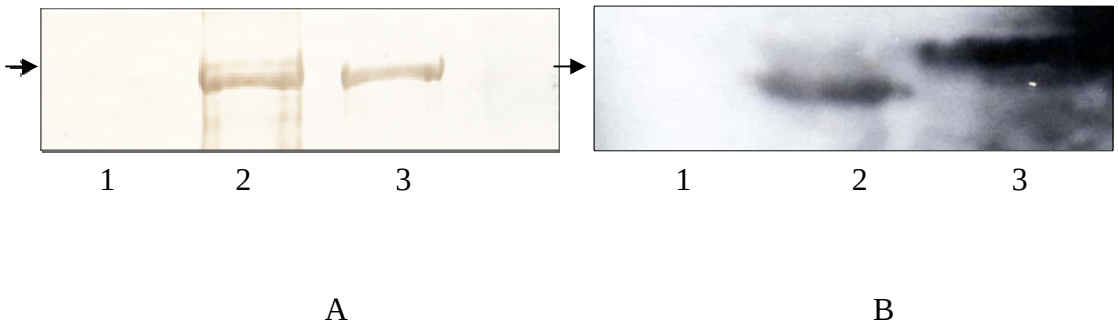


Figure 15: Detection of HEV ORF2 by the antibodies raised in rabbit. A. E coli expression. Lane 1, uninduced, lane 2, induced lane 3, purified HEV ORF2 protein. (B) Lane 1, Ad-GFP infected cell lysate. Lane 2, *E. coli* lysate expressing HEV ORF2 protein. Lane 3, Ad-ORF2 infected HEK293 cell lysate.

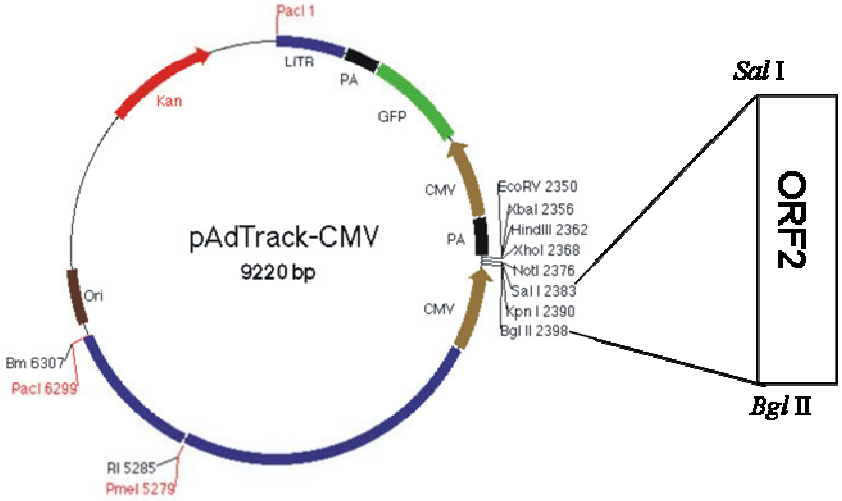


Figure 16: Vector map of pAdTrack-CMV-ORF2

2.3.3. Propagation of HEV: Cholesterol is required for the egress of HEV

Membraneous compartments are used by viruses to cross membrane barriers during infection assembly and release. How viruses use these membrane lipid composition and protein-lipid binding for its biological functions is an area emerging area of investigation (Schroeder, 2010). Compared to other membrane lipids cholesterol has special features. They don't form membrane of its own and penetrates less deeply in the hydrophobic layer of membrane. It is also one of the most diffusible lipid and analogous to small-molecule drugs in structure and function. Pathogenic process of viruses are interfered most effectively when they are concentrated into membrane rafts as cholesterol anchors on antiviral drugs and other inhibitors (Rajendran *et al.*, 2010).

Virus egression involves different pathways and most of these pathways are associated with lipid rafts (Noisakran *et al.*, 2008; Yasuda *et al.*, 2003). Cholesterol is associated with the egress of various viruses like sindbis virus (Hafer *et al.*, 2009), bluetongue virus (Bhattacharya & Roy, 2008) and HIV (Zheng *et al.*, 2003). A proposed model for HEV replication and egress is given in Fig. 5. Simvastatin is a member of the statin family of drugs that inhibit 3-hydroxy-3-methylglutaryl CoA reductase. Statins lower plasma cholesterol levels, resulting in reduction of the risk of cardiovascular disease. Simvastatin has been shown to suppress various viral infections including HIV, HCV etc (Delang *et al.*, 2009; Giguere & Tremblay, 2004). Recently, an efficient cell culture system for HEV in PLC/PRF/5 and A549 cells using a genotype 3 HEV (strain JE03-1760F) was developed (Yamada *et al.*, 2009). I used this system to study HEV propagation and to understand the role of cholesterol in its egress.

As shown in Fig. 9, linearized construct pJE03-1760/wt was used to make infectious viral RNA. After transfection in PLC/PRF/5 cells the virus egress started at ~17 dpt as can be seen in Fig. 17 lane 4. The viral RNA level kept increasing and could be detected at 19 dpt. To check if the infection can persist up on splitting of the cells followed by media change PLC/PRF/5 now infected with HEV was trypsinized and splitted into different plates. HEV RNA was detected even up to 30 dps, Fig. 17, lane 14. Up on treating with simvastatin, a drug which inhibits cholesterol synthesis, in PLC/PRF/5 cells infected with HEV (30dpt) there was considerable decrease in the amount of viral RNA. RNA level was decreased below half after egress, i.e. viral RNA in the media (0.45 and 0.53 fold change in case of 20 and 50 µM treated, respectively) and slightly less for cellular viral content (about 0.75 and 0.57 fold change in case of 20 and 50 µM treated, respectively) Fig. 18A and B. Untreated control samples were normalized. This shows that simvastatin has some effect in the egress of HEV and shows the possibility of using lipid rafts for its egress.

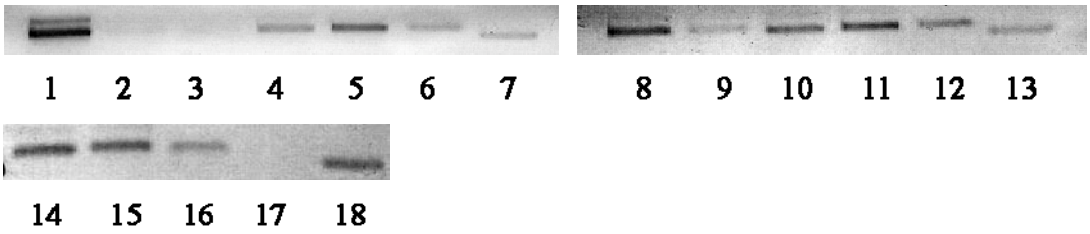
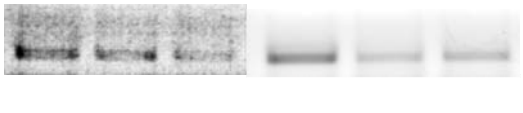
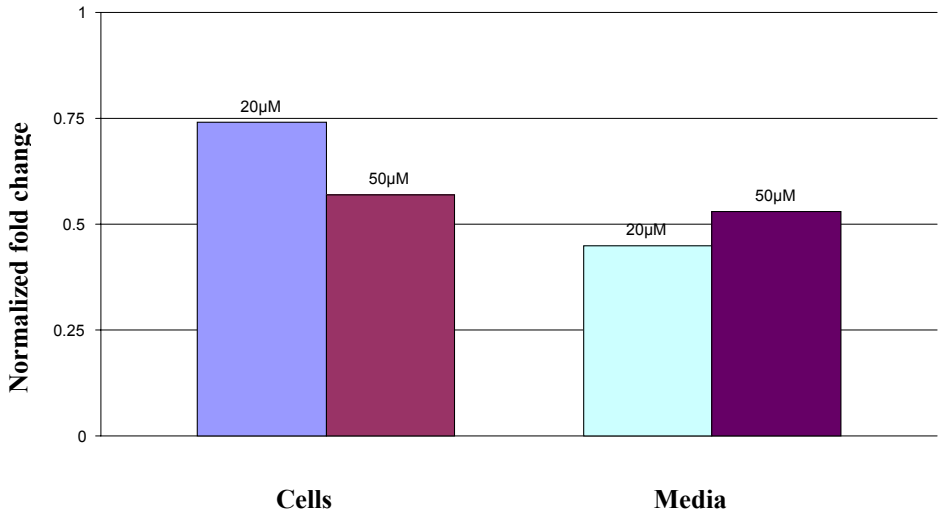


Figure 17: Propagation of HEV in cell culture. RT-PCR from the RNA isolated from PLC/PRF/5 cells after transfection with HEV-RNA. Lane1, 2 dpt, (day post transfection), lane 2, 3 dpt, lane 3, 7 dpt. Lane 4 17 dpt, lane 5 and 6 positive controls, lane 7, DNA ladder, lane 8, 19 dpt, lane 9, 3 dps (days post splitting), lane 10, 5 dps, lane 11, 7 dps , lane 12 positive control, lane 13, DNA ladder, lane 14, 30 dps, lane14, 22 dps, lane 15, positive control, lane 16, DNA ladder.



A



B

Figure 18: Effect of simvastatin on the HEV egress or replication. (A) Lane 1-3 RT-PCR of total RNA isolated from cells. Lane 1 contol, lane 2 treated with 20 µM simvastatin, lane 3, 50 µM of simvastatin, lane 4 -6, RT-PCR of RNA isolated from media, lane 4 control, lane 5 treated with 20 µM of simvastatin, lane 6, 50 µm simvastatin, (B) Graphical representation of normalized fold change after quantification of the bands using image J.

2.4. Discussion

2.4.1. Detection of HEV in swine

Hepatitis E virus is widespread in many countries mainly in tropical and developing countries (Chandra *et al.*, 2008). Here in the present study, out of 50 samples screened, 16 were found positive for HEV RNA presence which comes to about 32%. The samples collected from different parts in Germany showed the diversified infection of domesticated swine. Detection of HEV RNA in the serum of domesticated pigs points to the view that HEV is a zoonotic disease and that domesticated pigs in Germany might be an important reservoir for the virus. Previous reports have shown the presence of HEV RNA in German wild boars (Schielke *et al.*, 2009) with about one third of the HEV cases in Germany may have been acquired in Germany. This is the first report of the presence of HEV RNA in domesticated pigs in Germany. Interestingly the closest related sequence is found to be swine HEV isolate from Japan of genotype 3 (accession no AB481226.1), after sequencing one of the PCR product and analyzing the sequence.

HEV is introduced to new areas in the world due to the globalization of food markets in developed countries. It remains unclear if autochthonous HEV is imported from countries like China, Taiwan and the United States, which is having HEV genotype III and IV circulating or is from a wild reservoir. Here the evidence shows that HEV is zoonotic in countries where it is not endemic (Huang *et al.*, 1992).

In US and Taiwan zoonotic spread of HEV may be occurring as the human and swine HEV isolates belongs to same genotype very much related to each other and in these places HEV is not endemic (Huang *et al.*, 2002; Li *et al.*, 2000). There are reports about detection and sequencing of HEV in domestic pig livers sold in groceries in Japan and United States (Yazaki *et al.*, 2003). HEV was also detected in raw pig livers from grocery shops in the Island of Hokkaido, Japan and these belonged to genotype 3 or 4. Liver specimens from 7 (1.9%) of 363 packages detected HEV RNA and had 99.5–100% sequence identity with a HEV infected patient in Hokkaido. Among 10 patients who had acute and fulmminent hepatitis during 2001-2002 in Hokkaido 9 had history of consuming under-cooked pig liver few

weeks before the disease onset. A study by Feagins *et al* shows that in US among 127 pakckages of pig liver screened 14 were positive for HEV RNA, and belonged to genotype 3 of HEV. There are also reports (Matsubayashi *et al.*, 2004; Mizuo *et al.*, 2005) confirming the the connection of domestic pigs in spreading of HEV to humans. Death of a 69-year old patient is reported due to fulminant hepatits during an HEV outbreak after eating barbecued pig liver/intestine (Mushahwar, 2008). In the same way, Mizuo *et al*. (Mizuo *et al.*, 2005), also reported on 32 patients who consumed undercooked pig livers and/or intestines, 1– 2 months before the commencement of hepatitis E. All the above reports show that zoonotic foodborne transmission of HEV has a key role in the HEV epidemiology. This raises major health concerns and confirms that pigs form animal reservoirs for human HEV (Erker *et al.*, 1999; Meng *et al.*, 1998). There are also reports of people having acute autochthonous hepatitis E virus infection in Germany who didn't had a history of travelling abroad (Brost *et al.*, 2010) This shows the vulnerability of German population to Hepatits E virus and necessity of stringent screening of swine farms in Germany for the detection of HEV. It is also advisable that swine meat be properly cooked to sterilize it from the presence of virions.

