# General Introduction

Hepatitis E virus (HEV), the causative agent of hepatitis E, has been recognized as a major cause of enterically transmitted non-A, non-B hepatitis in many developing countries (Arankalle et al., 1994; Purcell, 1996; Mushahwar and Dawson 1997). The disease, which is characterized by acute hepatitis, tends to affect young adults with a reported mortality of up to 25% in pregnant women (Purcell and Ticehurst, 1988; Khuroo, 1991). Recently sporadic cases of acute hepatitis E have been reported in industrialized countries without the association of known risk factors. The first animal strain of HEV was isolated in pigs by Meng et al. (1997). This animal strain of HEV was later characterized and designated swine HEV (Meng et al., 1998). Swine HEV appears to be ubiquitous in pig herds in the United States. Numerous studies have demonstrated that swine HEV is genetically and antigenically related to human HEV, and that hepatitis E may be a zoonosis and pigs an animal reservoir. The discovery of swine HEV affords us the opportunity to study HEV infection in a swine model system. Results from previous unpublished studies of natural and experimental infection of pigs with swine HEV demonstrated that HEV may replicate extrahepatically. The underlying hypothesis of my thesis research is that HEV replicates in tissues and organs other than the liver, which is the target organ.

The pathogenesis and replication process of HEV is poorly understood. Because HEV is presumably transmitted by the fecal-oral route, it is unclear how the virus reaches the liver and an extrahepatic site(s) of replication could provide a possible explanation. Identification of extrahepatic sites of replication could significantly contribute to our understanding of the pathogenesis and replication of HEV, provide information to aid in the generation of an *in vitro* cell culture system, and could have important implications for xenotransplantation.

The objective of this research was to identify sites of extrahepatic HEV replication using a sensitive and strand-specific RT-PCR assay to detect the replicative, negative strand viral RNA. By utilizing this assay to test tissue samples collected from pigs experimentally infected with swine HEV and the US-2 stain of human HEV, we would be able to demonstrate the presence of replicating virus in specific tissues or organs. As part of this

objective, we aimed to generate synthetic negative-strand HEV RNA and use it as a positive control to develop and standardize the negative strand-specific RT-PCR assay.

# Literature Review

## Human hepatitis E virus

### History

Hepatitis E was first recognized as a separate clinical condition in 1980 when serological studies of water-born epidemics during the mid 1950s in India demonstrated the presence of another enterically-transmitted viral agent (Khuroo, 1980). Originally, these outbreaks were believed to be caused by the hepatitis A virus (HAV). This form of viral hepatitis proved to be distinct from hepatitis A and was first referred to as enterically-transmitted non-A, non-B hepatitis (ENANBH) (Arankalle et al., 1994; Khuroo et al., 1983).

In 1983 the first experimental evidence of this additional viral hepatitis was produced. Using fecal suspensions from patients with hepatitis A -like symptoms in Tashkent, Uzbekistan, fecal-oral transmission of the disease was demonstrated in a human volunteer (Balayan et al., 1983). Immune electron microscopy (IEM) of the feces collected from the volunteer between 28 and 45 days after exposure revealed virus-like particles of 27 to 30 nm in diameter (Balayan et al., 1983). The patient developed antibodies to these virus particles; however, serologic response to HAV or hepatitis B virus (HBV) was not present. Severe clinical hepatitis was also evident 36 days after ingestion of the fecal suspension. Similar results in cynomolgus monkeys were also reported (Balayan et al. 1983). This agent was subsequently identified as the cause of many water-born epidemics of hepatitis in developing countries (Khuroo et al., 1983; Arankalle et al., 1993).

A significant contribution to the identification of the viral agent was achieved when Reyes et al (1990) successfully cloned a portion of the viral genome. Subsequent experiments determined the complete sequence of the genome and the virus was named hepatitis E virus (HEV) (Purcell and Ticehurst, 1988).

## Biology of HEV

### *Morphology*

HEV has been described as a spherical particle between 27-34 nm in size. HEV has similarity with caliciviruses on its surface showing isohedral symmetry with cuplike depressions and no envelope. HEV can not be reliably distinguished from other "small round viruses" present in feces based on morphological characteristics alone (Purcell, 1996).

### *Genomic organization*

The genome of HEV is a single stranded, positive sense, polyadenylated RNA molecule of 7.5 kb in length (Tam et al., 1991). Short non-coding regions (NCR) are present at the 5' and 3' end and are 27 and 68 nucleotides respectively in length (Aggrawal and Krawczynski, 2000). Three overlapping open reading frames (ORF) are identified (Tam et al., 1991).

ORF 1 is believed to code for nonstructural proteins based upon extensive sequence analysis and comparison of conserved motifs in other known viral proteins. The ORF 1, consisting of 5,079 nucleotides, encodes a methyl transferase, a Y domain of unknown function, a papainlike cysteine protease, a proline rich hypervariable region, an X domain of unknown function, a helicase-like motif and an RNA-dependent RNA polymerase. The presence of the methyl transferase suggested that the 5' end of the genome may be capped which was later proved by Kabrane-Lazizi et al (1999) using a monoclonal antibody to 7-methylguanosine ($m^7G$). Although it's function is unknown, the sequence of the Y domain is similar to those in other viruses including the rubella virus (Purcell, 1996). The papainlike cysteine protease is a type of protease which is predominantly found in alphaviruses and rubella virus (Gorbalenya et al., 1991). The proline-rich region of hypervariability is thought to be important for the flexibility of the molecule acting in a hinge-like fashion (Reyes et al., 1991; Tsarev et al., 1992). Sequences matching the X domain of ORF 1 flank papainlike proteases in other positive-strand RNA viruses (Gorbalenya et al., 1991). The helicase-like motif is most closely related to the superfamily I (Gorbalenya et al., 1988, 1989), while the RNA-dependent RNA Polymerase motif is most closely related to supergroup III viral RNA ploymerases (Koonin et al., 1991).

The ORF 2 is 1,980 nucleotides in length and begins 38 nucleotides before the end of ORF 1 (Aggrawal and Krawczynski, 2000). ORF 2 encodes the viral capsid protein of HEV. A signal sequence is present at the 5' end which is immediately followed by a 300 nucleotide region rich in arganine (Purcell, 1996). The ORF 2 protein is apparently synthesized in the endoplasmic reticulum and then transported to the cell surface in it's mature glycosylated form (Jameel et al., 1996). ). Three potential glycoslylation sites are present in ORF 2, however, it is not known which of these sites are functional (Bradley et al., 1993). The major immunogenic epitope is present at the 3' end of ORF 2 (Tam et al., 1991).

ORF 3 is 369 nucleotides long and overlaps both ORF 1 and ORF 2 by 1 and 328 nucleotides, respectively. The ORF 3 protein has a cystein-rich region near it's amino terminus which has the ability to bind HEV RNA and form a complex with the ORF 2 protein (Zafrullah et al., 1997). It appears that the ORF 3 protein may serve as a cytoskeletal anchor where the major capsid protein and HEV RNA can bind to begin the process of viral nucleocapsid assembly (Zafrullah et al., 1997).

### *Replication*

Little is known about the replication process of HEV. HEV is not closely related to other well characterized viruses and successful propagation of the virus in cell culture has been limited. ORF 1 contains particular motifs present in other viruses; however, the functionality of these motifs is unknown. Like other positive-strand RNA viruses, HEV replicates through a negative-strand RNA intermediate and uses viral RNA polymerase to synthesize genomic and messenger RNA. Subgenomic RNA transcripts of 2.0 and 3.7 kb have been reported but the significance of these subgenomic RNAs is not known (Tam et al., 1991). The virus most likely uses common cellular mechanisms for translation and cleavage.

### *Propagation in cell culture*

Strains of HEV from both China and Russia have reportedly been replicated in cell cultures (Huang et al., 1990; Huang et al., 1992; Kazachkov et al. 1992). The Chinese strain produced cytopathic effect (CPE) and hemagglutination in embryonic lung cells (Huang et al., 1990). The Russian strain, which was isolated from primary kidney cells of experimentally infected cynomolgus monkeys, was propagated by cocultivation with FRhK cells. No CPE was evident in cultured cells; however, chronic infection was detected by

dot-blot hybridization as well as indirect immunofluorescence. Neither report has been independently confirmed. Replication of HEV in primary cynomolgus monkey hepatocytes has been reported (Tam et al., 1996). However, presently there is no adequate *in vitro* cell culture system for HEV.

