Chapter Five

Discussion
Successful replication of a virus in its host is a complex process which involves multilevel interactions. Viruses and host evolve together and this co-evolution often leads to species specificity. Presence of specific attachment proteins/receptors and availability of cellular factors required for viral multiplication in a host cell are the important host factors. While, structural proteins are involved in receptor binding (capsid/envelope), proteins involved in replication are the viral factors which contribute significantly in deciding host/tissue specificity. Lack of appropriate cell culture model or small animal model have been major hurdles in understanding molecular mechanisms of HEV replication, tissue/species specificity, cell surface receptors and immunopathogenesis of HEV. Reverse genetics has provided us with a tool to study viruses that cannot be grown efficiently in cell culture system. Genotype 3 and 4 HEV strains were shown to grow efficiently in PLC/PRF5 cells (Tanaka et al. 2007 and 2009), however, genotype 1 virus still lacks efficient cell culture system. Infectious cDNA clones have been developed successfully for the genotypes 1, 3 and 4 of the mammalian strains of HEV (Panda et al. 2000, Emerson et al., 2001, Yamada et al., 2009, Cordoba et al., 2012). These clones have helped shedding some light on the virus replication. There are number of studies that have addressed the issue of viral determinants and host specificity (Cordoba et al. 2012, Feagins et al., 2011, Graff et al., 2005), but the question is still open. A recent report from Dr. Emerson’s group suggests that the host restriction for the genotype 1 virus could be due to restricted entry in the host cells and efficiency of the virus to generate ORF2 protein in the given host (Nguyen et al., 2014). Lack of compatibility between cell surface receptor and receptor binding region (rbc) (456-605 amino acids) in the capsid protein of virus was suggested to be deciding factor. They used an efficiently replicating genotype 3 virus replicon (P6), developed from the Kernow C-1 virus (Shukla et al., 2012), for developing chimeric genotype 1/3 viral genomes. P6 has 171-nucleotide insertion (from human S17 ribosomal protein encoding gene) in the HVR region. It was previously reported by this group that P6 virus, isolated from a chronic HEV case was to able cross species barrier (Shukla et al., 2011). Further, SAR55/S17 chimera could replicate in LLC-PK (pig) cells. Use of P6 virus clone to develop SAR55/P6 (genotype 1/3) chimeras in the first place was not very suitable model to study
species specificity since the virus had ability to infect cells from a broad spectrum of species, ranging from rodent to primate. Further, selective growth of SAR55/P6-rcp chimera developed in this study could grow in HepG2/C3A cells but not in LLC-PK cells suggesting possible role of additional factor/s than just the receptor binding region in the capsid protein in deciding host specificity.

We attempted to answer this question by constructing genotype 1/4 chimeras. In the first phase our major aim was to construct full genome cDNA clones of HEV genotype 1 and 4 which are replication competent. Subsequently, construct chimeras by replacing parts of genotype 1 with corresponding parts of genotype 4 and check their species specificity using human and swine cell lines.

Construction of full genome infectious clones had many difficulties associated with it, major being single nucleotide mutations introduced by conventional cloning. We initially cloned both genotype 1 (T1FG) and genotype 4 (T4FG) viral genomes by making use of natural / modified restriction sites in the viral sequences. On checking replication competence of these constructs we understood that they were not replicating. On personal discussion with Dr. Suzanne Emerson (NIH) we understood that even one nucleotide change in HEV genome can result into tremendous decrease in replication efficiency. Under such conditions our genotype 1 clone, T1FG: 50nt changes with 25 amino acid changes, 18 in the ORF1 region and 7 in the ORF2 region as compared to the prototype sequence, GenBank accession no. DQ459342; genotype 4 clone, T4FG: 14nt changes as compared to the prototype sequence, GenBank accession no. AY723745 would hardly replicate. However, for T1FG this problem was sorted out by adopting fusion PCR technique to join individually amplified genomic fragments. The same technique did not work for type 4, as several attempts to stitch PCR amplicons either failed or generated unexpected products. We decided to continue with the initial T4FG clone and repaired the ORF1 region by doing extensive site directed mutagenesis. Overall, it was our experience that type 4 full genome cDNA construct is extremely unstable as compared to type 1 clone. Tired of sequencing and with a hope of finding even one infectious clone, we did in
vitro transcription of 20 different clones obtained in the last ORF1 correction mutagenesis experiment, transfected them individually in S10-3 cells and assayed for positive replication. We could get one clone which was positive in both nsRNA assay and IFA done for ORF2 protein.