2.4.2. Cloning and expression of HEV ORF2 in various expression systems, ORF2 as a vaccine candidate

HEV ORF2 protein which has the potential as a vaccine candidate has been studied for expression in various expression systems. An experimental vaccination study with cynomolgus monkeys tested a baculovirus-expressed ORF2 of MW 55000 daltons, encompassing amino acids 112 to 609 of ORF2 from a Pakistani HEV isolate (Tsarev *et al.*, 1994). This protein, synthesized in insect cells, was shown to be more efficient in generating antibody responses in experimentally infected monkeys (Tsarev *et al.*, 1994) than fusion proteins made in E. coli. A chronological titration study shows that animals given from a dose of this 0.4-mg recombinant ORF2 protein protected them from hepatitis while challenging with high doses of the HEV. Primates were shown resistant to heterologous challenge with the Mexican strain of HEV after given a 50-mg dose of the recombinant protein (Tsarev *et al.*, 1997). In an independent experiment, recombinant baculovirus was engineered to contain 112–660 amino acids of the pORF2 that expressed as a stable and soluble

protein of MW 62,000 daltons. The 62 kDa protein was purified to near homogeneity and was used as an immunogen in experimental vaccination of cynomolgus monkeys (Aggarwal & Jameel, 2008). Protective immunity to HEV by using ORF2 depends on the native confirmation mimicking the HEV proteins expressed during infection. As a result, different studies using various recombinant proteins show contradictory results. Immunization with a bacterially expressed peptide of MW 23,000 daltons elicited a vigorous antibody response preventing experimental infection in two of three rhesus monkeys and reduced viral excretion in one (Im *et al.*, 2001). This protein form homodimers and thus mimics added tertiary structure of the capsid proteins. A vaccine candidate containing baculovirus expressed recombinant capsid protein showed protection in macaques infected with HEV. It passed a phase I clinical trial and was scheduled for phase II/III clinical trials (Robinson *et al.*, 1998; Stevenson, 2000). Bacterially expressed peptide corresponding to aa 368–aa 606 of HEV ORF2 was used as a vaccine candidate (Li *et al.*, 2005). When vaccinated twice with a 10 µg or a 20 µg formulation of this vaccine rhesus monkeys showed relatively high antibody response. The response to a 5 µg formulation was belated but reached comparable antibody levels. All the three vaccine formulations afford complete protection against infection with 10^4 genome equivalent dose of the homologous genotype 1 virus. At higher virus dose of 10^7, the same vaccine formulation partially protected against the infection and completely protected against hepatitis. This vaccine candidate had undergone phase 3 trial (Zhu *et al.*, 2010).

Adenovirus vectors offer several important advantages from a vaccine perspective. They have an exceptional safety record compared to live viral vaccines (Polo & Dubensky, 2002). Some of the promising reports pertaining to non-human primate models of the Ebola virus (Sullivan *et al.*, 2000) *Mycobacterium tuberculosis (Mtb)* (Ronan *et al.*, 2009) Papilloma virus (Berg *et al.*, 2005) highlight the potential of Ad-based vaccination approach. However, one concern against adenovirus for human use is the preexisting antibodies to adenoviruses which may compromise the efficacy of Ad-based vaccines. Works on Ad-based human immunodeficiency virus (Casimiro *et al.*, 2003) and Ebola vaccines (Yang *et al.*, 2003) has suggested that DNA priming followed by vector boosting can effectively overcome the effect of preceeding Ad immunity.

To investigate the utility of the Ad vector in developing HEV vaccines, I constructed a replication-defective recombinant Ad (rAd) vector by inserting the HEV ORF2 protein-encoding gene into the early region 1 (E1) of the Ad genome. As the HEV ORF2 has been proposed as a vaccine candidate I looked for its expression in various expression systems with clear emphasis on adenovirus based expression system. Its expression was observed through RT-PCR and Western blot (WB). In WB, the size of ORF2 band was slightly higher than the band of ORF2 expressed in *E.coli*, which may be associated with post-translational modifications. Our main interest was to explore the utility of replication defective adenovirus type 5 (rAdV5) vectors as HEV vaccine carriers. The ORF2 protein, which is exposed on the surface of the virus, is the dominant virus antigen that interacts with host cell receptors (Kalia *et al.*, 2009) and represents the major target of neutralizing antibodies (Acharya *et al.*, 2003; Robinson *et al.*, 1998; Srivastava *et al.*, 2007).

2.4.3. Cholesterol is required for the egress of HEV

Lacks of a good infectious system have hindered the HEV research for instance in the search for antiviral drugs. The recent introduction of pJE03-1760 by (Yamada *et al.*, 2009) provides many promises to take HEV research to the next phase. They used a faecal suspension with high load of HEV (2.0 $x10^7$ copies/ml genotype 3). This was used to develop an efficient cell-culture system for HEV in a hepatocarcinoma cell line (PLC/PRF/5). Serum samples containing IgM-HEV antibodies isolated from HEV infected patients of genotype 1, 3 or 4 neutralized the genotype 3 infectious virus demonstrating that HEV antibodies are broadly cross-reactive. Serum samples obtained from patients 8.7 or 24.0 years after the commencement of HEV infection also prevented the propagation of HEV in PLC/PRF/5 cells, signifying the presence of lifelong HEV antibodies with neutralizing activity in individuals with past HEV infection. I was also able to establish an infectious system using this clone. PLC/PRF/5 cells transfected with HEV RNA were able to propagate the virus continuously as can be seen in Fig. 18A. I studied the egress of HEV in this system by depleting cholesterol in cells and by treating it with simvastatin.

Non-enveloped viruses entry and exit are mostly associated with lipid rafts (Briggs *et al.*, 2003; Tsai, 2007). Lipid rafts are made of a mixture of glycosphingolipids and protein receptors structured in glycolipoprotein microdomains (Thomas *et al.*, 2004). Signalling cycles at the plasma membrane involve reversible merging and disassembly of rafts driven by activation states of the raft proteins (Rajendran *et al.*, 2010) However, during virus morphogenesis, rafts merge irreversibly into microscale platforms of assembly and budding (Schmitt & Lamb, 2005; Schmitt *et al.*, 2005; Waheed *et al.*, 2008; Waheed & Freed, 2009; Waheed *et al.*, 2009). Cholesterol-viral proteins characterized till now include that of HIV, influenza virus and Semliki Forest virus (Ahn *et al.*, 2002; Nayak *et al.*, 2004; Ono, ; Ono, ; 2010). They have key role in virus entry of egress. Glyco protein 41 associated with HIV fusion has cholesterol binding is considered to a cholesterol recognition consensus (CRAC) motif in a flexible domain of the ectodomain to introduce the trans-membrane segment (Vishwanathan *et al.*, 2008). Viral fusion at the hemifusion stage and the ability of the isolated CRAC peptide to induce segregation of cholesterol in artificial membranes were effected due to mutation in this motif (Greenwood *et al.*, 2008; Vishwanathan *et al.*, 2008). Influenza A virus M2 protein is shown to co-purify with cholesterol (Schroeder *et al.*, 2005). Its ability of proton translocation needed for virus uncoating is not depended on cholesterol. Its transmembrane channels are also too short for integral raft insertion (Schroeder *et al.*, 2005). CRAC motifs in the flexible post-TM domain may be responsible for Cholesterol binding. This domain has three factors for binding to membrane rafts. Virualnce in mice is also associated with mutation of the CRAC motif. They have affinity to the raft–non-raft interface and can target M2 protein to lipid rafts, the site for virus assembly (Chen *et al.*, 2008). Cholesterol depletion slows down apical transport via sphingolipid-cholesterol rafts and causes mis-sorting of hemagglutinin (HA) to the basolateral membrane (Keller & Simons, 1998; Simons *et al.*, 1998). In a heterologous expression system it was observed that cholesterol is needed for the maturation and stability of M2. Ultrastructure of the Golgi was altered and interfered with cytotoxicity of expressed M2 during cholesterol depletion due to the cytotoxicity of expressed M2 (Cleverley *et al.*, 1997). In alpha and flaviviruses the pH-dependent fusion of the viral and the endosomal membrane during virus infection is through class II fusion proteins, E1 and E (Heinz & Allison, 2000; Kielian, 1995). The replication of Semliki Forest virus (SFV) can be restricted 1000-fold In cholesterol-depleted insect cells (Phalen & Kielian, 1991), whereas a

less cholesterol-dependent point mutant, P226S, is only restricted 40-fold (Vashishtha *et al.*, 1998).

I hypothesize that HEV, a non-enveloped virus egress is associated with lipid rafts. Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis in the liver catalyzing the conversion of HMG-CoA to mevalonic acid (Bocan, 2002). Cholesterol depletion by 30 or 50 µM of simvastatin treatment drastically reduced the titer of HEV RNA in cell culture supernatant (Fig. 18B lane 4 - 6). Because the level of HEV RNA inside the cells remained largely unchanged (Fig. 18B, lane 1-3), simvastatin prevented the egress of viral particles. It is possible that cholesterol depletion through simvastatin leads to lipid raft disruption and thus affecting virus egress. Thus HEV may be using a pathway associated with lipids for its egress. It also points to the possiblility that cholesterol is associated with HEV egress and it may be binding to the viral particles. More studies are required to investigate this possibility. Other possibilities such as blocking the encapsidation, inactivation of particle assembly etc. can also be not ignored. Simvastatin (SIM) has been shown to exhibit a strong *in vitro* anti-HBV activity. Moreover, a combination of SIM with each of the individual nucleos(t)ide analogues lamivudine (LMV), adefovir (ADV), tenofovir (TEN) and entecavir (ETV), showed synergistic antiviral activity (Bader & Korba). Among the statins, simvastatin and mevastatin display the strongest *in vitro* anti-HCV activity, while fluvastatin and lovastatin have moderate inhibitory effects, and pravastatin is without an antiviral effect. Combinational therapy involving interferon-alpha (IFN-alpha) or HCV nonstructural (NS)5B polymerase or NS3 protease inhibitors and statins showed a good antiviral activity (Delang *et al.*, 2009). Simvastatin produces vesicles using internal membranes which cannot sustain viral RNA synthesis and thus reducing infection through reduced transcription (Liu *et al.*, 2006). Although this is a preliminary work as it was not repeated more than once due to technical problems, it shows that simvastatin treatment at this range depleted the cholesterol level. As a result, hepatitis E virus egress was hindered, but not much on its replication. This also makes simvastatin a promising drug candidate against HEV.

Chapter 2

3. Chikungunya virus

3.1. Introduction

The reappearance of Chikungunya disease (Chik) caused by Chikungunya virus (ChikV) in the Indian Ocean Islands and India brought worldwide attention due to its explosive nature, high morbidity and complex clinicopathological manifestations. The disease caused by the ChikV is already involved in many outbreaks in Africa and Asia ever since its discovery in 1952 (Edelman *et al.*, 2000; Powers & Logue, 2007).

ChikV, an alphavirus belonging to the *Togaviridae* family, was first isolated from a febrile individual in Tanzania in 1952 (Bodenmann & Genton, 2006). Chikungunya means “that which bends up” from the Makonde root verb kungunyala, in reference to the stooped position developed due to the severe joint and muscle pain and other rheumatologic manifestations. Analysis of patients sera and the antigenic characterization collected during the outbreak found the agent to an alphavirus closely related to Mayaro and Semliki Forest virus and not dengue (Powers & Logue, 2007).

ChikV is transmitted to humans by several species of mosquitoes, with *Aedes aegypti* and *Aedes. albopictus* being the two main vectors. The symptoms of Chik normally start 4–7 days after the mosquito bite. Acute infection lasts 1–10 days and is characterized by a painful polyarthralgia, high fever, asthenia, headache, vomiting, rash, and myalgia (Pialoux *et al.*, 2007).

3.1.1.Geographic distribution of ChikV

The latest outbreak of Chik fever which started in 2005 drawn in several countries in Africa, Asia and recently Europe. The first outbreaks were reported from Kenya and the Comoros. The south Western Indian Ocean region was hit seriously, including Mauritius, the Seychelles, Madagascar, Mayotte and La Reunion. In La Reunion, the most affected island, 266,000 cases were reported (34% of the population) along with 200 mortality cases associated with more severe form besides the classical

form of the disease. After reaching Indian subcontinent in October 2005 it caused more than 1.3 million cases (Bonn, 2006; Charrel *et al.*, 2007; Pialoux *et al.*, 2007).

Sequencing of various ChikV isolates isolated from different geographical areas have shown the prevalence of three lineages with distinct genotypic and antigenic characteristics (Arankalle *et al.*, 2007). Two genotypes are reported in Africa viz. West African and East Central South African (ECSA) genotypes. These two genotypes caused all the epidemics while the Asian genotype represented for whole of Asia (Powers & Logue, 2007). Introduction of ECSA genotype to the Asian continent for the first time was reported during the 2005-06 epidemic (Yergolkar *et al.*, 2006). Chik cases were also reported recently from Europe, USA and Australia through travelers returning from affected areas (Bonilauri *et al.*, 2008). The geographic distribution of ChikV showing its dispersal from Africa to otherparts of the world is shown in figure 1.