### *Genetic heterogeneity*

In the past few years many novel strains of HEV have been genetically identified. Modern molecular biology techniques have assisted in the identification of novel strains from endemic areas such as China, Pakistan, and Nigeria. Noteworthy is the fact that these novel strains differ significantly in nucleotide sequence when compared to known strains. In China, six novel strains have been identified from patients who had previously tested negative for antibodies to HEV (Wang et al., 1999).

While identification of variant strains of HEV in endemic areas is not surprising, this development is not limited to endemic regions. Novel strains of HEV have also been identified in industrialized nations such as the United States, Italy, Taiwan and Greece (Shlaudler et al., 1999; Hsieh et al., 1998, 1999).

Most HEV strains identified thus far are only partially sequenced. Phylogenetic analyses performed on these novel strains are based on partial sequences and are therefore not conclusive. Isolates from Asia, Africa, Mexico and the United States have been fully sequenced and subsequent phylogenetic analyses from these fully sequenced isolates identifies three major genotypes: Burmese-like genotype consisting of Asian and African strains, the Mexican genotype, which consists of a single Mexican strain, and the U.S. genotype, consisting of the U.S. swine HEV and two strains of human HEV (US-1 and US-2). When using strains with partial sequence for phylogenetic analyses, Shlauder et al (1999, 2000) reported at least eight different genotypes of HEV. Together with the three established genotypes of HEV, 5 additional genotypes were identified. Genotype 4 consists of several variant Chinese strains, genotype 5 consists of an Italian isolate, genotype 6 and 7 consist of two Greek isolates and genotype 8 of two Argentine isolates.

Separate phylogenetic analysis of three different genomic regions of HEV by Haqshenas et al (2001) produced a different grouping consisting of 5 genotypes. European strains including the two Greek isolates and Italian isolate were determined to be most closely related to genotype 3 which includes human HEV strains in the U.S., the swine HEV strains from the U.S. and New Zealand. Genotype 1 consists of most Asian and the well known Burmese strain as well as African strains which were determined to be distinct

but still related. Genotype 2 consists of the single Mexican strain of HEV. Novel strains of HEV isolated from patients in China and Taiwan encompass Genotype 4. The fifth genotype includes a potential avian form of HEV and the chicken big liver and spleen disease virus (BLSV).

The deviation of nucleotide sequences between genotypes is approximately 20 to 30%. Most variation in the nucleotide sequences represent silent mutations, which do not affect the deduced amino acid sequence.

### *Antigenic factors and serologic heterogeneity*

HEV encodes antigens which are reactive in IEM, enzyme-linked immunosorbant assay (ELISA), Western blot, and immonofluorescence (Purcell, 1996). Until the first successful cloning of a portion of the HEV genome in 1990, the only assay avaailable for detection of ant-HEV antibodies was IEM, using covalescent sera from hepatitis E patients. Antigens expressed from cloned cDNA were first used for serological tests in 1991 (Ichikawa et al., 1991; Yarbough et al. 1991). The major immunogenic epitopes of HEV exist at the carboxyl ends of ORF 2 and ORF 3 (Ichikawa et al., 1991). The carboxyl end of the putative capsid protein (ORF 2) is fairly conserved among known HEV isolates. Studies have demonstrated that the antibody response to HEV infection in the liver is primarily directed against the capsid protein (Krawczynski, 1993). ORF 3 is less conserved among known HEV isolates and consequently diagnostic test performed using expressed proteins from ORF 3 appear to be more strain specific than those utilizing expressed protein from ORF 2 (Purcell, 1996). The conservation in the carboxyl end of ORF 2 partially explains the fact that all known strains of HEV appear to be of a single serotype.

### *Experimental animal infection and host range*

A variety of primate species have been used for studying HEV. The most useful species have been cynomolgus and rhesus monkeys. Both intravenous (IV) and oral routes of infection have been used, but the IV route of infection has proven far more reliable. Similar results have also been demonstrated in studies involving the hepatitis A virus (HAV). The course of infection, including viremia, enzyme levels, antibody response and virus shedding, in experimentally infected primates is similar to that of humans infected by HEV (Purcell, 1996).

Pigs and mice have also been reported to be susceptible to HEV infection. Pigs in particular may serve as a useful animal model for HEV study. A strain of human HEV recovered from a patient in the U.S. (US-2) has shown the ability to infect pigs (Meng et al., 1999; Halbur et al., 2001).

## Pathogenesis

The pathogenesis of HEV is poorly understood. The route of entry into a host is believed to be oral by drinking feces-contaminated water. Person to person contact seems to be a less likely source because of the high titer of virus that is believed to be necessary for infection. It had been assumed that sites of virus replication other than the liver existed, possibly in the intestinal tract. However, no sites of extrahepatic replication had previously been identified. How the virus reached the liver from it's primary site of replication was also unknown, although travel by way of the portal vein was suspected (Purcell, 1996). HEV replicates in the cytoplasm of hepatocytes and is then released into bile and blood.

Clinical disease appears predominantly in young adults from 15-40 years of age. The average incubation period is about 6 weeks. Specific IgM and IgG immune responses occur early in the disease progression (Purcell, 1996). IgM response quickly disappears, however, IgG response is more persistent and may be detected in a patient up to 14 years after infection (Bryan et al., 1994; Khuroo et al., 1993).

In human infection, virus particles were detectable in the feces about 34 days after infection (Chauhan et al., 1993). Virus shed in feces of infected individuals is believed to be the source of infection. It is believed that the major source of virus in the intestinal tract is from the liver; however, other sources such as virus replication in the GI tract can not be excluded.

The reported mortality of HEV infection varies but has been noted to be as high as 1% (Purcell et al., 1989). The severity of HEV infection in pregnant women is of particular concern. Mortality rates for pregnant women have been reported to be as high as 25% and the risk increases with each trimester (Purcell, 1988; Khuroo, 1991). The reason for this high mortality rate during pregnancy is still unknown.

Changes to the liver during HEV infection include focal necrosis with no localization to a particular zone of the lobule (Purcell, 1996). Inflammation, although modest, consisting of Kupffer cells and polymorphonuclear leukocytes is present (Purcell, 1996). It is theorized that liver damage may be immune mediated and not a result of viral replication based on the time discrepancy in the appearance of replicating virus and

histopathological and biochemical evidence of hepatitis. No extrahepatic or sequelea effects of HEV infection have been recognized.

HEV infection can not be differentiated by clinical presentation alone. Clinical signs are very generalized such as flu-like symptoms including headache, fatigue, nausea, vomiting, and diarrhea. Jaundice is evident in some cases. Not all HEV infections are clinically evident.

## Epidemiology

Epidemics of hepatitis E have been reported from Southeast and Central Asia, the Middle East, Northern and Western Africa, and Mexico (Fig. 1) (Purcell, 1996). While contaminated water is thought to be the most common mode of transmission, some food-born epidemics have been demonstrated (Meng et al., 1987; Bai et al., 1987). Person to person contact represents a relatively low risk for transmission. Travel to an endemic area is a well documented risk factor (DeCock et al., 1987; Roberts and Whitlock, 1992, Fletcher, 1993; Koshy et al., 1994). One hospital outbreak was also reported (Robson et al., 1992).

Serologic studies of the prevalence of anti-HEV antibodies show surprising results. In endemic regions, the seroprevalence of was much lower than expected (3% to 26%) (Purcell, 1996). In nonendemic regions, the prevalence of antibodies to HEV was higher than expected (1% to 3%) (Purcell, 1996). In the U.S., 1% to 2% of blood donors are positive for anti-HEV. The seroprevelance of HEV is age-specific. Young adults appear to be the age group at highest risk. Virtually identical age-specific seroprevelance patterns are present in the same populations over a span of 10 years and no difference in seroprevelance between men and women was detected (Purcell and Ticehurst, 1988). The prevalence of anti-HEV in areas where HEV is not considered endemic suggests the existence of subclinical infections and possibly an animal reservoir for the virus.