Even though in-vitro transcribed RNA was rendered negative for template DNA, quantitative PCR (qPCR) was not helpful. Present cell culture systems for genotype 1 HEV are not so robust and in presence of large amount of input RNA in cell transfections there is a high background of this RNA and its degradation products. Any marginal change due to newly synthesized RNA is not significant and hence we did not use real-time PCR to evaluate replication efficiency of the replicons. Low RNA transfection efficiency; poor virus replication with undetectable levels of capsid protein in the cells increase difficulties further in detecting the replicating status of the virus. Moreover, virus particles of HEV genotype 1, generated after RNA transfections remain cell associated (Emerson et al., 2001). Our attempts to develop quantitative methods for virus replication miserably failed. Since ORF2 protein detection is the accepted method in HEV replication studies, we decided to use immunofluorescence assay. We could use polyclonal antibodies (human serum) successfully, for detecting both type 1 and type 4 capsid proteins in IFA. Our group has previously shown that ORF2 proteins from both genotype 1 and 4 viruses are useful in detecting antibodies against either virus (Arankalle et al., 2007). However, monoclonal antibodies, against genotype 1 partial ORF2 protein (458-607 amino acids) were not useful. Other option was to use PCR based detection of negative sense replicative intermediate RNA. We optimized a new assay for nsRNA detection by using tagged primer based PCR (Chatterjee et al., 2012). Use of tagged primers prevented self priming during cDNA synthesis. However, this assay was not completely safe as it yielded non-specific positivity in presence of $>10^6$ positive sense RNA copies in the nsRNA reaction. Before proceeding with the nsRNA detection we processed all RNA samples from cells for positive sense HEV RNA quantitation. Samples were diluted to reduce input RNA copies to $<10^6$ copies/reaction to avoid false positivity.
We developed one full genome cDNA clone for each genotype 1 and genotype 4 viruses. ORF2 encoding regions of these both clones still contained mutations (7 in T1FG and 7 in T4FG) at amino acid level, however all three glycosylation sites (Asn137, Asn310 and Asn562) were intact in both the clones. ORF1 region sequences, both cis acting 5’- and 3’-NCR as well as the ORF1-ORF3/2 junction region sequences had 100% identity with the respective prototype sequences. With ongoing difficulties in developing replicative clones we had requested Dr. Suzanne Emerson for giving genotype 1 infectious cDNA clone (pSK-HEV2) developed in her laboratory. We also received clonal Huh-7 cells (S10-3) developed in her laboratory, which were more permissive for HEV than the parental Huh-7 cell line. We were able to check replication efficiency of pSK-HEV2 replicon in S10-3 cells as there were optimized assays in place. On comparing T1FG and pSK-HEV2, later was found to be better replicating. It also showed comparatively intense IFA signals than the T1FG. Emerson et al (Emerson et al. 2013) have recently reported presence of two highly conserved stem loop structures, ISL1 and ISL2, in the centre of ORF2 encoding region that are essential for capsid protein synthesis. Silent mutations in this region were shown to have negative effect on capsid protein synthesis. Though none of the ORF2 mutations from T1FG and T4FG constructs were present in these SL structures, we still believe that removal of mutations in ORF2 region would increase replication efficiency of T1FG and T4FG replicon constructs.

We could not get any swine liver cell line and used swine kidney, testis and embryo cell lines for testing replication. Various hepatoma cells of human origin, two non-liver cell lines A549 and Caco2, and three swine cells lines were tested for their permissiveness by using transcripts from T1FG, pSK-HEV2 and T4FG clones. Human hepatoma cells supported both genotype 1 and 4 virus relication as seen by positive signals in nsRNA and ORF2 detection assays (table 8). Swine embryo cells (ESK-4) and kidney cells (PK-15) were positive for nsRNA with T4FG. PK-15 cells were positive for both nsRNA and ORF2 protein. All three swine cell lines remained negative for both nsRNA and ORF2 till the 12th day after transfection with T1FG or pSK-HEV2
replicon transcripts, confirming that swine cells do not support HEV genotype 1 virus replication.