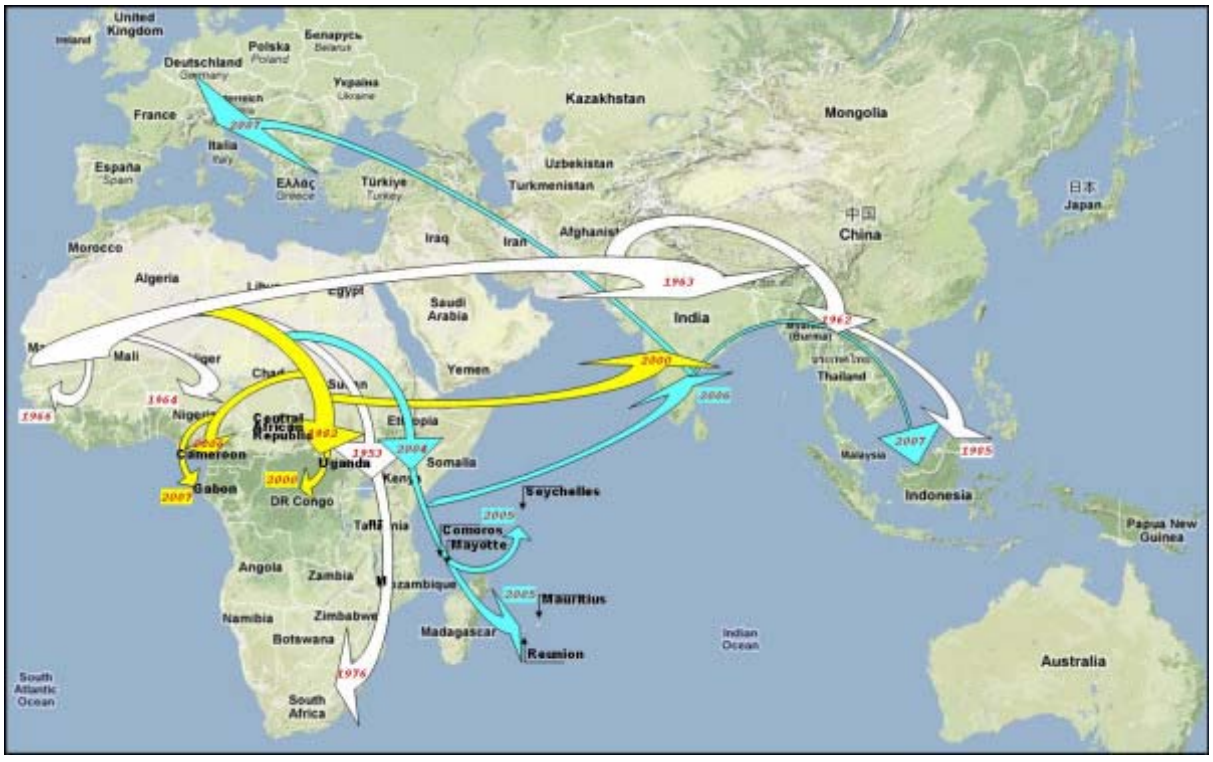


Figure 1: Geographical distribution of ChikV showing dispersal pattern from Africa to the Indian Ocean and Europe during the past 20 to 50 years (de Lamballerie *et al.*, 2008).

3.1.2. Molecular biology of ChikV

Alphaviruses are enveloped positive stranded RNA viruses consisting of approximately 12,000 nucleotides (Fig. 2). The virus has its 5' capped RNA with a 7-methylguanosine while the 3' end is polyadenylated. 5' two-thirds of the genomic RNA forms the non-structural proteins translation site. From the Negative-stranded RNA intermediate a sub-genomic positive-strand RNA referred to as 26S RNA, is transcribed. Viral structural proteins are translated from this RNA (Strauss & Strauss, 1988). ChikV has a genomic organization similar to other alphaviruses, consisting: 5' cap-nsP1-nsP2-nsP3-nsP4-(junction region)-C-E3-E2-6K-E1-poly(A) 3' (Khan *et al.*, 2002; Rice & Strauss, 1981; Strauss *et al.*, 1984).

Sequences in 5' and 3' regions of alphaviruses are highly conserved. It also possess conserved repeated sequence elements (RSEs) in the 3' non translated region (NTR) (Khan *et al.*, 2002)

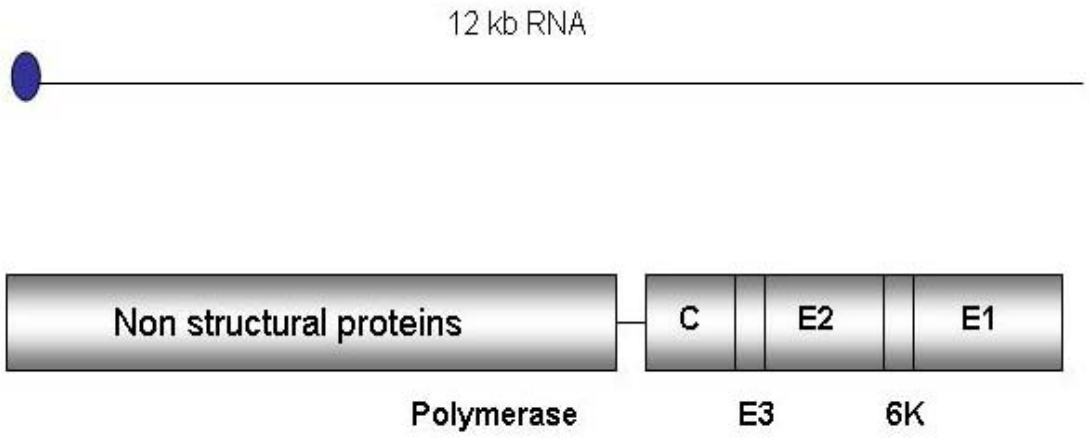


Figure 2: Structure of the ChikV genome along with translational proteins

3.1.3. Non-structural proteins

The non-strucutural protein produces a big protein named P123. It is short-lived and accumulates during the early stage of infection. They are found localized along with

viral replication complex in endosomes and lysosomes. The viral genome replication to antigenome starts by its interaction with nsP4. This follows the P123 processing in which its cleaved into nsP1, nsP2 and nsP3. RNA polymerase complex membrane association is facilitated by this late processing.

NsP1 is the viral RNA capping enzyme. This enzyme is important for the virus as all viral RNA synthesis and replication takes palce in the cytoplasm, and host encoded methyl transferase is restricted to the nucleus.

Eukaryotic capping enzymes form covalent binding with GMP, where as for NSP1 it's a covalent link between 7-methyl-GMP and nsP1. This capping is associated through various steps, 1. methylation of GTP takes place and thus forms m7GMp-nsP1 complex. 2. This 7-methyl-GMP complex is transferred from NSP1 to the mRNA to create the cap structure. NSP1 is palmiyolated and is also associated with host cell cytoskeleton remodeling. This process induces filopodium-like structural arrangement at the surface of the host cell.

The second non-structural protein NSP2 has two different domains having separate functional activities. The N-terminal region forms RNA polymerase complex and encodes trisphosphatase and RNA helicase activity. The second domain of the protein in C-terminal encodes a protease which specifically cleaves the P123 to release other four proteins.

The third protein NSP3 functions are not well defined but are thought to be related to negative strand and sub genomic 26S mRNA synthesis.

The fourth protein NSP4 encodes an RNA dependent RNA polymerase (RdRp). The synthesis of genomic and sub genomic RNA takes place through the recognition of replication specific signals. A 26S sub genomic mRNA trnanscription is also associated with NSP4 were initiation of RNA synthesis takes place internally on sub genomic RNA. The structural proteins of ChikV are translated from this (Ahola *et al.*, 1997; Froshauer *et al.*, 1988; Hardy & Strauss, 1989; Khan *et al.*, 2002; Laakkonen *et al.*, 1998; Lemm *et al.*, 1994; Malet *et al.*, 2009; Strauss & Strauss, 1994).

3.1.4.Structural proteins

The structural poly-proteins are associated with the virus structure. The viral capsid protein (VCP) has autocatalytic protease activity which results in the cleavage from rest of the structural protein. After the self-cleavage, the VCP interacts with ribosomes followed by viral RNA binding and assembly into icosahedric core particles. This nucleocapsid protein (NCP) then interacts with the cytoplasmic domain of envelope protein 2 (E2) at the cell membrane, and thus results in budding and formation of mature virions. These new virions attach to cell surface through an uncharacterized cell receptor using endocytosis. The membrane proteins fuse with cell membrane during the process. This eventually results in the release of NCP to cytoplasm, followed by ribosome binding leads to uncoating of the genomic RNA to become accessible. Ribosome also binds with E3 protein while its function is unknown.

The envelope protein 2 (E2) is a by products of structural protein p62. The processing of p62 takes place through cellular protease furin which eventually releases the E2. Processing p62 precursor takes place in the end mostly to avoid E1 fusion activation before its final export to cell surface. Structural protein processing by furin produces E2-E1 heterodimer. This E2-E1 heterodimer is unstable and dissociates at low pH while, p62-E1 heterodimer is stable. The E2 protein contain transitory transmembrane region which is disrupted by palmitoylation. This takes place in the C terminal region. This palmitoylation results in re-arrangement of the protein from the luminal to the cytoplasmic side. This is essential as it forms important structure for the virus budding by interacting with CP. The release of E2 C-terminus takes place as a later part of protein export in cytoplasm. The E2 gets attached to the cells by binding to unknown cellular receptor.

The small structural protein 6K is a membrane protein and is associated with virus glycoprotein processing. It is also found to be important for membrane permeabilization, and the budding of viral particles. Due to this lipophilic properties, the selection of lipids that interact with the transmembrane domains of the glycoproteins is proposed to be mediated by the 6K protein. This also affects the deformability of the membrane which is important for the budding process.

Envelope protein 1 (E1) is a class II viral fusion protein. This fusion activity is inhibited while E1 is bound to E2 in mature virion. The dissociation of E1/E2 heterodimer and associated trimerization of the E1 subunits occurs during the acidification of endosomes, a process which occurs after the virus attachment to the target cell and endocytosis. The E1 trimer activates the release of viral nucleocapsid to cytoplasm during the cell and viral membrane fusion. This fusion is activated during the presence of cholesterol and sphingolipid. (Choi *et al.*, 1996; DeTulleo & Kirchhausen, 1998; Khan *et al.*, 2002; Sanz *et al.*, 2003; Smit *et al.*, 1999; Strauss & Strauss, 1994).

3.1.5. Sequence requirements for the activity of alphavirus capsid protease

The alphaviral single-stranded RNA genome is packaged into a nucleocapsid made up of 240 copies of the basic capsid protein (Cheng *et al.*, 1995). The nucleocapsid is surrounded by a lipid bilayer containing two integral glycosylated membrane proteins (E2 and E1) and a small peripheral protein E3. The alphavirus capsid is multifunctional and plays a key role in the viral life cycle. The capsid is made up of two domains, the unstructured, RNA binding N-terminal segment (residues 1–118) and a C-terminal globular protease domain (residues 119-267); (Choi *et al.*, 1996). The serine protease domain consists of a β-barrel motif with a typical serine-histidine-aspartic acid catalytic triad with the active site interspersed between two subdomains (Choi *et al.*, 1997; Owen & Kuhn, 1997).

The first cleavage event in SFV is executed by the serine protease domain of the capsid protein. Cleavage occurs between tryptophan 267 and serine 268 of the polyprotein and releases the capsid protein. After the autoproteolytic cleavage, the free carboxylic group of W267 interacts with the catalytic triad (H145, D167 and S219) and thus leads to self inactivation. Even though the capsid protease is a chymotrypsin like protease, there is an important structural difference to this protease family. The viral protease lacks the C-terminal α-helix present in chymotrypsin and instead forms two short β-strands. These β-strands lead to bending of the C-terminus towards the centre of the capsid protein that allows the

last W267 side-chain to reach the active site for auto-cleavage to occur (Choi *et al.*, 1997).

3.1.6. Nuclear Import and export of ChikV capsid protein

The bi-directional transport of molecules between nucleoplasm and cytoplasm occurs through the nuclear pore complex (NPC), a supra molecular structure of the nuclear envelope (NE) (Vasu & Forbes, 2001). Nuclear pore complexes allow passive diffusion of ions and small proteins up to size of 40 kDa or less than 9 nm in diameter, but restrict passage of larger molecules to those containing an appropriate targeting signal (Paine *et al.*, 1975). Nucleocytoplasmic transport is a tremendous activity that is mediated by the interaction of transport cargoes with karyopherins. Karyopherins are adaptor proteins that recognize cargo proteins containing a nuclear localization signal (NLS) and which also interact with the transport receptor importin β. Together, these proteins build up a ternary complex that interacts with the nuclear pore complex and translocates into the nucleus (Lange *et al.*, 2007). The NLS used by the classical nuclear import pathway is a short stretch of positively charged amino acids (aa), arginines and lysines that lack strict consensus sequence (Dingwall & Laskey, 1991). A monopartite NLS, like that of SV40 large T antigen, is composed of a cluster of five to seven basic amino acids and a typical bipartite NLS contains two clusters of basic amino acids separated by a linker of 10–11 amino acids (Dingwall *et al.*, 1982; Kalderon *et al.*, 1984; Lanford & Butel, 1984).