# Swine hepatitis E virus

## History

In 1990, Balayan et al demonstrated the first successful infection of domestic swine with a human HEV strain recovered from Central Asia. Clayson et al (1995) reported the detection of HEV antibodies from sera in 18 of 55 pigs and the detection of HEV RNA from sera and feces in 3 of 47 pigs in the Kathmandu Valley.

In 1997, Meng et al identified and characterized the first animal strain of HEV, swine HEV, from pigs in the U.S. Swine HEV was isolated in a prospective study on a commercial swine farm in Illinois (Meng et al., 1997). Twenty piglets, born to sows seropositive or seronegative for anti-HEV antibodies, were selected, tagged and mixed with other piglets in the same herd. The piglets were monitored for 5 months for HEV infection by using both ELISA to examine for seroconversion to anti-HEV and RT-PCR with degenerate HEV primers to test for fecal virus shedding. Seroconversion first occurred at 14 weeks of age and spread in a pattern consistent with that induced by an infectious agent. Sixteen of 20 piglets in the study had seroconverted by 21 weeks of age. A virus genetically related to human HEV, designated swine HEV was identified from serum and fecal samples of the piglets (Meng et al., 1997).

## Genomic organization

The complete sequence of swine HEV has been determined (Meng et al., 1997, 1998). The genomic organization of swine HEV is very similar to that of human HEV. The polyadenylated, single-stranded, positive sense, RNA genome is about 7.5 kb in length. Three open reading frames are present as well as a 5' and 3' non-coding region. ORF 1 encodes for nonstructural proteins including a methyl transferase, followed by a Y domain of unknown function, a papain-like cystein protease, a proline-rich domain, a hypervariable region, a helicase and an RNA-dependent RNA polymerase. ORF 2 encodes the putative capsid protein. ORF 3 encodes a protein of unknown function although it is likely similar in function to the ORF 3 protein of human HEV.

## Pathogenesis

Analogous to human HEV, little is known about the pathogenesis of swine HEV. Transmission is presumably by fecal-oral route and feces from infected pigs appear to be the main source of virus. Contaminated water and feed are believed to be the source of infection. However, experimental infection via the oral route has been unsuccessful. Kasorndorkbua et al (2001) showed that even a $10^{4.5}$ 50% pig infectious dose of swine HEV was not sufficient to cause infection by the oral route.

In pigs naturally infected with swine HEV, a subclinical infection was observed. Naturally infected piglets were necropsied at early stage infection and nineteen different tissues and organs were examined. No gross lesions were apparent although microscopically all four piglets had evidence of hepatitis characterized by mild to moderate focal hepatocellular necrosis. All piglets had lymphoplasmacytic enteritis and three had mild multifocal lymphoplasmacytic interstitial nephritis (Meng et al., 1997).

In a study performed by Halbur et al (2001), pigs experimentally infected with swine HEV or the US-2 strain of human HEV showed no clinical signs of infection (clinical symptoms, elevated liver enzyme levels or biliruben). The gross lesions observed were mild to moderately enlarged hepatic and mesenteric lymph nodes in pigs inoculated with swine or human HEV between 7 and 55 days post inoculation. Both lymphoplasmacytic hepatitis and hepatocellular necrosis were observed in groups of experimentally infected animals as well as the pigs in the uninoculated control group. However, lymphoplasmacytic lesions were significantly more severe in human HEV and swine HEV inoculated pigs than compared to control animals. A significant difference in hepatocellular necrosis was also observed in pigs that were inoculated with human HEV when compared to the control group (Halbur et al., 2001).

## Seroepedemiology

Seroepidemiological studies have demonstrated that swine HEV is ubiquitous in the Midwestern United States. Approximately 80% to 100% of pigs in commercial farms were infected (Meng et al., 1997). The infection appears to occur around 2 to 3 months of age. Similar results have been discovered in both endemic and nonendemic areas. In Australia, 92% to 95% of 4 month old commercial pigs tested positive for anti-HEV antibodies (Chandler et al., 1999).

## Experimental Infection of Non-human Primates With Swine HEV

Both rhesus monkeys and chimpanzees have been experimentally infected with swine HEV (Meng et al., 1998). Two rhesus monkeys inoculated IV with $10^{4.5}$ 50% pig infectious dose of swine HEV seroconverted to anti-HEV about 4 weeks post inoculation. Swine HEV excretion in the feces was demonstrated and viremia was detected. Changes consistent with mild hepatitis were observed in the liver near the time of slight liver enzyme elevations. Chimpanzees inoculated with swine HEV also became infected. Seroconversion occurred about 6 weeks post inoculation and swine HEV RNA was detected in the feces of the chimpanzee. Infection of non-human primates demonstrates the ability of swine HEV to cross species barriers and possibly infect humans.

## Novel Human HEV Strains Closely Related to Swine HEV

Recently, novel strains of human HEV closely related to swine HEV have been identified. In the United States, two strains of human HEV isolated from patients with acute hepatitis E (US-1 and US-2) are closely related to each other as well as the U.S. strain of swine HEV (about 98% sequence identity in ORF 1) (Schlauder et al., 1998). These two novel human HEV strains are distinct from other strains of HEV worldwide showing only about 80% sequence similarity in ORF 1. Similar cases have been reported in Taiwan where several novel strains of human HEV have been isolated and are also distinct from other known strains of human HEV (about 75% sequence similarity) (Hsieh et al., 1998; Wu et al., 2001). However, these Taiwanese human HEV strains are very closely related to a swine HEV strain recovered in Taiwan (about 97% sequence homology). Similar results have also been reported in Spain (Pina et al., 2000). These data indicates that swine HEV does indeed infect and cause hepatitis in humans.

## Experiments Performed in This Study

The work comprising this study is described in the article "Evidence of sites of Extrahepatic Replication of the Hepatitis E Virus in a Swine Model" submitted to the journal of clinical microbiology, which is shown in the subsequent text of this thesis.

# Evidence of Extrahepatic Sites of Replication of the Hepatitis E Virus in a Swine Model

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## Abstract

Hepatitis E virus (HEV) is the major cause of enterically transmitted non-A, non-B hepatitis in many developing countries, and is also endemic in many industrialized countries. Due to the lack of an effective cell culture system and a practical animal model, the mechanisms of HEV pathogenesis and replication are poorly understood. Our recent identification of swine HEV from pigs affords us an opportunity to systematically study HEV replication and pathogenesis in a swine model. In an early study, we experimentally infected specific-pathogen-free (SPF) pigs with two strains of HEV: swine HEV and the US-2 strain of human HEV. Eighteen pigs (group 1) were each inoculated intravenously with swine HEV, nineteen pigs (group 2) with the US-2 strain of human HEV, and seventeen pigs (group 3) as uninoculated controls. The clinical and pathological findings have been previously reported. In this expanded study, we aim to identify the potential extrahepatic sites of HEV replication using the swine model. Two pigs from each group were necropsied at 3, 7, 14, 20, 27, and 55 days post inoculation (DPI). Thirteen different types of tissues and organs were collected from each necropsied animal. Reverse transcriptase PCR (RT-PCR) was used to detect the presence of positive strand HEV RNA in each tissue collected during necropsy at different DPIs. A negative strand-specific RT-PCR was standardized and used to detect the replicative, negative-strand of HEV RNA from tissues that tested positive for the positive strand RNA. As expected, positive strand

HEV RNA was detected in almost every type of tissue at some time point during viremic period between 3 and 27 DPI. Positive-strand HEV RNA was still detectable in some tissues in the absence of serum HEV RNA from both swine and human HEV inoculated pigs. However, replicative, negative strand of HEV RNA was detected primarily in the small intestine, lymph nodes, colon, and liver. Our results indicate that HEV replicates in tissues other than the liver. The data from this study may have important implications for HEV pathogenesis, xenotransplantation, and the development of an in vitro cell culture system for HEV.