Knowing that there is species barrier for genotype 1 virus we decided to retain pSK-HEV2 as the backbone and developed 10 chimeric constructs by replacing different genomic regions of the pSK-HEV2 clone with the T4FG. There are previous attempts to identify viral determinants responsible for restricting host range in HEV. Besides above described genotype 1/3 clones, Dr. Emerson’s group had developed chimeric genotype 1/3 construct by incorporating 3’-terminal sequences from the genotype 3 swine virus into the genotype 1 clone and shown that this chimera could not establish infection in pigs (Graff et al., 2005). Cordoba et al (2012) constructed four chimeras (Jr+ORF2+3’NCR) of genotype 4 human and genotype 3 swine HEV in the backbone of genotype 1 human HEV and vice versa. Faegins et al (2011) reported five chimeras: Jn+ORF2 and Jn+ORF2+3’NCR of genotype 4 human HEV in pSK-HEV2, Jn+ORF2+3’NCR of genotype 3 swine HEV in pSK-HEV2, ORF2 and Jn+ORF2+3’NCR of genotype 4 human HEV in genotype 3 swine HEV(pSHEV-3). None of these constructs were shown to successfully cross the species barrier.

RNA virus genomes often contain functionally active RNA structures that are critical during the various stages of viral replication. Error prone copying by the RdRp of these viruses restricts genome size of RNA viruses and in many cases only a small part of viral genome is functionally relevant as RNA. Secondary and tertiary structures in such RNA elements play essential regulatory roles during translation, RNA replication, and assembly of virions. Cis-acting 5’ and 3’ non-coding genomic regions are important signatures of RNA viral genomes which help viral RdRp to discriminate between cellular and viral mRNAs. RNA secondary structures are sensitive to point mutations which may cause large changes in the secondary structures. Graff et al., (2005) have shown that there is a significant decrease in HEV replication with a single nucleotide change at 7106nt in the 3’NCR of pSK-HEV2 proving that sequences or structures in the 3’terminal region are critical for HEV replication. Similarly, with specific binding of ORF2 protein to the 5’end of HEV genome and its role in encapsidation was demonstrated by Surjit et al (2004).
These viral regulatory elements are also responsible for interacting with various cellular proteins at different stages of replication.

Considering these, we developed type 1/4 chimeras by replacing ORF1, ORF2/3 encoding regions and the cis-regulatory genomic elements, 5’NCR, 3’NCR and putative subgenomic promoter (Jn) (currently called as the junction region rather than subgenomic promoter since it is not functionally confirmed) with type 4 sequences. Separate chimeras were constructed by swapping regulatory elements and ORF2/3 encoding region alone or along with adjacent junction region and/or 3’NCR. Establishment of the ORF1 chimera was very difficult and we could only manage its cloning along with 5’NCR. There was no chimera with type 4 ORF1 without 5’NCR.

All the constructs were initially checked for their replication competence in S10-3 cells. These cells supported replication of all the constructs as evidenced by immunofluorescence and nsRNA assays (table 9 and 10). The most interesting observation we came across while doing IFA was that the replication efficiencies of these chimeras were different. To our surprise, it was seen that chimeras T4 (Jn +ORF2 +3’NCR) and T4 (ORF2+3’NCR) were replicating with 5- and 3- folds higher efficiency as compared to the parental clone pSK-HEV2 (figure 36 and 37). Swapping of the ORF2, Jn+ORF2 or 3’NCR (chimeras T4 [ORF2], T4 [Jn+ORF2], and T4 [3’NCR]) did not change replication efficiencies of the clones and were comparable to the parental clone. On contrary to T4 (3’NCR) clone, there was a significant reduction in the replication efficiency of the T4 (5’NCR) clone, indicating major contribution of the 5’NCR during virus replication and its probable association with species specific factors during this. It is likely that host proteins probably interact with the 3’end of the negative sense RNA intermediate (complementary to the 5’NCR of the genome) to help initiate synthesis of genomic positive sense molecules. Unfortunately, due to very low replication efficiency of the parental T4FG clone, we could not include it in the quantitative analysis.