Contrariwise, nuclear export signals (NES) are recognized by exportins and allow proteins to be transported actively from the nucleus to the cytoplasm through the NPC (Turner & Sullivan, 2008). NES are specifically bound by exportin known as exportin 1 (XPO1 or CRM1). This transport is mediated via binding to the GTP-bound form of the guanine nucleotide-binding protein Ran (RanGTP). The most commonly identified NES are short leucine-rich signals, although other hydrophobic residues, such as isoleucine, methionine, phenylalanine and valine, can contribute to the signal (Nigg, 1997).

Alphaviruses of the *togaviridae* family contain capsid protein localized both in the cytoplasm and nucleus (Atasheva *et al.*, ; 2010; Favre *et al.*, 1994). Chikungunya

virus(ChikV) capsid protein (CP) is of particular importance due to the explosive nature, high morbidity and complex clinicopathological manifestations of ChikV disease (Pialoux *et al.*, 2007). The disease caused by ChikV has already been involved in many outbreaks in Africa and Asia since its discovery in 1952 (Edelman *et al.*, 2000; Powers & Logue, 2007). ChikV capsid protein exhibits many functions. It selectively packages the viral genome, but not cellular or viral subgenomic RNA into viral particles, and encodes protease activity required for processing of the structural polyprotein (Thomas *et al.*, 2010).

Alphaviruses of the family *togaviridae* contain an icosahedral nucelocapsid which is localized both in cytoplasm and nucleus (Atasheva *et al.*, ; Favre *et al.*, 1994). The ChikV capsid is of particular importance, due to the explosive nature, high morbidity and complex clinicopathological manifestations of ChikV disease (Pialoux *et al.*, 2007). The disease, caused by the ChikV has already been involved in many outbreaks in Africa and Asia ever since its discovery in 1952 (Edelman *et al.*, 2000; Powers & Logue, 2007). ChikV capsid protein exhibits many functions. It selectively packages the viral genome, but not cellular or viral subgenomic RNA into viral particles. Capsid protein also encodes protease activity required for processing of the structural polyprotein.

As nuclear and cytoplasmic trafficking may play an important role in the regulation of ChikV function, I have sought to further define the nuclear localization and nuclear export signals in ChikV capsid. Here, I demonstrate that ChikV capsid binds to karyopherin α (kar α) for its nuclear translocation and that the C-terminal NLS domain is enough for this interaction. I am also showing that ChikV capsid encodes a CRM1 mediated nuclear export signal which is mapped to a leucine rich region. Deletion of 90 aa from N-terminal region of ChikV capsid made the protein purely nuclear, which shows that this region is important for its cytoplasmic localization or export.

3.2. Materials and Methods

3.2.1. Materials

Materials and devices if not mentioned here are written in chapter 1.

3.2.1.1. Devices

Confocal microscope	Leica Confocal Microscope, Wetzlar,

3.2.1.2. General material

Coverslips 12 mm (round), 18 mm x 18 mm (square)	Gerhard Menzel GmbH, Braunschweig
Microscope slides (26 mm x 76 mm x 1 mm)	Engelbrecht, Edermünde

3.2.1.3. Chemicals

DRAQ5	Cell Signaling Technology, Danvers, USA
Leptomycin B	Sigma Aldrich, St. Louis, USA
Mounting medium	Dako, Carpinteria, USA

3.2.1.4. Antibodies

Anti-FLAG (Mouse)	Sigma Aldrich, St. Louis, USA
Anti-GFP (Rabbit)	Acris antibodies, Herford

3.2.1.5. Vectors

pSMART LCKan	Kind gift of Christian Drosten, Universität Bonn
pChikcap	Produced in lab, GenBank: HM369441.1
pCAGGS α 1, 2, 3, and 4	Kind gift of P. Palese, Mount Sinai School of Medicine, New York.
pCR 2.1 TA cloning vector	Invitrogen, Karlsruhe
pET28a	Novagen, Merck Chemicals Ltd, Nottingham, UK
pEGFP-N1	Clonetech, Mountain view, USA

3.2.2. Isolation of ChikV RNA from patient serum

Chikungunya viral RNA was isolated from serum of patients who travelled to the Indian Ocean during the 2007 outbreak, using Roche high pure viral RNA kit according to the manufacturer's instruction. RNA was reverse transcribed and amplified using the primers chik_cap_fwd 5'-atggagttcatcccaacccaaac-3' and chik_cap_rev 5'-tccactcttcggctccctcag-3'. The PCR product was cloned to pCR 2.1 TA cloning vector (Invitrogen) according to manufacturer's instruction and positive clones were screened using restriction digestion. They were further sequenced at both forward and reverse direction. The final ChikV capsid sequence was submitted to NCBI genebank (GenBank: HM369441.1).

3.2.3. Alphavirus capsid protease: sequence requirements for the capsid protease activity

3.2.3.1. Cloning and mutagenesis

ChikV capsid protein (1 to 261) was cloned as fusion protein with FLAG tag (DYKDDDDK) at the N-terminus and GFP tag at the C-terminus in pEGFP-N1 vector (Clontech) using primers Flagchik cap_fwd and ChikcapXmaI_WT_rev (table 1). The PCR products were cloned between the *Hind*III and *Xma*I sites of the vector pEGFP-NI. All clones were verified by sequencing in both forward and reverse direction. Deletion mutant Δ46, was constructed using primers FlagChik cap_fwd and ChikcapΔ46_rev (table 1). Other mutations were incorporated by adding mutations in the reverse primer in the C-terminal of the protein (see table 1, no 4-10). As template for PCR, plasmid pSMART LCKan containing ChikV structural genes (Kowalzik *et al.*, 2008) or pChikcap (GenBank: HM369441.1) containing the ChikV capsid gene was used.

Serine 213 in the catalytic triad was mutated to alanine by fusion PCR (Szewczyk *et al.*, 2006) using primers [5'-atggactacaaagacgatgacgataagatggagttcatcccaac-3', 5'-aagatcggtctac**cgg**cgtcccctggttt-3'] and [5'-aaaccaggggac**gcc**ggtagaccgatctt, atcccgggtccactcttcggctc-3']. Error-prone PCR was performed using oligos FlagChik cap and ChikcapXmaI_WT (table 1) as primers. Reaction mixtures contained 10x PCR buffer (Qiagen), 5 mM Mg^{2+}, 10 ng of template DNA, 10pM of each primer, and

2.5 U of Taq polymerase. The reaction conditions were as follows: step 1, 5 min, 95 ºC ; step 2, 30 s, 94 ºC ; step 3, 30 s, 58 ºC ; step 4, 45 s, 72 ºC ; and step 5, 7 min, 72 ºC ; steps 2 to 4 were repeated 35 times.

Table 1: Primers used to mutate the capsid protease of Chikungunya virus.

Mutation	Primer	Nucleotide sequence (5'-3')
Wt	Flagchik cap_fwd	aagcttatggactacaaagacgatgacgataagaccatggagttcatccc
EGAEEW (wt)	ChikcapXmaI_WT_rev	atcccgggtccactcttcggctc
Δ46 AA (deletes aa 216-261)	ChikcapΔ46_rev	atcccgggttctaccgctgtcccc
W261A (EGAEE**A)**	ChikcapW-A_rev	atcccgggt**cgc**ctcttcggccc (4)
A258E (EG**E**EEW)	ChikcapA-E_rev	atcccgggtccactcttc**ttc**cccctcgg (5)
A258S (EG**S**EEW)	Chikcap3A-Sr_rev	atcccgggtccactcttc**gga**cccctcgg (6)
E256A (**A**GAEEW)	Chikcap_E-A_rev	atcccgggtccactcttcggcccc**ggc** (7)
A258T (EG**T**EEW)	ChikcapA-Tr_rev	atcccgggtccactcttc**ggt**cccctcgg (8)
E260D (EGAE**D**W)	Chikcap_E-D_rev	atcccgggtcca**atc**ttcggccc (9)
E260A (EGAE**A**W)	Chik_cap_E-A_rev	atcccgggtcca**cgc**ttcggccc (10)

3.2.3.2. Cell culture and transfection

HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin. For transfection, HEK293 cells were seeded in 12-well plates at a density of $2x10^5$ cells and transfection was performed using turbofect (Fermentas) transfection reagent according to the manufacturer's instruction

3.2.3.3. Western blot

48 hours post transfection cells were washed once with PBS followed by lysis, using RIPA buffer (Sigma). Protein samples were subjected to 12% SDS-PAGE and transferred to PVDF membrane (Amersham) by using the Xcell II blot module (Invitrogen) and blocked overnight at 4 ºC with 3% BSA in PBST. The membranes were probed with monoclonal anti-FLAG antibodies (Sigma) followed by anti-mouse secondary antibodies conjugated with horseradish peroxidase (Santacruz). Blots

were visualised with the enhanced chemiluminescence (ECL) detection reagents (Amersham, UK).

3.2.3.4. Protein modelling and analysis

The structure of the capsid protease was modeled using automated homology modelling server 3D-JIGSAW (http://bmm.cancerresearchuk.org/~3djigsaw/) (Offman *et al.*, 2008). The template, Semliki Forest virus capsid protein (PDB ID 1vcp_C) is 91% identical in sequence to the ChikV capsid protease domain, which makes the model more reliable. Comparison of WT and mutant structures were done using SPDBV software (Guex & Peitsch, 1997). The figures were generated using pymol (http://www.pymol.org)**.** Energy computations were done with the GROMOS90 implementation of Swiss-PdbViewer in collaboration with Dr. Jagdish Rai, Beckman Institute, Illinois, USA.

3.2.4. Nuclear Import and export of ChikV capsid protein

3.2.4.1. Cloning and mutagenesis

ChikV capsid full-length and mutants were cloned between *Hind*III and *Xma*I sites of the multiple cloning site of pEGFP-N1 vector using primers, HindIIIChikcap_fwd and Chik capXma1_rev (table1, no1 and 2). Expression vector pet28a (Invitrogen) was used to clone ChikV capsid sequence amplified by PCR using primers chk_petcapsid_fwd (5'-ggatccatggagttcatcccaacc-3', chk_petcapsid_re 5'-ctcgagtccactcttcggctc-3') from pChikcap (GenBank: HM369441.1) containing ChikV capsid gene as template or using ChikkcapSDM_S-A between *BamH*1 and *Xho*1 sites.

Deletion mutants were constructed using the primers given in table 1. Construction of Chikcap Δ46 has been described previously. To mutate leucine at position 149 and 152 to alanine, an overlap PCR was performed using primers 5'-aagcttatggagttcatcccaacccaaac-3", 5'-cttaaaggccgctttggccgcgtccgc-3' and 5'-gcggacgcggccaaagcggcctttaag-3', 5'-atcccgggtccactcttcggctc-3' both the fragments were having 27 nucleotides complementary in the overlapping region. Mammalian expression vector pCAGGS containing karyopherin α 1, 2, 3 and 4 with an N-terminal FLAG tag were the generous gift of Dr. P. Palese, and has been described

previously (Shaw *et al.*, 2005; Wang *et al.*, 1997). Deletion mutant pCAGGS Δ 259 karyopherin 4 was constructed by digesting the full-length vector with *Tth*111I followed by ligation.

Table 2: Primers used for the the FL or deletion constructs

Protein	Primer	Nucleotide sequence (5'-3')
Full-length	HindIIIChikcap_f	aagcttatggagttcatcccaacccaaac
"	ChikcapXmaI_WT_rev	atcccgggtccactcttcggctc-3'
Δ118 aa from C-terminal	Chikcap143_rev	atcccgggtccccttacgtgtgctgg

3.2.4.2. Protein expression and purification

N-terminal His tagged pet28aChik cap was transformed to *Bl21* cells (Invitrogen) and the protein was expressed in 500 ml culture containing kanamycin. The bacterial culture was grown at 37 °C until OD600 had reached 0.6, induced with IPTG at a concentration of 1 mM and kept overnight at 21 ºC in a rotary shaker. The cells were pelleted and resuspended in 6 ml of buffer containing PBS and 5% glycerol, and lysed by sonication for 3 min. The cell lysate was spun at 5,000 rpm for 20 min and imidazole was added to the supernatant at a concentration of 10 mM. Precharged and prewashed NiNTA beads (Invitrogen) of 0.6 ml of bed volume was mixed with the lysate at 4 °C for 1 h. It was then loaded to column and washed with 50 ml of PBS containing 20 mM imidazole and 5% glycerol. Protein was eluted using elution buffer containing PBS, 5% glycerol and 300 mM imidazole. The eluents were dialyzed against buffer containing PBS and 5% glycerol. Eluents were analysed on SDS-PAGE and confirmed by Western blot using anti-His antibodies. Protein estimation was done using the BCA kit.