# Introduction

Hepatitis E virus (HEV), the causative agent of hepatitis E, has been recognized as a major cause of enterically transmitted non-A, non-B hepatitis in many developing countries (1, 30, 37, 39). Transmission of the virus occurs primarily by the fecal-oral route through contaminated drinking water in areas with poor sanitation. The disease affects mainly young adults with a reported mortality rate of up to 25% in pregnant women (10, 14, 37, 39). In the U.S., sporadic cases of acute hepatitis E without known risk factors have been documented and anti-HEV antibodies have been detected in a significant proportion of healthy individuals (21, 28, 32, 37, 39, 45). HEV is a positive, single-stranded, RNA virus without an envelope. The genome, which is about 7.5 kb in size, contains 3 open reading frames (ORFs) and a short 5' and 3' nontranslated region (NTR) (13, 18, 31, 38). ORF 1 is the largest of the three and encodes for non-structural proteins such as methyltransferase, helicase and RNA-dependent RNA polymerase. ORF 2 encodes the putative capsid protein and ORF 3, which overlaps with ORF 1 and ORF 2, encodes a cytoskeleton-associated, phosphoprotein (13, 37-39, 55). HEV was originally classified in the *Caliciviridae* family. However, recent studies have demonstrated the unique genomic organization of HEV, and therefore it has been declassified and designated in an unassigned genus "hepatitis E-like viruses" (3, 18, 35).

In 1997, a novel virus, closely related to human HEV, was discovered in swine (24). This virus, designated as swine HEV, was extensively characterized (9, 24-29). In the U.S., two strains of human HEV identified from patients with acute hepatitis E (US-1 and US-2) have shown a striking genetic similarity to swine HEV (6, 40). The two U.S. strains of human HEV share ≥97% amino acid identity with swine HEV in ORFs 1 and 2, but are genetically distinct from other known strains of HEV worldwide (26). It has been shown that the US-2 strain of human HEV infects pigs and the swine HEV infects non-human primates (9, 26). Similar findings were reported in Taiwan as well, where a novel strain of swine HEV was isolated from Taiwanese pigs. This Taiwanese strain of swine HEV shared 97.3% nucleotide sequence similarity with a strain of human HEV isolated from a retired Taiwanese farmer, but is genetically distinct from the U.S. strain of swine HEV and other HEV strains worldwide (12). Numerous genetically distinct strains of human HEV have also been identified in many other industrialized and developing countries (41, 42, 49, 50).

Due to the lack of a practical animal model and an in vitro cell culture system for HEV, the mechanisms of HEV pathogenesis and replication are poorly understood. With the discovery of swine HEV, we now have a homologous animal model system to study HEV infection. It has been suspected that HEV might replicate in tissues and organs other than the liver (2). Recent results from studies performed with rats infected with human HEV have suggested that the virus may replicate extrahepaticaly (20). The objectives of this study are to utilize swine as a model system to systematically study HEV replication, and to identify potential extrahepatic sites of HEV replication.

## Materials and Methods

**Viruses.** The swine HEV used in this study was recovered from a pig in Illinois (24). The swine HEV inoculum has an infectious titer of $10^{4.5}$ 50% pig infectious dose ($PID_{50}$) per ml of inoculum (26). The US-2 strain of human HEV (kindly provided by Dr. Isa Mushahwar of Abbott Laboratories, North Chicago) used in this study was first recovered from a hepatitis patient in Tennessee and then transmitted to cynomolgus monkeys (6, 40). The US-2 strain of human HEV has an infectious titer of $10^5$ 50% monkey infectious dose ($MID_{50}$) per ml of inoculum (Purcell et al., unpublished data).

**Experimental infection.** The experimental infection of pigs with swine and human HEV and the clinical and pathological findings from the study have been reported previously (9). Briefly, fifty-four, cross-bred, SPF pigs of 3-4 weeks old, were randomly assigned into 3 groups. Group 1, eighteen pigs were inoculated intravenously (I.V.) with $10^{4.5}$ $PID_{50}$ of swine HEV. Group 2, nineteen pigs were inoculated I.V. with $10^{4.5}$ $MID_{50}$ of the US-2 strain of human HEV. Group 3, seventeen pigs were used as uninoculated controls. Two pigs from each group were necropsied at 3, 7, 14, 20, 27, and 55 days post inoculation (DPI), respectively. Thirteen different types of tissues and organs were collected from each necropsied animal including liver, mesenteric, tracheobronchi and hepatic lymph nodes, small intestine, colon, stomach, pancreas, spleen, salivary gland, tonsil, heart, lung, kidney, and skeletal muscle. Samples of tissues and organs were frozen immediately at -80°C until used for analysis.

**Tissue homogenates.** Samples of each tissue and organ collected at necropsy were homogenized in 10% (W/V) sterile PBS buffer. Mesenteric, tracheobronchi, and hepatic lymph nodes were pooled and homogenized in 10% PBS buffer. The tissue homogenates were clarified by centrifugation at 3,000 rpm for 15 min at 4°C. The supernatants of the tissue homogenates were harvested and stored at -80°C until use.

**RNA extraction and reverse transcriptase PCR (RT-PCR).** Total RNA was extracted with TriZol Reagent (GIBCO-BRL) from 100 µl of 10% tissue homogenates or serum samples. The total RNA was resuspended in 11.5 µl of DNase, RNase and proteinase-free water (Eppendorf, Inc). Reverse transcription was performed at 42°C for 60 min. with 1µl of the R1 reverse primer (5'-CTACAGAGCGCCAGCCTTGATTGC-3'), 1µl of superscript II reverse transcriptase (GIBCO-BRL), 0.5 µl of 0.1 M dithioteritol, 4 µl of 5X RT buffer and 1µl of 10 mM dNTPs. Ten µl of the resulting cDNA was amplified in a 100 µl nested PCR reaction with ampli*Taq* gold DNA polymerase (GIBCO-BRL). The PCR parameters included an initial incubation at 95°C for 9 min to activate the ampli*Taq* gold DNA polymerase, followed by 39 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 1.5 min, with a final incubation at 72°C for 7 min. The first round of PCR produced an expected fragment of 404 bp using the forward primer F1 (5'- AGCTCCTGTACCTGATGTTGACTC-3') and the reverse primer R1. For the second round of PCR, the forward primer F2 (5'-GCTCACGTCATCTGTCGCTGCTGG-3') and the reverse primer R2 (5'-GGGCTGAACCAAAATCCTGACATC-3') were used to produce an expected product of 266 bp (Fig. 2). The PCR parameters for the nested PCR essentially the same as those in the first round PCR. The primers were designed in conserved regions of the ORF 2 to amplify both swine HEV and the US-2 strain of human HEV.

**Standardization of a negative strand-specific RT-PCR**

***Cloning of an ORF 2 fragment.*** To standardize a negative strand-specific RT-PCR to detect replicative, negative strand HEV RNA in infected tissues, a negative strand of HEV RNA transcript had to be generated for use as a positive control. Briefly, total RNA was extracted from 100 µl of feces collected from a SPF pig experimentally infected with swine HEV (25). The Qiagene 1 step RT-PCR kit was used to amplify an ORF 2 fragment of swine HEV according to the protocol supplied by the manufacturer. Total RNA was reverse-transcribed and PCR was performed with 10 µl of resulting cDNA in a 100 µl reaction. The PCR reaction was carried out for 31 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min, followed by a final incubation period at 72°C for 7 min. Primers F5526 (5'-GGGGGATCCAGCTCCTGTACCTGATGTTGACTC-3') and R5955 (5'-GGCCTCGAGCTACAGAGCGCCAGCCTTGATTGC-3') were used in the RT-PCR. The sense primer F5526 has an introduced BamHI restriction site and the antisense primer R5955 has an introduced XhoI restriction site to facilitate the subsequent cloning steps. The resulting PCR product (418 bp) was excised from an agarose gel and purified with the glass milk procedure using the GENE CLEAN II Kit (BIO 101, Inc). The purified PCR product was first ligated into a TA vector using T4 DNA ligase (Stratagene) at 12°C overnight. The recombinant plasmid was transformed into DH5α competent *E.coli* cells (GIBCO-BRL). Plasmids containing the insert were identified and confirmed by restriction enzyme digestions. The insert was subsequently subcloned into pBluescript II SK (+) plasmid (PSK II) (Stratagene) by directional cloning using BamHI and XhoI (Fig. 3). The recombinant PSK II plasmid containing the ORF 2 fragment was isolated, and confirmed by DNA sequencing.