Improvement in the replication efficiency after inclusion of the 3’NCR in the chimera T4 (5’-3’ NCR) made the analysis further complicated. We speculate that the HEV 5’- and 3’-NCRs may either directly interact with each
other or via host-cell proteins during replication. Comparable levels of replication efficiencies of T4 (5’NCR-Jn-ORF2-3’NCR) and pSK-HEV2 but lower level replication of chimeras T4 (5’NCR-Jn-ORF2) and T4 (Jn-ORF2) suggest minor role of the subgenomic promotor region in deciding host specific replication efficiency. However, significantly higher replication of chimeras T4 (Jn-ORF2-3’NCR) (5 fold higher) and T4 (ORF2-3’NCR) (3-folds) higher (table 13) suggested critical role of 3’NCR in regulating replication efficiency in the host specific manner. In addition, binding of host proteins was made more effective in the presence of respective ORF2 from the same genotype (probably via RNA elements such as SL1 and SL2) since replication of the chimera T4 (3’NCR) was though comparable to pSK-HEV2, it was significantly less than the above two clones bearing type 4 3’NCRs along with adjacent ORF2 regions. We cannot comment on the receptor specificity at this juncture since all infections were established by RNA transfections. Role of junction region cannot be completely neglected since chimera T4 (Jn-ORF2-3’NCR) replicated efficiently than the T4 (ORF2-3’NCR). Junction region has been shown to influence HEV replication (Cao et al 2010), but two more chimeras T4 (Jn) and T4 (Jn+3’CR) would perhaps shed more light on this matter.

These results indicated that host proteins can bind to 3’NCR alone and/or require compatible 5’ and 3’ NCR. Our results suggest compatibility of 5’-NCR of the genotype 1 and 3’NCR of the genotype 4 viral genomes. Reverse interaction is probably not very successful. Hence, chimera T4 (5’NCR) probably replicated with very low efficiency. Other chimeras, T4 (3’NCR+5’NCR), T4 (5’NCR+Jn+ORF2) and T4 (5’NCR+Jn+ORF2 +3’NCR) harbouring 5’NCR could replicate better perhaps due to presence of type 4 3’NCR, Jn and ORF2 regions. We could not include T4 (5’NCR+ORF1) in this analysis since it replicated with very low efficiency.

T4FG was positive for both nsRNA and IFA in PK-15 cells. However, for the chimera T4 (5’NCR-ORF1) though S10-3 cells were positive for both nsRNA and IFA, PK-15 cells always showed only nsRNA and no ORF2 protein. We could not get T4 ORF1 chimera in the limited period and assume that it would have replicated better than T4 (5’NCR-ORF1) in PK-15 cells due
to compatibility of 5'- and 3'-NCRs in the clones. We think ORF1 is the region responsible for deciding the species barrier. ORF1 encodes non-structural proteins of the virus. Viral non-structural proteins are known to interact with host cell proteins and modulate host cell environment (NS1 protein of influenza virus, NS5A protein of HCV). It is possible that some specific RNA elements or proteins from the ORF1 interact with host cell proteins and help replication. 5'NCR structurally is involved in early stages of replication and ORF1 proteins are directly or indirectly (in association with other cellular proteins) involved during the entire process of replication. Whether this phenomenon is due to ORF1 alone or in combination with 5'NCR can be solved with another chimera T4 (ORF1).

For generating virus particles from the clones, S10-3 cells were used. These viral particles were used to infect both HepG2/C3A and PK-15 cells. We took both the parental clones pSK-HEV2 and T4FG and three representative chimeras, T4 (Jn +ORF2 +3’NCR), T4 (ORF2- 3’NCR) and T4 (5’NCR+ORF1) for these experiments. HepG2/C3A infected with T4 (Jn +ORF2 +3’NCR) were positive in IFA. pSK-HEV2 and T4 (Jn +ORF2 +3’NCR) were positive for replicative intermediates. We think either we failed to generate viruses for T4FG and chimera T4 (5’NCR+ORF1) or they were too less in quantity for infection as both were negative for nsRNA and IFA. None of the chimeras showed signs of infection in PK-15 cells. For pSK-HEV2, it is known not to infect swine cells (Nguyen et al., 2013). It was surprising for T4 (Jn+ORF2 +3’NCR) to behave similarly as it had the capsid region of type 4. Also the chimera was not positive for replicative intermediates when capped genomes were transfected in PK-15 cells. Overall, we were not satisfied with these lateral infection experiments. It is known that cell culture generated virions have ORF3 protein coat in association to lipids (Emerson et al., 2010). It is also quite likely that ORF3 directly or indirectly is masking the epitopes recognised by cellular receptors if any. We speculate that the barrier is both at the receptor level and non-conducive cell milieu. It would be interesting to see how T4FG, and chimeras T4 (5’NCR+ORF1), T4 (ORF1) behave. We will have to wait to generate these viruses in quantity enough to produce a successful infection.