3.2.4.3. Pull-down assay

Pull-down assay was performed as described before (Haenni *et al.*, 2008). Purified His-tagged ChikV capsid protein was incubated with whole cell extracts from HEK293 cells; either untransfected or transfected with FLAG tagged karyopherinα 1-4, Δ 259 karyopherin 4. Ni-NTA beads of 10 µl resuspended volume were added to

this protein lysate mix and incubated further at 4 ºC. Samples were centrifuged and the supernatant was discarded. The beads were washed further using RIPA buffer and finally 40 µL of Laemmli buffer was added to the beads and boiled for 10 min. The samples were subjected to SDS–PAGE and transferred to nitrocellulose membrane. The membranes were probed with monoclonal anti-FLAG antibodies (Sigma) followed by anti-mouse secondary antibodies conjugated with horseradish peroxidase (Santacruz). Blots were visualized with the enhanced chemiluminescence (ECL) detection reagents (Amersham, UK).

3.2.4.4. Co-imunoprecipitiation

For Co-IPs, HEK293 grown in Petri dishes (10 cm) were transfected with FLAG karyopherin α-4 and pEGFP-ChikcapFL, using Turbofect (Fermentas) transfection reagent. Cell lysates were prepared 48 h after transfection in 1.5 ml of RIPA lysis buffer. Aliquots which contain about 500 µg of total protein were incubated with 1 µg of anti-FLAG mouse monoclonal antibody (Sigma) or a non-specific monoclonal antibody for 1 h at 4 ºC. A 50% suspension of protein A–Sepharose (Sigma), blocked by incubation with 1% BSA in PBS, was added to the lysate and incubated for 2 h at 4 ºC with shaking. The beads were collected by centrifugation, followed by three washes with RIPA lysis buffer. After the final wash, the supernatant was discarded, 20 µL of Laemmli buffer was added to the beads and boiled for 10 min. The samples were subjected to SDS–PAGE and analyzed by Western blotting using anti-GFP antibody.

3.2.4.5. Cell culture and transient transfections, treatment with LMB and immunofluorescence

HEK293 cells were maintained in DMEM supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. For transfection, HEK293 cells were seeded in 12-well plates at a density of 2×10^5 cells and transfection was performed using Turbofect (Fermentas) transfection reagent according to the manufacturer's instructions. For the experiments with Leptomycin B (LMB), cells were treated with a final concentration of 20 ng/ml LMB, for 4–16 h. For detection of overexpressed proteins by immunofluorescence, HEK293 cells were fixed for 10 min in a solution containing 4% formaldehyde in PBS. Cell were washed twice in PBS for five min and incubated with 5 µM DRAQ5 (Cell Signaling Technology) according to

manufacturer's instruction. Finally, coverslips were mounted on slides with anti-fade mounting media (Dako). Samples were analyzed on a Leica confocal microscope.

3.2.4.6. Nuclear localization analysis

NIH Image J v1.30 software was used to quantify the nuclear localization of GFP fusion proteins. All confocal images were quantified using settings where the intensity of GFP fluorescence was linear and ranged from 10 to 160 pixel values (Sheng *et al.*, 2004). To examine the nuclear localization of GFP fusion proteins, a square with an area of 225 pixels was used to measure the mean intensities of three different regions in the nucleus and the cytoplasm of a representative cell from each of 3 transfections. Thus, 3 representative cells yielded a total of 9 areas from the nucleus and 9 areas from the cytoplasm. Relative nuclear localization is reported as the ratio of the mean intensity of GFP fluorescence in the nucleus and cytoplasm (nuclear/cytoplasmic ratio, N/C ratio).

3.3. Results

3.3.1. Alphavirus capsid protease: sequence requirements for the activity

Alphaviral replication depends on the efficient and precise cleavage after tryptophan 261 executed by the capsid protease. (Melancon & Garoff, 1987) This tryptophan site is conserved in all alphaviruses analyzed including ChikV (Fig. 3A). In order to determine sequence requirements of the ChikV capsid protease I cloned the ChikV capsid in fusion with GFP. Figure 3B shows that the ChikV capsid protease efficiently cleaved the capsid from the GFP tag, which also shows that S262 is not important for the cleavage. A mutation of S213 to alanine inactivated the protease and thus prevented cleavage of GFP from the capsid protease Fig. 3B, lane 8. Since S213 is an essential part of the autocatalytic triad, this cleavage was executed by the protease domain of the ChikV capsid protease. Thus, the capsid protease activity monitored in the assay depended on the integrity of the catalytic triad of the protease.

I next analyzed if a deletion at the C-terminus retained its protease activity. As shown in Figure 3B, lane 2, a deletion of 46 aa from the C-terminus completely abolished the protease activity. While E256, G257 and E259 in ChikV capsid are conserved among alphavirus capsid, the aa in positions 258 and 260 seem to vary specifically between virus species (Fig. 3A). Thus, I set out to analyze the influence of the C-terminal aa sequence for the activity of the ChikV capsid protease. As expected, mutating tryptophan in the 261 position to alanine (W261A) completely inactivated the protease (Fig. 3B, lane 9). I then analysed whether amino acids near tryptophan in the cleavage site of the protease domain will have any effect on enzyme activity. As shown in the Western blot (Fig. 3B, lanes 3-6 and 10 to 11), mutations at these sites were not found to have any effect on the protease activity. Even the substitution of E256 conserved in all alphaviruses to alanine did not have any effect on the activity of the capsid protease.

To identify additional motifs important for the protease activity, I performed an error-prone PCR to introduce mutants. I obtained and analyzed mutants with substitutions of P61L, Q86R, A207V, or I227K. Mutants P61L, Q86R, and A207V of the capsid did

not have any effect on the activity of the protease (data not shown). However, an exchange of isoleucine 227 to lysine completely inactivated the protease activity (Fig. 3B, lane 7).

To our knowledge the importance of I227 for the activity of alphaviral capsid proteases has not been reported before. Thus, I further analyzed the loss of activity by molecular modelling and comparison of WT and mutant structures. Figure 4 indicates that the lysine introduced at position 227 is spatially very close to the catalytic triad and may disrupt electrostatic interactions in the catalytic site. Rotamers of lysine 227 can make a salt-salt bridge with aspartic acid 161. This rotamer is a preferred conformation of lysine 227 considering the electrostatic interaction with surrounding residues and the absence of steric hindrances (Fig. 4B). The 3.38 Å distance between the side chain nitrogen atom NZ of lysine 227 and the side chain oxygen atom OD1 of ASP 161 is the ideal length of a salt bridge which is less than 4 Å. This distance between OD1 and NZ is almost the same before (3.38 Å) and after energy minimization (3.49 Å). Here PDB nomenclature is used in denoting the side chain atoms of amino acids. NZ is the nitrogen atom of amino group of side chain of lysine. The OD1 is one of the oxygen atom of carboxyl group of side chain of aspartic acid.

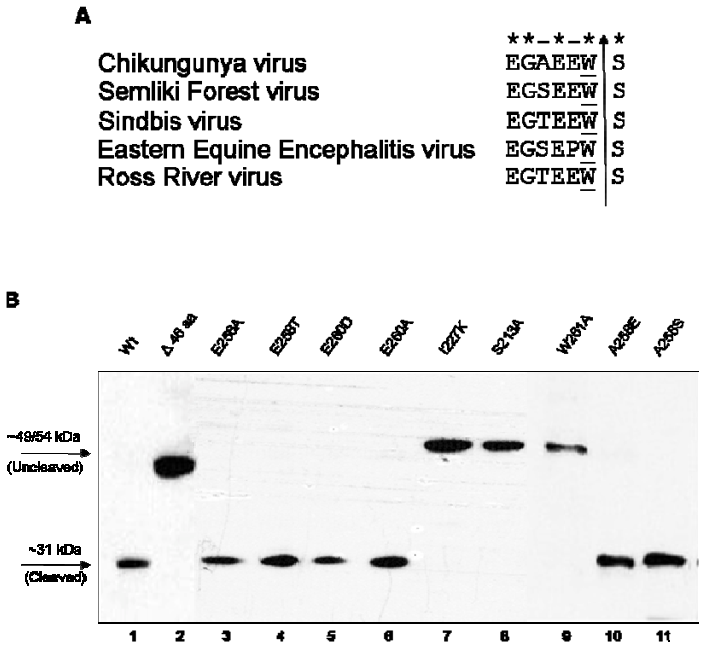


Figure 3A: Conserved amino acids near the cleavage site between tryptophan and serine at the C-terminus of the capsid protein of alphaviruses. The arrow indicates the cleavage site. Figure 3B: Mutations affecting the protease activity of the ChikV capsid protease. Capsid-GFP fusion constructs were transfected into HEK293 cells.

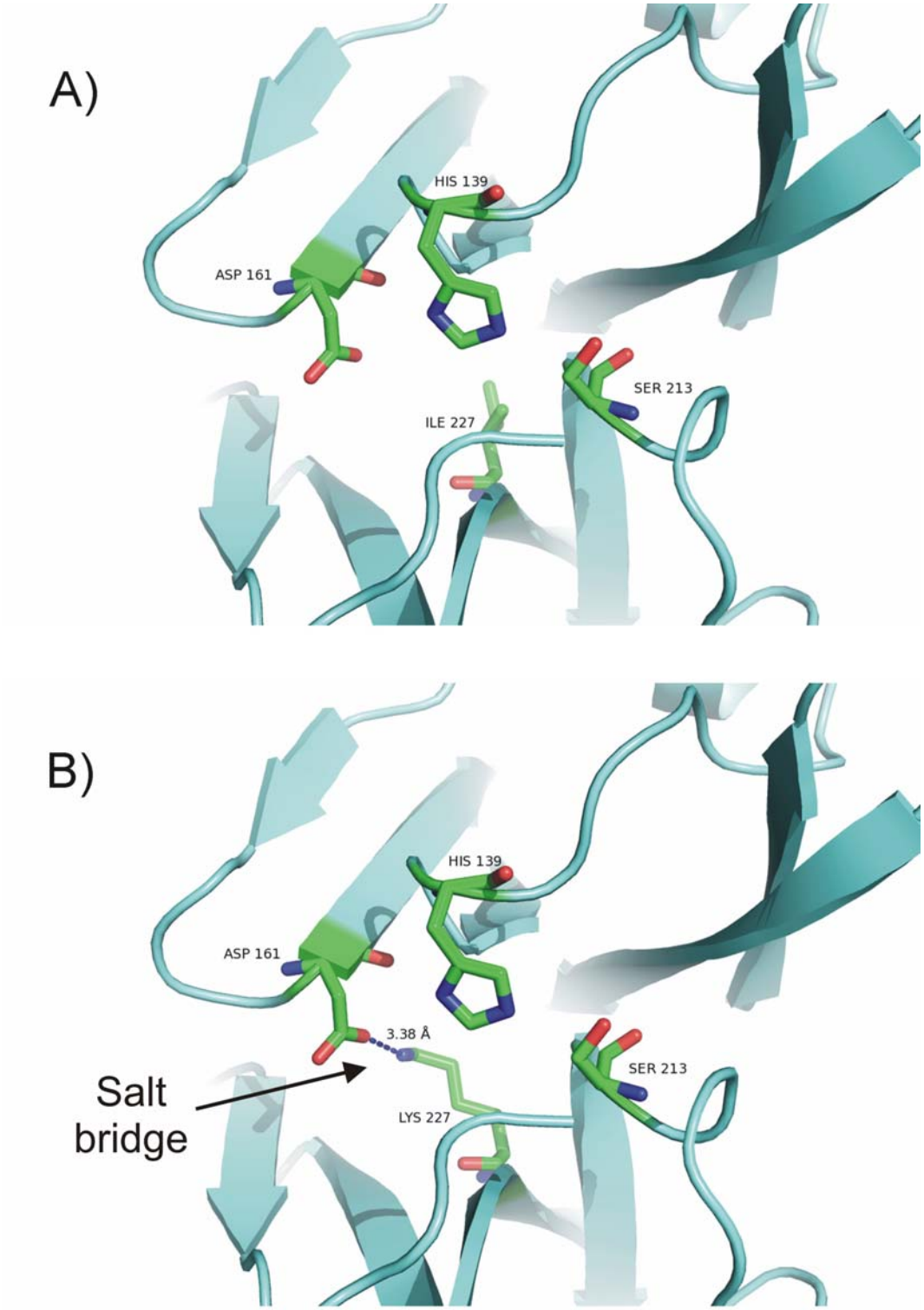
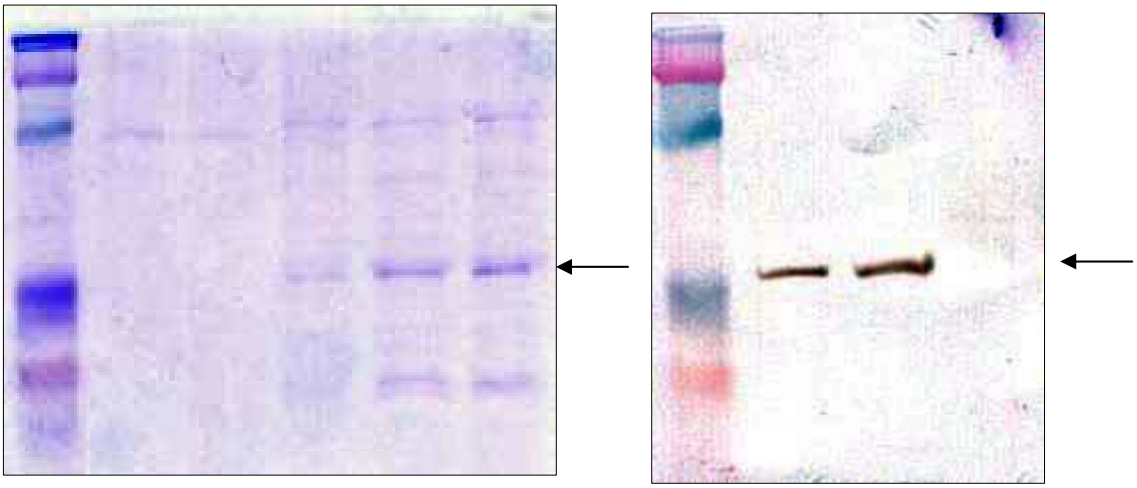