***In vitro transcription of negative strand HEV RNA.*** Recombinant PSK II plasmid containing the ORF 2 fragment of swine HEV was linearlized by restriction enzyme digestion with BamHI. Synthetic negative stranded RNA was transcribed in vitro by activation of the T7 promoter of the PSK II plasmid using Ampliscribe T7 high yield transcription kit (Epicentre Technologies) according to the manufacturer's protocol (Fig. 4). The transcribed negative strand RNA was separated in a 1.5% agarose gel and the

expected RNA band was excised and purified using RNaid isolation kit (Bio 101 Inc.) to remove plasmid DNA. The gel-purified RNA was further treated with DNase for 60 min at 37°C. The RNA was then extracted with TriZol Reagent, and only the top half of the aqueous layer was collected to further eliminate potential plasmid DNA contamination. A nested PCR reaction with no reverse transcription step using Ampli*Taq* gold DNA polymerase and the external F1, R1 set of primers and the internal F2, R2 set of primers was performed to ensure that no plasmid DNA remained in the purified synthetic negative-strand RNA.

**Specificity and sensitivity of the negative strand-specific RT-PCR.** To standardize a negative strand-specific RT-PCR for HEV, the synthetic negative-strand HEV RNA was serially diluted from 100 ng to 10 ag. Negative strand-specific RT-PCR was performed on each dilution. Reverse transcription was performed with the forward primer F1, 1 µl of superscript II reverse transcriptase, 0.5 µl of 0.1 M dithioteritol, 4 µl of 5X RT buffer and 1µl of 10 mM dNTPs. A nested PCR reaction with ampli*Taq* gold DNA polymerase was subsequently performed with 5 µl of the cDNA in a 50 µl reaction. The first round PCR reaction used primers F1 and R1 with an expected product of 404 bp. The second round PCR reaction used 5 µl of the first round PCR product in a 50 µl reaction with internal primers F2 and R2 with an expected product of 266 bp. To demonstrate the specificity of the RT-PCR assays, a serum samples known to contain positive stranded HEV RNA was tested by both the positive and negative strand-specific RT-PCR assay.

## Results

**Tissue distribution of positive stranded HEV RNA.** All pigs from both HEV-inoculated groups seroconverted to antibodies for HEV (9). Positive strand HEV RNA was detected from 3 to 27 DPI in various tissues including liver, lymph nodes, colon, small intestine, stomach, spleen, kidney, tonsil, salivary gland, and lung from pigs inoculated with swine HEV (Table 1) (Fig. 5). Viral RNA was not detected from tissues collected at 55 DPI. In the absence of viremia, swine HEV RNA was still detectable in tissues from 20 to 27 DPI (Table 1). Swine HEV viremia disappeared after 14 DPI, and

positive strand viral RNA was not detectable from 20 to 55 DPI in the serum of swine HEV inoculated pigs.

In pigs inoculated with the US-2 strain of human HEV, viral RNA was detected in numerous tissues from 3 to 27 DPI (Table 1). The tissue distribution of positive strand viral RNA is similar to that in swine HEV inoculated pigs. Positive strand HEV RNA was not detected in the serum of pigs from 27 through 55 DPI. Again in the absence of viremia at 27 DPI, positive strand viral RNA was still detectable in a number of tissues (Table 1).

All control pigs in group 1 remained seronegative for anti-HEV antibodies throughout the study. In addition, negative results were observed in various tissue samples from animals which were experimentally infected, which served as an internal negative controls for the RT-PCR assays. Therefore, RT-PCR was not performed on the seronegative control animals.

**Standardization of the negative strand-specific RT-PCR.** The negative strand-specific RT-PCR for HEV was standardized with the synthetic negative strand HEV RNA transcript. By using the synthetic negative strand RNA as a positive control, we showed that the test can detect negative strand HEV RNA to the dilution level of 10 pg in the first round of the PCR reaction and 1 fg in the second round of the nested PCR reaction (Fig. 6). The dilution level of 1 fg correlates to approximately 4,500 viral genome copies. The negative strand RT-PCR is specific as it fails to detect positive strand viral RNA from serum samples that contain only positive strand HEV RNA (Fig. 7).

**Evidence for extrahepatic replication of HEV.** Detection of positive strand HEV RNA in various tissues of inoculated pigs does not mean that HEV replicates in these sites. Therefore, a negative strand-specific RT-PCR was performed to re-test all the tissues that tested positive for positive strand HEV RNA in the traditional RT-PCR. In swine HEV inoculated pigs, replicative, negative strand viral RNA was detected in the liver, lymph nodes, colon, small intestine and spleen between 7 and 27 DPI (Table 2) (Fig. 8). In pigs inoculated with the US-2 strain of human HEV, negative strand HEV RNA was detected in the liver, lymph nodes, colon, small intestine, stomach, spleen, kidney, tonsil and salivary gland between 3 and 27 DPI (Table 2).

## Discussion

The first animal strain of HEV, swine HEV, was genetically identified and characterized from a pig in the United States (24). Subsequently, several other strains of swine HEV were identified from pigs in Taiwan (12, 53). In Spain, strains of HEV from patients with acute hepatitis E were found to have a 92 to 94% nucleotide identity with a strain of HEV recovered from sewage of a slaughter house which was primarily of swine origin (34). A variant strain of HEV has also been recovered from tissue and fecal samples of wild-trapped rodents in Nepal. Phylogenetic analysis revealed that the HEV strain recovered from these rodents is most closely related to human strains of HEV from hepatitis patients in Nepal (48).

Serological studies also support the theory of an animal reservoir(s) for HEV. Anti-HEV antibodies have been identified in pigs from industrialized countries such as the U.S. (25), Canada (27), Korea (27), Taiwan (12, 52, 53), and Australia (4). Anti-HEV antibodies have also been detected in pigs from endemic regions, such as Nepal, China and Thailand (5, 27). In addition to pigs, 77% of rats from Maryland and 90% of rats in Hawaii are positive for anti-HEV antibodies (17). The prevalence of antibodies in rats occurs in both urban and rural areas and increases in proportion to estimated age of the animals (8, 17). Anti-HEV antibodies were also detected in wild-caught rhesus monkeys (47). In Vietnam, where HEV is endemic, anti-HEV antibodies have been detected in 44% of chickens, 36% of pigs, and 27% of dogs (46). Approximately 42 to 67% of sheep and goats have tested positive for anti-HEV antibodies in Turkmenistan where HEV is also endemic (7). In the U.S., anti-HEV antibodies have been detected in about 1to 2% of blood donors, with up to 20% detected in areas such as Baltimore, Maryland and Northern California (21, 22, 32, 45). Similar results have been reported in other industrialized countries such as the Netherlands (54), Italy (56), Greece (36), England (23, 30), Spain (15), Germany (19), Sweden (16), Finland (30) and Taiwan (11, 33, 52). Although the sensitivity and specificity of these various serological tests are unknown, these results suggest that HEV infection may be underestimated, especially in industrialized countries, and that hepatitis E may be a zoonosis.

The lack of a practical animal model has hindered HEV research. The identification of swine HEV in pigs affords us an opportunity to study HEV pathogenesis and replication in a swine model. The U.S. strain of swine HEV has shown the ability to infect non-human primates (26). In an earlier report, we showed that pigs could be experimentally infected with both swine HEV and the US-2 strain of human HEV (9). The clinical and

pathological findings of HEV infection in pigs were reported previously (9). In the current study, we attempted to use the samples of tissues and organs collected from this previous study to identify the potential extrahepatic sites of HEV replication.

It has been hypothesized that liver damage induced by HEV infection may be due to the immune response to the invading virus, and may not be a direct cause of viral replication in hepatocytes (37). Since HEV is presumably transmitted fecal-orally, it is unclear how the virus reaches the liver, and an extrahepatic site(s) of replication would be a possible explanation (2, 37). Primary hepatocytes are the only known sites of HEV replication (43, 44). In a preliminary study with naturally infected pigs, we found that positive strand HEV RNA was detectable in a number of tissues, even after viremia was cleared (Meng et al., unpublished data). Furthermore, in experimentally infected pigs, we also found that the relative genomic titer of HEV in the feces was at least 10 fold higher than that in bile collected from the same and prior day (Meng et al., unpublished data), suggesting HEV may replicate in the gastrointestinal tract.

In this study, we first tested by RT-PCR for the presence of positive strand HEV RNA from various tissues and organs of both swine HEV and human HEV infected pigs. We showed that positive strand HEV RNA was detectable in numerous tissues. The positive strand viral RNA detected in tissues from 3 to 14 DPI in swine HEV-inoculated pigs, and from 3 to 20 DPI in human HEV-inoculated pigs may not be attributable to the replicating virus, since viral RNA was also detected in the sera. However, after viremia disappeared, positive strand viral RNA was still detectable in various tissues between 20 to 27 DPI in swine HEV-infected pigs and at 27 DPI in human HEV infected pigs. Detection of positive strand HEV RNA from various tissues and organs in the absence of detectable serum viral RNA, indicated that the virus detected from these tissues represents replicating virus and is not due to contamination of the tissue samples by circulating virus in the blood.

HEV is a positive strand RNA virus, and therefore HEV replication produces an intermediate negative strand RNA. To further confirm that the HEV RNA detected in extrahepatic tissues are due to HEV replication, we re-tested by the negative strand-specific RT-PCR assay, all tissues that were positive for the positive strand viral RNA. The negative strand RT-PCR was standardized to detect only the replicative, negative strand HEV RNA in infected tissues. We showed that the negative strand-specific RT-PCR is sensitive and specific for the replicative, negative strand HEV RNA. Our results indicate that extrahepatic replication of HEV does occur. We were able to detect negative-strand HEV RNA in a variety of extrahepatic tissues. It appears that pigs inoculated with the US-

2 strain of HEV had positive results in more tissues than in pigs inoculated with swine HEV. No replicating virus was detected in the kidney, stomach, pancreas, lung, heart, muscle, salivary gland, or tonsil of pigs inoculated with swine HEV. Similarly, negative strand HEV RNA was also absent in pancreas, lung, heart and muscle tissues from human HEV-inoculated pigs. The detection of replicating virus primarily in small intestine and colon supports our earlier preliminary observations that the feces contains more virus than bile.

In human HEV-infected pigs, HEV replication was found in tonsil, small intestine and colon tissues as early as 3 DPI. In swine HEV-infected pigs, replicative viral RNA was first detected in small intestine, colon, lymph nodes and liver. Both swine HEV and human HEV replicate longer in liver, lymph nodes, small intestine, and colon than in other tissues. Other extrahepatic tissues such as kidney, tonsil, and salivary gland only had transit detectable replicative HEV RNA. It appears that lymph nodes and the intestinal tract are the main extrahepatic sites of replication. The I.V. route of inoculation used in this study prevents us from identifying the initial site of HEV infection, since the natural route of HEV infection is presumably fecal-oral. Our earlier studies showed that a much higher infectious titer of HEV is required to initiate an infection by the oral route of inoculation (Kasorndorkbua et al., unpublished data). The I.V. route of inoculation has been almost exclusively used in animal experiments with HEV and the hepatitis A virus.

The significance of identifying extrahepatic sites of HEV replication is unclear at this time. Since we demonstrated that tissues other than the liver support HEV replication, this could help identify an in vitro cell culture system to propagate HEV. Extrahepatic replication of HEV also raises additional concerns involving xenotransplantation with pig organs. Xenotransplantation is a potential solution for the shortage of human organs for transplantation. Pigs have been identified as favorable organ donors as they have similar anatomic and metabolic characteristics with humans (51). The possibility of transmission of swine HEV to immunosuppressed xenograft recipients has been a concern, especially in situations that would involve liver transplants. The findings from this study indicate that xenotransplantation with other organs may also pose potential risk for HEV xenozoonosis. In addition, damages to these extrahepatic tissues and organs resulting from HEV infection may render them useless for xenotransplantation.

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# General Conclusions

In conclusion, my colleagues and I were able to prove our hypothesis that HEV does replicate in extrahepatic tissues. We were able to demonstrate that our strand-specific RT-PCR assay was sensitive and specific for the replicative, negative strand of HEV RNA. We identified the intestinal tract and lymph nodes as the principal sites of extrahepatic HEV replication. The results obtained in this project will be further confirmed in the future by using *in-situ* hybridization with a probe synthesized from our cloned HEV fragment.

The identification of extrahepatic sites of HEV replication may have significant implications for the development of a cell culture system for HEV and it also raises concern about possible transmission of swine HEV to immunosuppressed xenotransplant patients. Our data also contributes significantly to the understanding of HEV pathogenesis and replication.

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**Table 1.** Detection of positive strand HEV RNA in pigs inoculated with swine HEV and the US-2 strain of human HEV

| | Days Post Inoculation | | | | | |
|---|---|---|---|---|---|---|
| **Tissues** | **3** | **7** | **14** | **20** | **27** | **55** |
| | | | **Swine HEV** | | | |
| Serum | NT | 2+[a] | 2+ | 0[b] | 0 | 0 |
| Liver | 1+[c] | 2+ | 2+ | 2+ | 1+ | 0 |
| Lymph Nodes[d] | 1+ | 2+ | 1+ | 2+ | 1+ | 0 |
| Colon | 0 | 2+ | 2+ | 1+ | 2+ | 0 |
| Small Intestine | 0 | 2+ | 2+ | 2+ | 0 | 0 |
| Stomach | 0 | 2+ | 2+ | 0 | 0 | 0 |
| Spleen | 0 | 2+ | 1+ | 1+ | 0 | 0 |
| Pancreas | 0 | 0 | 0 | 0 | 0 | 0 |
| Kidney | 0 | 0 | 0 | 0 | 1+ | 0 |
| Tonsil | 0 | 0 | 0 | 0 | 1+ | 0 |
| Salivary Gland | 0 | 0 | 0 | 0 | 1+ | 0 |
| Lung | 0 | 1+ | 1+ | 0 | 0 | 0 |
| Heart | 0 | 0 | 0 | 0 | 0 | 0 |
| Muscle | 0 | 0 | 0 | 0 | 0 | 0 |
| | | | **Human HEV** | | | |
| Serum | 0 | 2+ | 2+ | 1+ | 0 | 0 |
| Liver | 2+ | 2+ | 2+ | 2+ | 2+ | 0 |
| Lymph Node | 2+ | 2+ | 2+ | 2+ | 2+ | 0 |
| Colon | 2+ | 1+ | 1+ | 1+ | 1+ | 0 |
| Small Intestine | 2+ | 2+ | 0 | 0 | 0 | 0 |
| Stomach | 2+ | 2+ | 1+ | 1+ | 1+ | 0 |
| Spleen | 1+ | 2+ | 2+ | 1+ | 2+ | 0 |
| Pancreas | 1+ | 0 | 0 | 1+ | 0 | 0 |
| Kidney | 1+ | 1+ | 0 | 0 | 0 | 0 |
| Tonsil | 1+ | 0 | 1+ | 1+ | 1+ | 0 |
| Salivary Gland | 0 | 1+ | 1+ | 0 | 0 | 0 |
| Lung | 1+ | 0 | 1+ | 0 | 0 | 0 |
| Heart | 1+ | 2+ | 0 | 0 | 0 | 0 |
| Muscle | 1+ | 2+ | 1+ | 0 | 0 | 0 |

[a] 2+: Two out of two necropsied pigs at a given DPI, are positive for positive strand HEV RNA in the indicated tissue sample

[b] 0: Zero out of two necropsied pigs at a given DPI, are positive for positive strand HEV RNA in the indicated tissue sample

[c] 1+: One out of two necropsied pigs at a given DPI, are positive for positive strand HEV RNA in the indicated tissue sample