Figure 4: Molecular modelling of the catalytic triad of ChikV capsid protease. Capsid protease was modeled using automated homology modelling server 3D-JIGSAW using Semliki Forest virus capsid protein as template. A) Wt catalytic triad in native enzyme with isoleucine at 227. B) The catalytic triad in mutant enzyme with lysine at 227. The side chain of lysine 227 is very close to side chains of the catalytic triad that will disrupt the electrostatic interactions of catalytic triad. The distance between OD1 of aspartic acid 161 and NZ of lysine 227 is 3.38 Å which is the optimal distance to form a salt bridge between these residues.

3.3.2. Nuclear Import and export of ChikV capsid protein

3.3.2.1. The ChikV capsid has preferential binding to karyopherin α 3 and karyopherin α 4

Several karyopherins are known to directly interact with different cargo proteins in the cytoplasm and to carry the cargos through nuclear pores into the nucleus. To decipher the mechanisms of ChikV capsid nuclear translocation, I analyzed the interactions of ChikV capsid with various karyopherins. ChikV capsid protein was expressed in E.coli with His tag and purified using the Ni-NTA column (Fig. 5A).
Authenticity of the protein was confirmed through Western blot using anti-His tag antibody (Fig. 5B). Purified ChikV capsid was incubated with HEK293 cell lysate; transiently transfected with various FLAG tagged Karyopherins (kar α 1-4). It was found that ChikV capsid interacts with the karyopherin α 4 specifically and weakly with karyopherin α3 (Fig. 6A: lane 3and4).



A B

Figure 5: His tagged purification profile of ChikV capsid protein. ChikV capsid expressed in *E. coli* in native form was purified using Ni-NTA column. A shows the coomassie stained SDS-PAGE of His tagged ~37 kDa purified protein (~20 ng/µl). B shows the Western blot of the protein with ∝His antibody. Arrows indicate the protein position.

To confirm the interaction, I investigated the interaction between ChikV capsid and kar α 4 in mammalian cell line. HEK293 cells were transiently transfected with FLAG-kar α 4 and ChikV-cap-GFP. The protein complex was co-immunoprecipitated with an anti-FLAG antibody bound to protein A–Sepharose. The bound proteins were

subjected to SDS-PAGE followed by Western blot using anti-GFP antibody. As shown in Fig. 6B (lane 2), ChikV capsid co-immunoprecipitated with kar α 4. No binding was observed in the control experiments using only beads or by using a non-specific antibody.

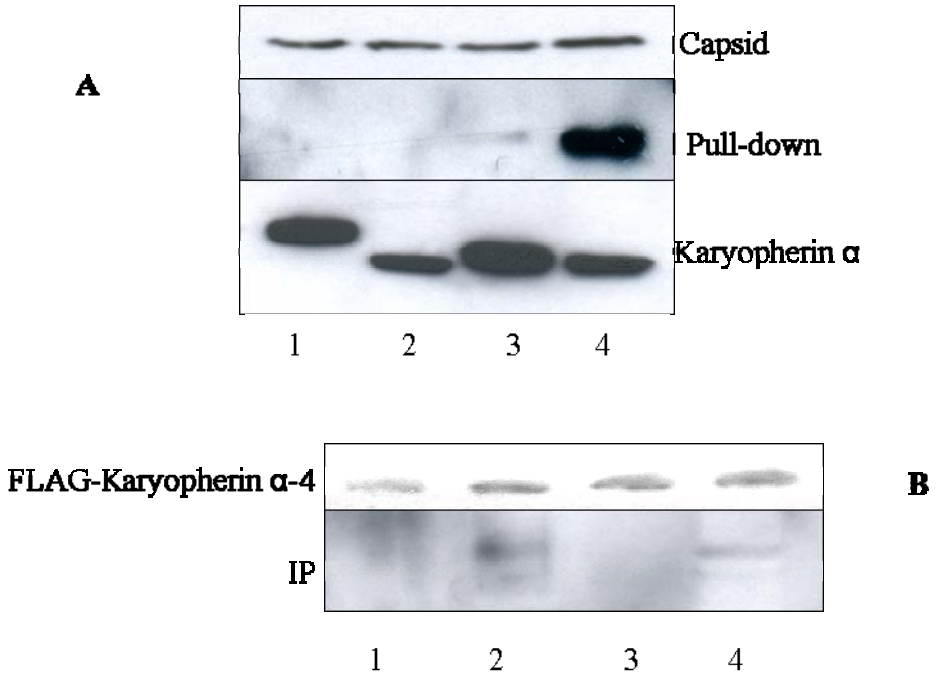


Figure 6: ChikV capsid protein interacts with karyopherins. A) Pull-down assay with *E. coli* expressed, purified ChikV capsid was performed. Lane 1-4 with karyopherin α 1, 2, 3 & 4. Karyopherins bound to capsid was detected with anti-FLAG antibody B) Immunoprecipitation to confirm the interaction of karyopherin α 4 with ChikV capsid. Lane 1, beads, lane 2 with anti-GFP antibody, lane 3, non-specific antibody, lane 4 Input.

3.3.2.2. NLS major binding site is adequate for the interaction between kar α 4 and capsid

Karyopherin α molecule contains two NLS-binding sites that directly recognize NLS sequences of cargo proteins. The primary NLS binding site is located at the N-terminal armadillo (alpha helices that form a hairpin structure); (arm) repeats 2–4, while the secondary NLS binding site is located at the C-terminal arm repeats 7–9 (Fig. 7A). NLS major binding site is associated in interaction with monopartite NLS, while minor binding site associated with bipartite NLS. (Conti *et al.*, 1998; Fagerlund *et al.*, 2005; Fontes *et al.*, 2000; Melen *et al.*, 2003).

To further characterize the mechanism of ChikV capsid binding to kar α 4 molecule, I created deletion mutant karα-4Δ 259, which lacks the minor binding site. I checked if the minor binding site is required for the interaction with ChikV capsid through pull-

down assay. Karα-4Δ 259, which lacks the minor binding site was able to interact with ChikV capsid alone (Fig. 7A lane 4) or in the presence of excess kar α 4-FL (Fig. 7B, lane 5). This shows that the N-terminal arm repeats of kar α 4 form the binding site for ChikV capsid.

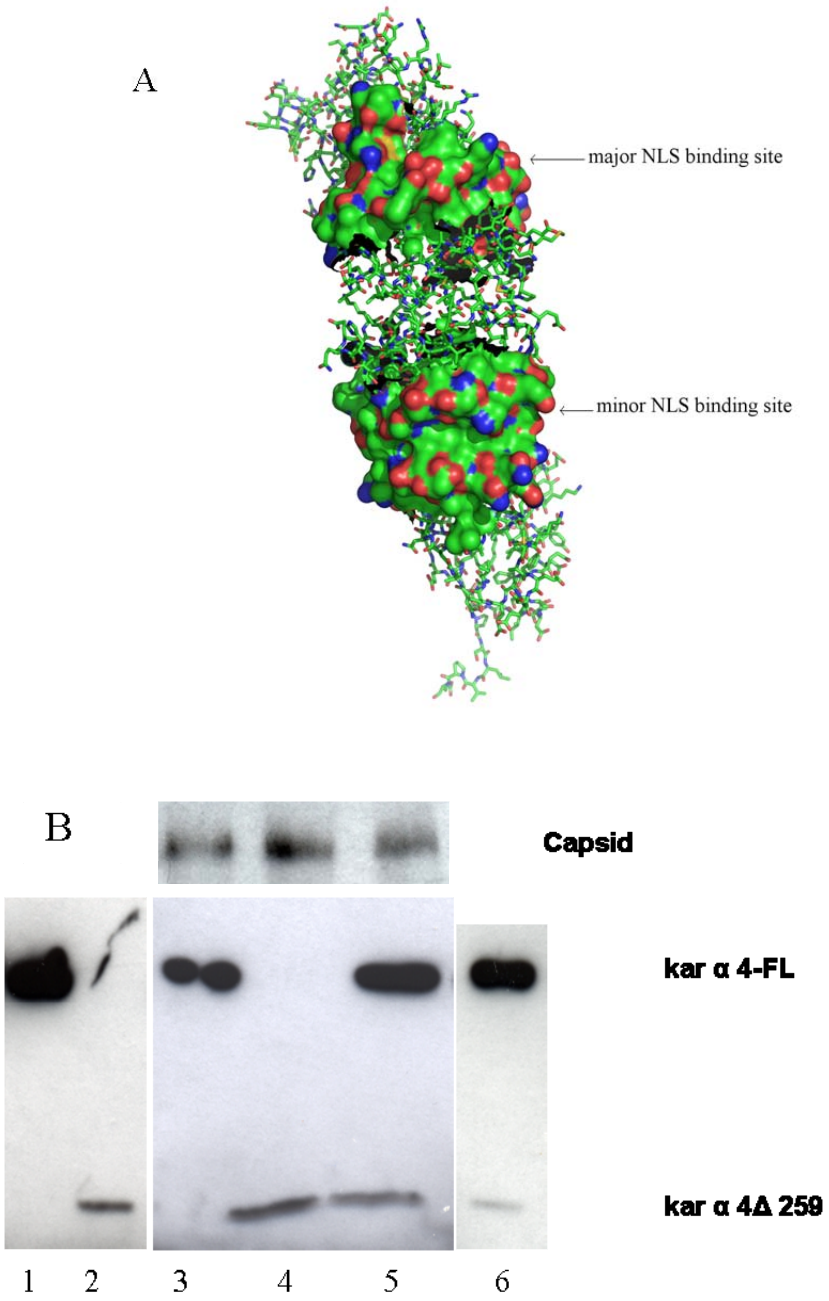
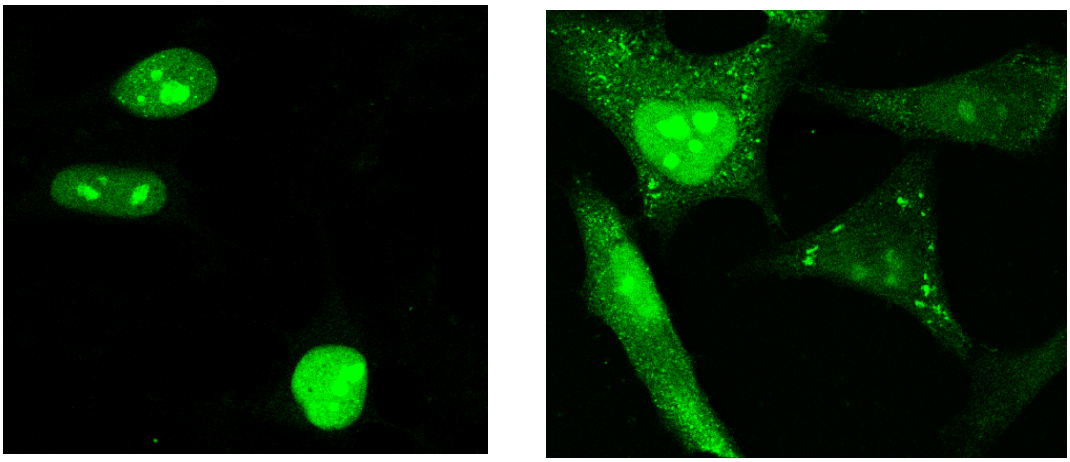


Figure 7A: Molecular model of karyopherin α 4. The major and minor NLS binding sites are marked. Fig. 7B: NLS major binding site is adequate for the interaction between kar α 4 and capsid: Lane 1 input of kar α 4-FL. Lane 2, input of kar α 4Δ 259. Lane 3 pull-down assay with kar α 4-FL. Lane 4 pull-down assay with kar α-4Δ 259. Lane 5 pull-down assay with mix of both kar α 4-FL and kar α-4Δ 259. Lane 6 input of kar α 4-FL and kar α-4Δ 259 mix.