[d] Lymph nodes: Pool of mesenteric, tracheobronchi, and hepatic lymph nodes

**Table 2.** Detection of replicative, negative strand of HEV RNA in pigs inoculated with swine HEV and the US-2 strain of Human HEV

| | Days Post Inoculation | | | | | |
|---|---|---|---|---|---|---|
| **Tissues** | **3** | **7** | **14** | **20** | **27** | **55** |
| | | | **Swine HEV** | | | |
| Liver | **0** | **2+**[a] | **2+** | **2+** | **0**[b] | **0** |
| Lymph Nodes[c] | **0** | **2+** | **1+**[d] | **0** | **0** | **0** |
| Colon | **0** | **1+** | **2+** | **1+** | **1+** | **0** |
| Small Intestine | **0** | **1+** | **2+** | **0** | **0** | **0** |
| Stomach | **0** | **0** | **0** | **0** | **0** | **0** |
| Spleen | **0** | **0** | **0** | **1+** | **0** | **0** |
| Pancreas | **0** | **0** | **0** | **0** | **0** | **0** |
| Kidney | **0** | **0** | **0** | **0** | **0** | **0** |
| Tonsil | **0** | **0** | **0** | **0** | **0** | **0** |
| Salivary Gland | **0** | **0** | **0** | **0** | **0** | **0** |
| Lung | **0** | **0** | **0** | **0** | **0** | **0** |
| Heart | **0** | **0** | **0** | **0** | **0** | **0** |
| Muscle | **0** | **0** | **0** | **0** | **0** | **0** |
| | | | **Human HEV** | | | |
| Liver | **0** | **2+** | **2+** | **1+** | **1+** | **0** |
| Lymph Node | **0** | **2+** | **2+** | **1+** | **0** | **0** |
| Colon | **1+** | **1+** | **0** | **0** | **0** | **0** |
| Small Intestine | **1+** | **2+** | **0** | **0** | **0** | **0** |
| Stomach | **0** | **2+** | **1+** | **0** | **0** | **0** |
| Spleen | **0** | **2+** | **1+** | **1+** | **0** | **0** |
| Pancreas | **0** | **0** | **0** | **0** | **0** | **0** |
| Kidney | **0** | **1+** | **0** | **0** | **0** | **0** |
| Tonsil | **1+** | **0** | **1+** | **0** | **0** | **0** |
| Salivary Gland | **0** | **1+** | **0** | **0** | **0** | **0** |
| Lung | **0** | **0** | **0** | **0** | **0** | **0** |
| Heart | **0** | **0** | **0** | **0** | **0** | **0** |
| Muscle | **0** | **0** | **0** | **0** | **0** | **0** |

[a] 2+: Two out of two necropsied pigs at a given DPI, are positive for replicative, negative strand HEV RNA in the indicated tissue sample

[b] 0: Zero out of two necropsied pigs at a given DPI, are positive for replicative, negative strand HEV RNA in the indicated tissue sample

[c] Lymph nodes: Pool of mesenteric, tracheobronchi, and hepatic lymph nodes

[d] 1+: One out of two necropsied pigs at a given DPI, are positive for replicative, negative strand HEV RNA in the indicated tissue sample

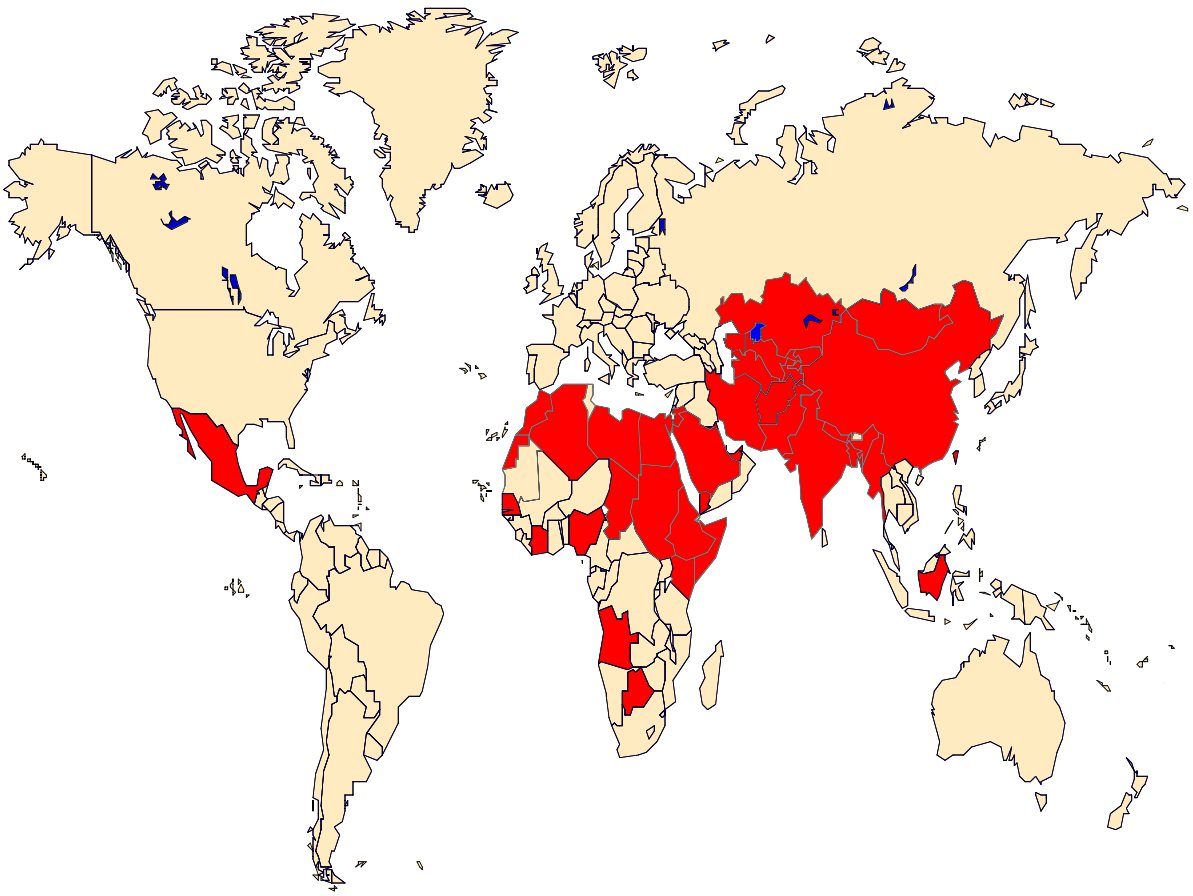

**Figure 1.** Countries in which there is serologic confirmation or strong epidemiological evidence of endemic and/or epidemic hepatitis E.

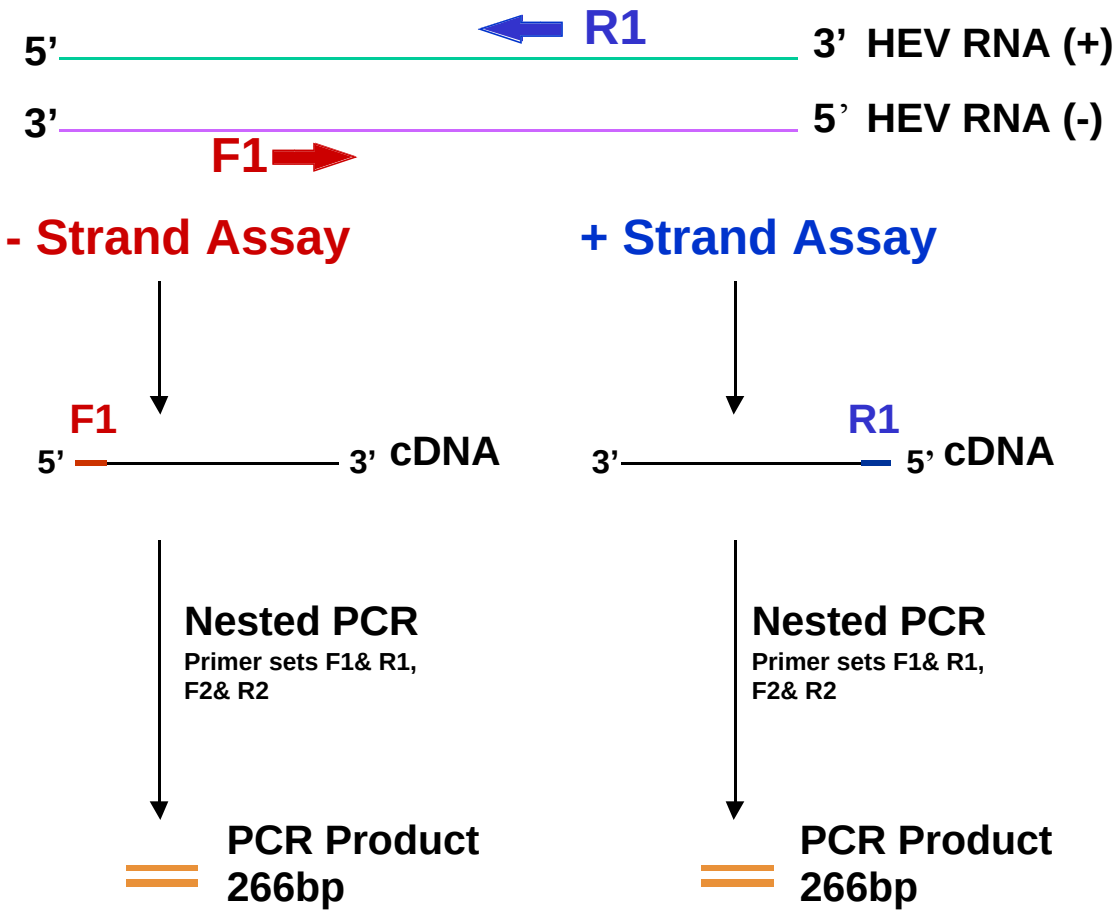


**Figure 2.** Diagram of RT-PCR assays used to detect the positive and negative strand RNA of HEV. Primers F1 and R1 bind to the negative and positive strand of HEV RNA respectively . Reverse transcription is performed to produce cDNA from each strand of HEV RNA. A nested PCR reaction using primers F1 and R1 as the outer primer set and primers F2 and R2 as the inner primer set is performed on the resulting cDNA. The size of the nested PCR product is 266 bp.

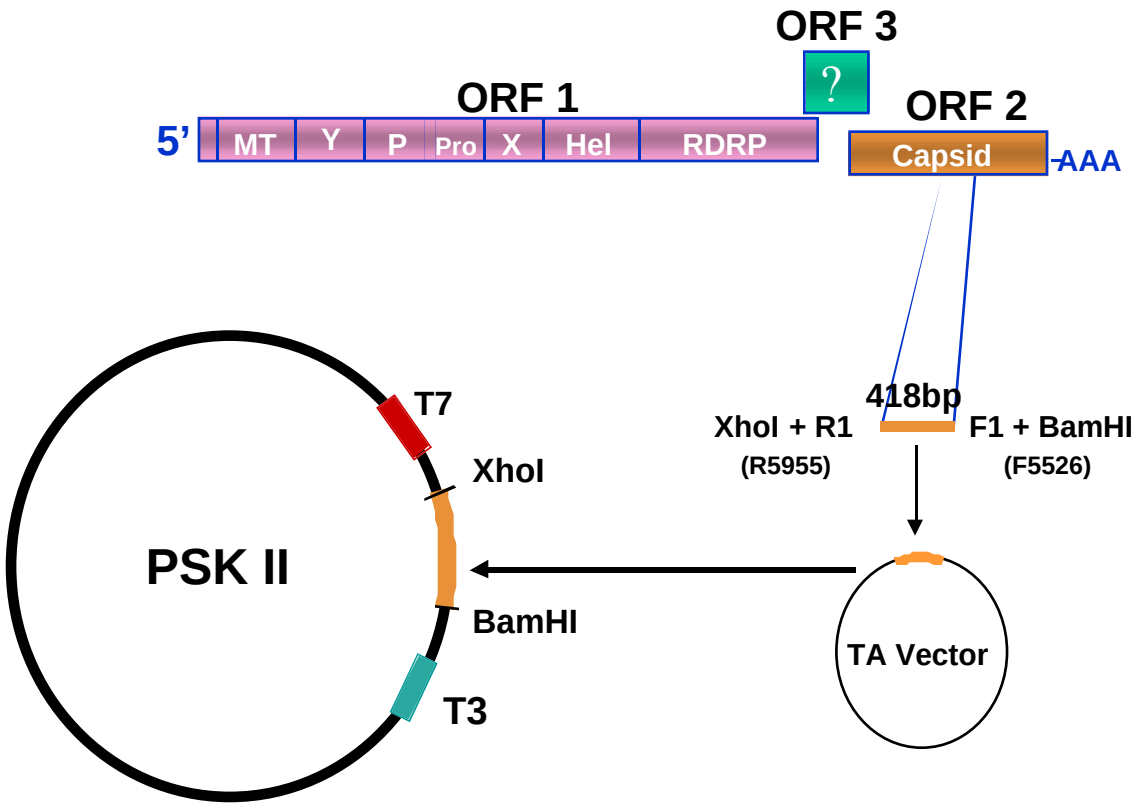


**Figure 3.** Diagram of the cloning of a 418 bp fragment of the ORF 2 of HEV into the plasmid pBluescript. A 418 bp of ORF 2 was first cloned into TA vetcor, then excised with restriction digestion by Bam HI and Xho I and subcloned into digested PSK II. The clone was used to generate synthetic HEV RNA.

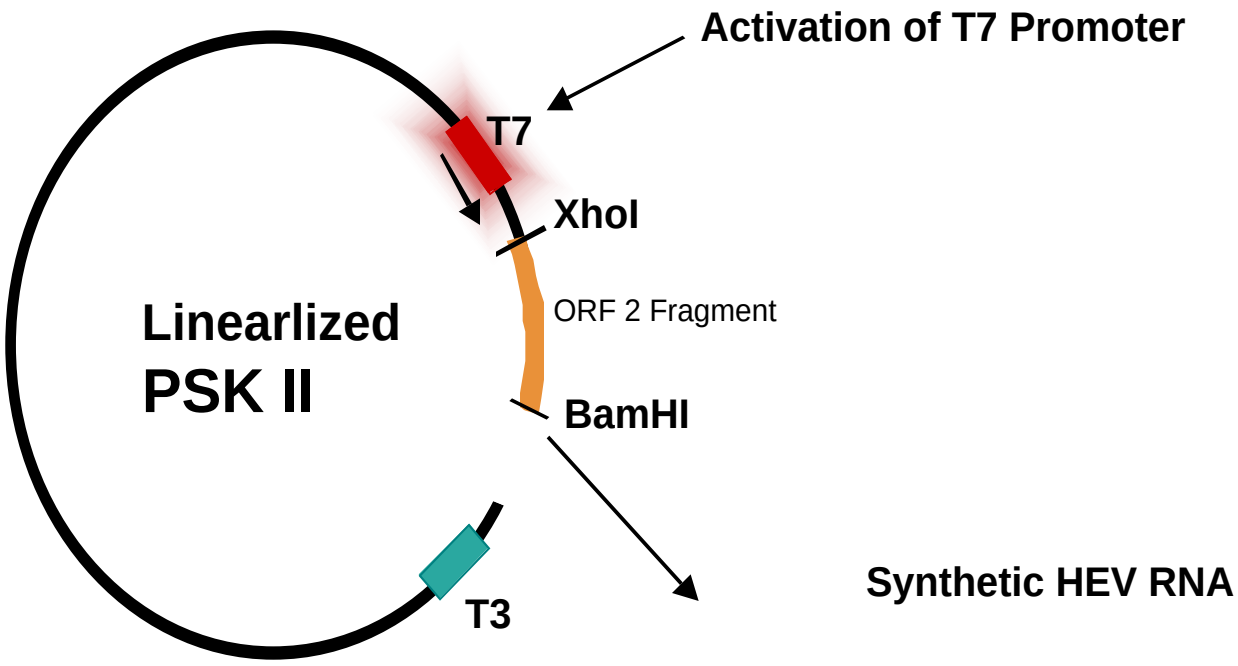


**Figure 4.** Diagram of the generation of synthetic negative strand HEV RNA. The T7 promoter of PSK II was activated with an in vitro transcription kit to produce negative strand HEV RNA. To ensure no contamination from plasmid DNA the RNA was gel purified, digested with DNase and isolated with Trizol reagent. This RNA was used to standardize the RT-PCR assay for detection of replicating virus.