3.3.2.3. ChikV capsid encodes a CRM1-dependent NES

The exit of proteins from the nucleus is mainly regulated via the nuclear export receptors. The XPO1 protein, also known as exportin1 and CRM1, is the most versatile of all export receptors, being involved in the movement of many different classes of proteins (Nigg, 1997; Turner & Sullivan, 2008). To check this I used the specific CRM1 mediated nuclear export inhibitor LMB (leptomycin B); (Kudo *et al.*, 1998). ChikV capsid fused with GFP was transfected to HEK 293 cells and treated with leptomycin B. As shown in Fig. 8A and C, leptomycin B treatment leads to total nuclear accumulation of ChikV capsid-GFP (15 ± 6 times nuclear) whereas in un-treated cells the protein was seen in both nucleus and cytoplasm (3 ± 1 times nuclear ; Fig. 8B and C).



A B

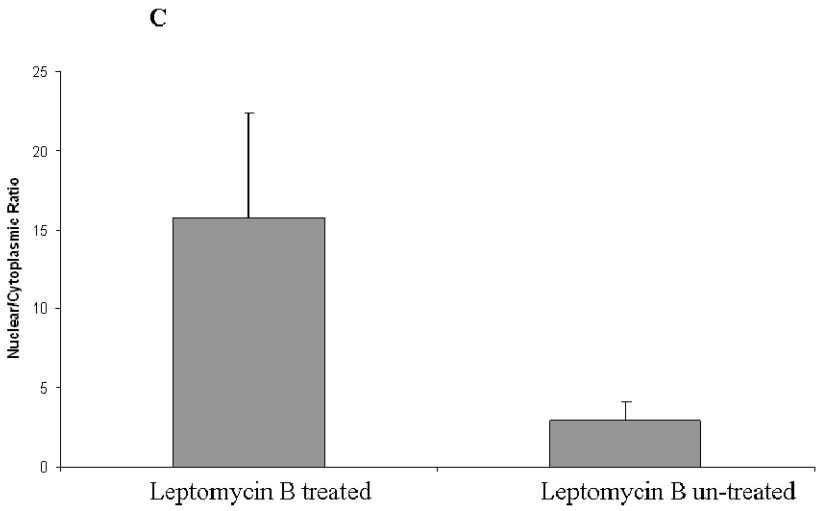


Figure 8: ChikV capsid encodes a CRM1-dependent NES. (A). Leptomycin B treated, (B) Un-treated (C) Graphical representation, showing the nuclear cytoplasmic ratio of both leptomycin B treated and un-treated cells.

Table 3: Amino acid sequence analysis of various alpha virus isolates.

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                                        167
ChikVQ8JUXI TIDNADLAKL AFKRSSKYDL ECAQIPVHM
ChikVQ5WQY  .......... .......... .........
ChikVQ5XXP  .......... .......... .........
SemlikiP03  V......... ...K...... .........
OckelboP27  ...HPV.S.. K.TK..A..M .F..L..N.
SindbisP03  ...HPV.S.. K.TK..A..M .F..L..N.
RossP13890  ....P..... TY.K...... .........
RossP08491  ....P..... TY.K...... .........
SagiyamaQ9  V...P..... TY.K...... .........
GetahQ5Y38  V...P..... TY.K...... .........
Middelburg  V...P..... ...K...... .........
OnyongO903  .......... .......... .........
OnyongP220  .......... .......... .........
IgboO90371  .......... .......... .........
BarmahP899  ....PE.... T..K...... ....V..C.
WEEP13897   K...EQ..AV KL.KA.M... .YGDV.QN.
EEVQ306W7   K...EQ..AI KL.KA.I... .YGDV.QC.
EEVQ306W5   K...EQ..AI KL.KA.I... .YGDV.QC.
EEVP08768   R...EQ..AI KL.KA.I... .YGDV.QC.
EEVP27284   R...EQ..AI KL.KA.I... .YGDV.QC.
EEVQ4QXJ7   R...EQ..AI KL.KA.I... .YGDV.QC.
VEEP36331   K...DV.SS. KT.KA..... .Y.DV.QS.
VEEP05674   K...DV..A. KT.KA..... .Y.DV.QN.
VEEP09592   K...DV..A. KT.KA..... .Y.DV.QN.
VEEP36329   K...DV..A. KT.KA..... .Y.DV.QN.
VEEP36332   K...DV..A. KT.KA..... .Y.DV.QN.
VEEP36330   K...DV..A. KT.KA..... .Y.DV.QN.
MayaroQ8QZ  V........R. SY.K...... .......A.
AURAQ86925  ...HPA.... K.TK..S..M .F.KL.TE.
```

3.3.2.4. Mapping of NES

To identify a region of ChikV capsid that may contain a nuclear export signal, deletion mutants were constructed (Fig. 9). Deletion of 46 aa from C-terminal do not have any affect in the nuclear cytoplasmic translocation as it can be seen distributed throughout the cell (Fig. 10B and E). Removal of 118 aa from C-terminal localized the GFP fusion protein entirely to the nucleus (8 ± 3 times more nuclear; Fig. 10C and E). Regions of aa 148 to 152 that are rich in leucine residues were examined as potential NES sites. For this leucine's at 149 and 152 were mutated to alanine, through overlap PCR. These amino acids were selected as it is conserved among most of the alphavirus isolates (table 3). Substitution of Leu to Ala in 149 and 152 positions (SDM) resulted

in decrease of cytoplasmic localization of protein (5 ± 1 times more nuclear Fig. 10D and E).

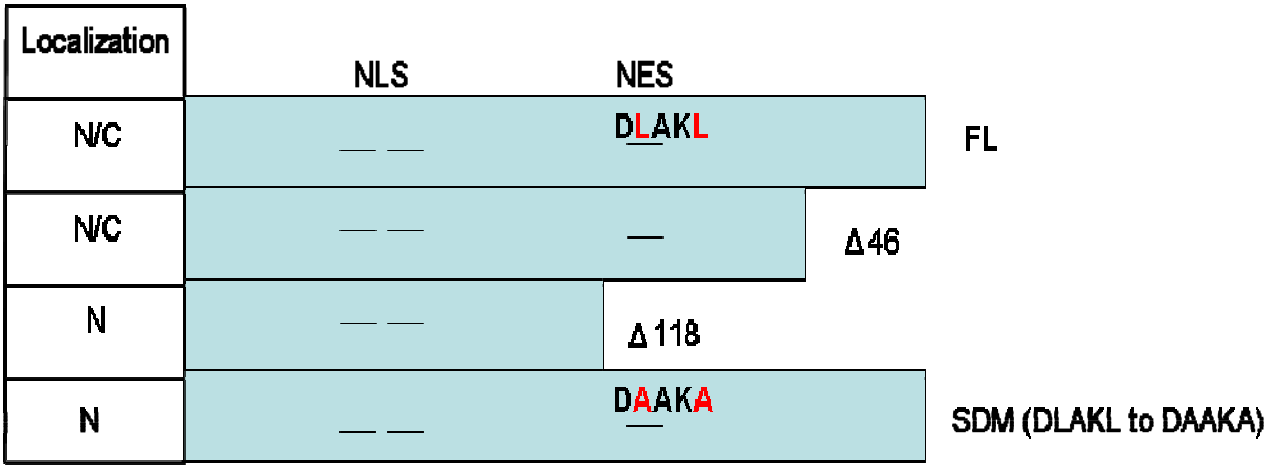
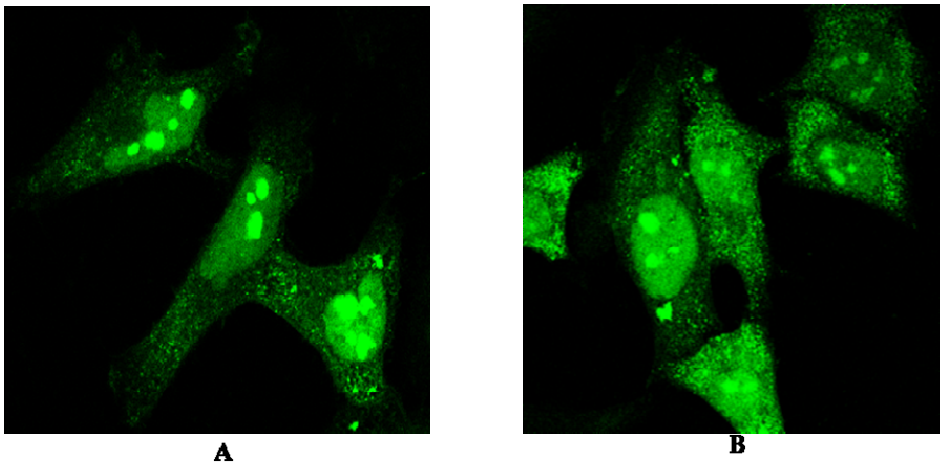
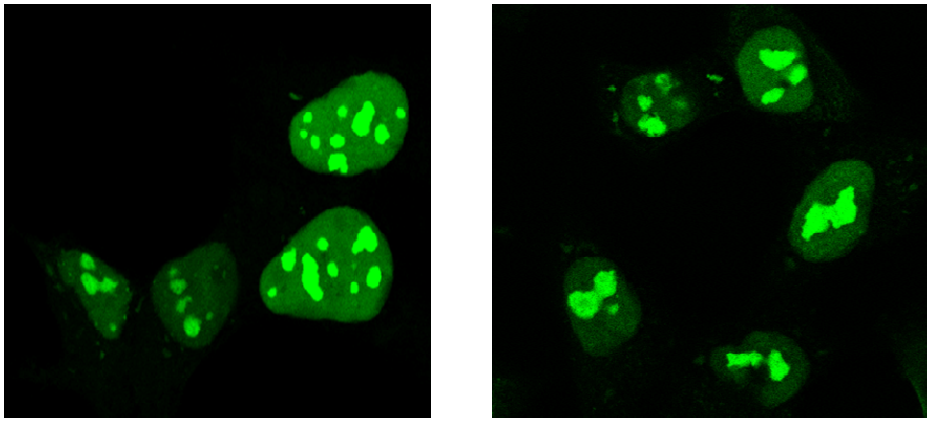


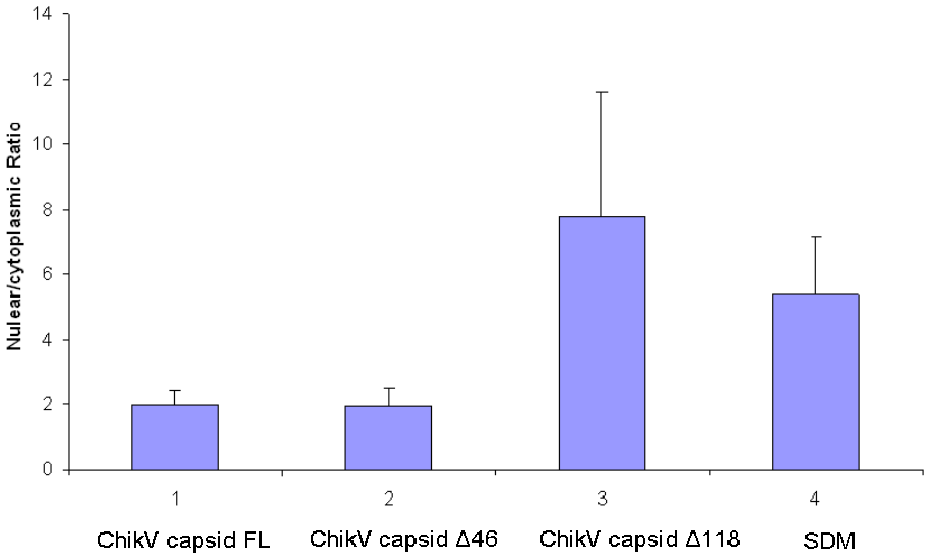
Figure 9: Various GFP fusion constructs used in this study to map the nuclear export signal along with their observed localization.



A B



C D



E

Figure 10: Mapping of NES. ChikV cap-GFP Mutants were constructed through deletion or through site directed mutagenesis and the localisation of the protein was analyzed in HEK 293 cells through confocal microscopy (A) ChikV capsid FL, (B) ChikV capsid Δ46 (C) ChikV capsid Δ118 (D) Site directed mutant (DLAKL to DAAKA) (E) Ratio of nuclear to cytoplasmic concentration of GFP-fusion proteins, as a measure of nuclear import and export.

3.4. Discussion

3.4.1. Alphavirus capsid protease: sequence requirements for activity

The cleavage specificity of alphaviral capsid proteases has been characterized partially for SFV and Sindbis virus (Choi *et al.*, 1996; Melancon & Garoff, 1987). The main determinants identified for site-specific cleavage is the conservation of W267 (W261 in ChikV) (Skoging & Liljestrom, 1998) and an intact catalytic triad (Melancon & Garoff, 1987). An exchange of threonine for serine (S262T) failed to prevent this cleavage, and showed that serine is not important for its activity. Deletion of 46 aa from C-terminus inactivated the protease either by a change (deletion) in the cleavage site or due to a change in the tertiary structure. These results also showed that amino acids with in the C-terminus of the ChikV capsid are important for the activity of the capsid protease.

It has been shown before in SFV capsid protease that W267 was essential for efficient viral growth (Skoging & Liljestrom, 1998). Mutating tryptophan 267 to alanine resulted in loss of cleavage activity and subsequent loss of viral replication. The dominant role of W267 for SFV replication leads us to analyze the role of other amino acids near tryptophan 261 in ChikV capsid. Mutation of other conserved or less conserved residues (Fig. 3A) close to tryptophan did not alter the activity of the ChikV protease (Fig. 3B). Our results further support the observation that the importance of tryptophan 267 in SFV is not related to a change in tertiary structure or improper folding but serves as sequence specific recognition signal for the autocatalytic triad (Skoging & Liljestrom, 1998).

One mutant introduced by error-prone PCR resulted in complete loss of protease activity when isoleucine at 227 position was changed to lysine. Molecular modelling showed that the formation of a salt bridge between mutant K227 and D161 potentially affects crucial functions of D161. D161 is one out of three conserved amino acid, forming the autocatalytic triad of the capsid protease. The probable salt bridge between K227 and D161 obviously disrupts the protease activity of the autocatalytic triad. In the native enzyme, D161 makes a low barrier hydrogen bond

(LBHB) with H139 (Frey *et al.*, 1994). Due to this LBHB, H139 acts as general base and abstracts the proton from the S213 hydroxyl group. De-protonated S213 will act as a nucleophile and will form a tetrahedral transition state with carbonyl carbon of the substrate. The disruption of LBHB by N-methylation of histidine (Henderson, 1971) or by mutation of D161 of the catalytic triad to asparagine has been shown to inactivate serine proteases (Craik *et al.*, 1987). The potential salt bridge between D161 and mutant K227 may also disrupt the LBHB between D161 and H139 and may consequently make the enzyme inactive as the salt bridge can be very stable in the hydrophobic environment of the active site (Anderson *et al.*, 1990).

In conclusion, the analysis shows that I227 is located close to the catalytic triad of the ChikV capsid and a mutation of this residue leads to disruption of the protease activity. I am also showing that amino acids other than the catalytic triads are important for the activity of this serine protease of the ChikV capsid.

3.4.2. Nuclear Import and export of ChikV capsid protein

The *Alphavirus* genus of the *Togaviridae* family contains a number of important human and animal pathogens. In that New World alphavirus CP induces apoptosis through transcription inhibition that is mainly determined by the amino-terminal fragment of the protein or by the positively charged RNA-binding domain. In the case of Old World alphaviruses which includes ChikV, CP doesn't induce transcriptional inhibition or apoptosis (Garmashova *et al.*, 2007). Despite the localization of ChikV CP in the cytoplasm and nucleus, the molecular mechanism underlying this intracellular transport is unknown. Similarly unknown is the regulation of its import and export. Although the alphavirus capsid protein has multiple critical functions attributed to it, some of its function remains indefinable. Alphavirus CPs with a size of ~30 kDa are smaller than the molecular size required (> 40 kDa) for kar mediated nuclear transport (Paine *et al.*, 1975). Still alphaviral CPs have been found to harbour NLS and the transport is energy dependent (Favre *et al.*, 1994; Michel *et al.*, 1990). To investigate the mechanism that determines the nuclear-cytoplasmic distribution of ChikV CP, we evaluated if it is interacting with karyopherins, the protein which shuttles NLS containing proteins to the nucleus. Using pull-down assays we identified karα4 as a specific binding partner of the viral protein. It is likely

that karα4 physically binds to ChikV CP in the cytoplasm, and then interacts with importin-β1 at nuclear pores followed by a Ran-GTP-dependent translocation process through nuclear pores into the nucleus.

Karα recognizes a variety of classical NLSs, like the very basic monopartite SV40 large T antigen NLS (PKKKRKV), the more hydrophobic monopartite c-myc NLS (PAAKRVKLD), and the bipartite nucleoplasmin NLS (VKRPAATKKAGQAKKKKLD) (Collas & Alestrom, 1996; Nadler *et al.*, 1997). Crystallographic analyses of karα bound to NLS peptides showed that the C-terminal minor binding site of karα comprising arm repeats 7 and 8 is able to bind NLS peptides. The crystal structure shows that the major NLS binding site of yeast karα molecule recognizes the classical monopartite NLS peptide of SV40 large T antigen and the larger downstream basic cluster of the classical bipartite NLS peptide. The minor NLS binding site has a supportive role in the binding of smaller basic cluster of the bipartite NLS (Conti & Kuriyan, 2000; Soisson *et al.*, 1998). The c-*myc* NLS peptide (PAAKRVKLD) binds to major as well as minor binding sites of yeast karα (Conti & Kuriyan, 2000). Crystal structure of the C-terminal domain of influenza virus polymerase PB2 subunit also shows similar binding (Tarendeau *et al.*, 2007). Mouse karα exhibits binding for the bipartite nucleoplasmin NLS as yeast karα, whereas SV40 large T antigen NLS peptide was shown to bind equally to the major and minor binding sites (Fontes *et al.*, 2000). Karα has two NLS binding sites that directly interact with the NLS containing protein. Arm repeats 2–4 comprise the N-terminal NLS binding site and arm repeats 7–9 the C-terminal NLS binding site. The C-terminal part of karα has been proposed to bind to various NLS. Through deletion analysis it has been shown that the NLS of Epstein-Barr virus (KRPRSPSS) and the matrix protein of HIV (KKKYKL) are bound through the C-terminal half of karα1 (Fischer *et al.*, 1997; Gallay *et al.*, 1996). Through site-directed mutagenesis it has been shown that the C-terminal arm repeats 8 and 9 of karα5 form binding site for STAT1 homodimers and STAT1-STAT2 heterodimers (Melen *et al.*, 2003). LEF-1 NLS, a human transcription factor (NLSKKKKRKREK) is recognized by karα1 and 5 and the binding was mapped to the C-terminus of karα1 (Herold *et al.*, 1998). In case of Influenza A virus nucleoprotein (NP), which has a nonconventional NLS in its N-terminal and is reported to be interacting with the C-terminal NLS binding site of karα5, comprising arm repeats 7–9. It also binds to karα3 via the C-terminal arm

repeats (Melen *et al.*, 2003). Semliki Forest virus CP, is proposed to contain 2 NLS, 66'KPKKKKTTKPKPKTQPKK'83 and 92'KKKDKQADKKKKKP'105 (Favre *et al.*, 1994) and with the putative 2 NLS sequences found in ChikV CP, 60'KPRRNRKNKKQKQK'73 and 84'KKQPPKKKPAQKKKKP'99 it was tempting to look for the karα domain responsible for this binding. Through pull-down assay we found that the N-terminal NLS binding site of karα4 is sufficient for the ChikV CP NLS interaction. A deletion of the C-terminal NLS binding site from karα4 did not have any effect on molecular interaction. Further studies are needed to show how ChikV CP specifically utilizes karα3 and 4 instead of all available importins for nuclear import.

The exit of proteins from the nucleus is mainly regulated via the nuclear export receptors. The CRM1 protein also known as XPO1, and exportin1, is the most versatile of all export receptors involved in the transport of many different classes of proteins (Nigg, 1997; Turner & Sullivan, 2008). CRM1 binding is often associated with leucine rich NES. The 10-15-residue LR-NESs was first identified in HIV-1 Rev and cyclic-AMP-dependent protein kinase inhibitor, and is recognized by the exportin CRM1. As the ChikV CP is present both in the nucleus and cytoplasm, we checked if it is CRM1 dependent. Leptomycin B has been shown to inhibit nuclear export of proteins due to alkylation of CRM1 (Kudo *et al.*, 1999). Here treatment with leptomycin B on cells transfected with ChikV CP-GFP resulted in the complete inhibition of nuclear export.

I further tested some deletion mutants fused to GFP to fine map the NES in the ChikV capsid. Removal of 46 aa from the C-terminus had no effect while removal of 118 aa made the protein exclusively nuclear. Analysis of the sequence revealed repeats of leucine rich regions between 148th and 162nd residues of ChikV capsid. Leucines in the 149th and the 152nd position were found to be highly conserved in different ChikV isolates and most of the alphaviruses (table 3). Substitution of these with alanine made the protein mostly nuclear and confirmed the CRM1 dependent NES. Thus the NES was mapped between aa 149 and 152 of the ChikV capsid. Even though region between aa 149 and 152 was found important for the CRM1 dependent nuclear export, the nuclear cytoplasmic ratio was only 5 ± 1 times more nuclear (Fig. 10D and E) compared to 15 ± 6 times nuclear (Fig. 8A and C) after the

leptomycin B treatment. This may be due to the fact that Chik V capsid may be encoding another CRM1 dependent NES other than that mapped between 149 and 152 positions. It is also possible that some other factors may be modulating the nuclear export of proteins. It may include phosphorylation as in the case of proteins like p53 were phosphorylation of ser15/20 in p53 in response to DNA damage leads to NES1 masking. Another mechanism consists in tetramerization of protein p53 within the nucleus in response to the DNA damage, NES2 is masked in tetrameric p53. Dissociation of this tetramer is required for the export of the protein from the nucleus (Stommel *et al.*, 1999). In case of androgen receptor, masking of NES/NLS as was shown in which NES is in the ligand-binding domain. In the presence of the ligand (androgen), NES is masked and Crm1 cannot recognize it. The receptor is translocated only after the dissociation of androgen (Saporita *et al.*, 2003). Here I am assuming that other regions may be important for the proper stability of the protein to be cytoplasmic or it is possible that the phosphorylation sites in it may be important for the modulation through the recognition by exportin 1 and thus the export of ChikV capsid.

To conclude we are showing that ChikV capsid is recognized by karyopherins and its export is mediated by CRM1 dependent nuclear export signal.

4. Summary

The main functions of viral capsids are to protect, transport and deliver the viral genome. I investigated some of these aspects of two positive stranded RNA viruses, Hepatitis E virus (HEV) and Chikungunya virus (ChikV), an alphavirus. I was able to detect HEV in swine sera collected from Western and Northern Germany. This shows the vulnerability of German population to HEV and the necessity for stringent screening of swine farms in Germany for HEV. I also expressed HEV capsid in various expression systems. The use of HEV capsid as a vaccine candidate is discussed using an adenovirus based expression system. One of the main problems that have hindered the HEV research is the lack of a good infectious system. Using clone pJE03-1760 I was able to establish an infectious system which was well propagating in the cells. This system was further used to analyse the role of cholesterol in virus egress. I found that simvastatin treatment hindered HEV egress.However simvastatin had no significant effect on HEV genome replication. Our data suggest simvastatin as a promising drug candidate for treating HEV.

Although the alphavirus capsid protein has multiple critical functions ascribed to it, some of its functions remain elusive. Alphaviral replication depends on the efficient and precise cleavage after tryptophan 261 executed by the capsid protease. I looked at the requirement of various amino acid sequences for the efficient cleavage of this protease using ChikV capsid. My analysis shows that amino acids other than the catalytic triads are important for the activity of the serine protease of the ChikV capsid. Despite the localization of ChikV capsid in both, cytoplasm and nucleus, its molecular mechanism is still unknown. Similarly unknown is the regulation of its import and export. Using various methods I was able to show that ChikV capsid is recognized by karyopherins and its export is mediated by a CRM1 dependent nuclear export signal.

These findings can be used for approach towards antiviral drugs or vaccines that target viral capsids.

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7.Publications

1**. Saijo Thomas**, Jagdish Rai, Lijo John, Christian Drosten, Brigitte M. Pützer, Stephan Schaefer. Functional dissection of the alphavirus capsid protease: sequence requirements for the activity. Virol J. 2010 Nov 18;7:327.)

2. **Saijo Thomas**, Brigitte M. Pützer, Stephan Schaefer. Nuclear import and export, of Chikungunya virus capsid protein. (Manuscript under preparation).

8. Erklärungen

Ich versichere hiermit an Eides statt, dass ich die vorliegende Arbeit selbstständig angefertigt und ohne fremde Hilfe verfasst habe, keine außer den von mir angegebenen Hilfsmitteln und Quellen dazu verwendet habe und die den benutzten Werken inhaltlich und wörtlich entnommenen Stellen als solche kenntlich gemacht habe.

Rostock, 27.10.2010 **Saijo Thomas**