Molecular and Serological Epidemiology of Swine Hepatitis E Virus from Pigs in Two Countries

by

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Molecular and Serological Epidemiology of Swine Hepatitis E Virus from Pigs in Two Countries

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Abstract

Hepatitis E virus (HEV), the causative agent of hepatitis E, is endemic in many developing countries. However, sporadic cases of acute hepatitis E have also been reported in industrialized countries including the United States. Increasing evidence suggested that hepatitis E is zoonotic. Swine HEV was discovered in 1997 from a pig in the United States and has the ability to cross species barrier and infect humans. There are four major genotypes of HEV worldwide and swine HEV identified to date in different countries belongs to either genotypes 3 or 4. Thus far, genotypes 1 (Asian strains) and 2 (a single Mexican strain) of HEV are exclusively found in humans. To determine if genotypes 1 and 2 of HEV also exist in pigs we tested serum and/or fecal samples for from pigs of different age groups in Thailand, and from pigs 2-4 months-of-age in two states (Sonora, Sinaloa) in Mexico. A universal RT-PCR was first standardized to detect all 4 different genotypes of HEV. Swine HEV RNA was detected from in 10/26 pigs at 2-4 months-of-age but not in pigs of 1-, 6-month old, adult/sow pigs from Thailand. In Mexico, swine HEV RNA was detected in 8 of 125 serum samples, 28 of 92 fecal samples of 2-4 month-old pigs. Antibodies to swine HEV were detected in 101 of 125 (80.8%) Mexican pigs. A total of 44 swine HEV isolates were amplified and sequenced for the ORF2 capsid gene region. Sequence analyses revealed that all the swine HEV isolates identified from pigs in Thailand and Mexico belong to genotype 3. Overall, the
Mexican swine HEV isolates shared 89-100% sequence identity to each other, and about 89-92% identity with the prototype genotype 3 US swine HEV. The Thailand swine HEV isolates displayed 97-100% nucleotide sequence identity with each other, and 90-91% identity with the prototype genotype 3 swine HEV. Phylogenic analysis revealed that minor branches do exist among Mexican swine HEV isolates. The results from this study indicated that genotype 1 or 2 swine HEV does not exist in pig from countries where human genotypes 1 and 2 HEVs are prevalent.
I would like to dedicate this thesis to my loved ones. I am truly thankful to all of the wonderful people that helped me through this experience. I would like to dedicate this to my loving parents, my dedicated friends (Sheila and Shannon), my faithful, patient, and affectionate Shane, and my loyal, unconditionally loving girls that were truly the bright stars in my life that I was so thankful to come home to.
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Abbreviations

bp: base pairs
DNA: deoxy-ribonucleic acid
ELISA: Enzyme-linked Immunosorbant Assay
HEV: hepatitis E virus
IgG: immunoglobulin G
kb: kilobase
kg: kilograms
NCR: noncoding regions
nm: nanometer
ORF: opening reading frame
RNA: ribonucleic acid
RT-PCR: reverse transcription polymerase chain reaction
SPF: specific pathogen free
US: the United States
General Introduction

Hepatitis E virus (HEV), the causative agent of hepatitis E, is endemic in many developing countries. However, sporadic cases of acute hepatitis E have also been reported in industrialized countries including the United States. Increasing evidence suggests that hepatitis E is a zoonotic infectious agent. A strain of HEV, designated swine HEV, was discovered in pigs from the United States in 1997. Swine HEV shares significant sequence identity to its human counterpart as well as viral morphology and protein function. HEV strains identified thus far worldwide form four major genotypes with swine HEV belonging to either genotype 3 or 4. So far, available data suggest that genotypes 1 (Asian strains) and 2 (a single Mexican strain) of HEV are exclusively found in humans. To determine if genotypes 1 and 2 also exist in pigs, we tested serum and/or fecal samples for evidence of swine HEV infection from pigs of different age groups in Thailand (a predominantly genotype 1 area), and from pigs of 2-4 months of age from different farms in two states (Sonora, Sinaloa) of Mexico (where the only strain of genotype 2 was reported). A universal RT-PCR test was standardized to detect all 4 different genotypes of HEV. A total of two hundred and ninety one fecal/serum samples were tested. Seventy-six were serum samples from Thailand and 215 samples were from Mexico (125 serum and 90 fecal). All Mexican serum samples were also tested for anti-HEV antibodies by ELISA. Swine HEV RNA was detected in 10 of 26 pigs of 2-4 month ages but not in 50 pigs of 1-, 6-month old, adult or sow pigs from Thailand. In Mexico, swine HEV RNA was detected in 8 of 125 serum samples and 28 of 92 fecal samples of 2-4 month-old pigs. Antibodies to swine HEV were detected in 101 of 125
(80.8%) Mexican pigs. A total of 44 swine HEV isolates were amplified and sequenced for the ORF2 capsid gene region. Sequence analyses revealed that all the swine HEV isolates identified from pigs in Thailand and Mexico belong to genotype 3. Overall, the Mexican swine HEV isolates shared 89-100% sequence identity with each other, and about 89-92% identity to the prototype genotype 3 US swine HEV. The Thailand swine HEV isolates displayed 97-100% nucleotide sequence identity with each other, and 90-91% identity with the prototype genotype 3 swine HEV. Phylogenic analysis revealed that minor branches do exist among Mexican swine HEV isolates. The results from this study indicated that genotype 1 or 2 swine HEV does not exist in pig from countries where human genotypes 1 and 2 HEV strains are prevalent and thus genotypes 1 and 2 HEV are unlikely to be zoonotic.
Literature Review

Hepatitis E:

In the mid 1950’s, a non-A, non-B enterically transmitted hepatitis was recognized in Delhi, India from a fecally contaminated, water borne epidemic. It wasn’t until the 1980’s that hepatitis E was discovered to be an infectious viral disease with clinical and morphological features of acute hepatitis (Krawczynski et al, 2000). This discovery was made from frozen serum samples collected during the 1955 epidemic in Delhi and sera collected during an epidemic in the Kashmir Valley in 1979 that proved negative to other known agents (Worm et al 2002, Viswanathan 1957, Wong et al 1980, Khuroo 1980). Since the first recorded incidence of this disease was not until 1955 and its frequency is low in industrialized countries, it is suggested that this is a new, emerging disease of developing countries. However, there are written records that suggest that there were outbreaks in the 18th and 19th centuries in Europe and the United States due to their similar features to hepatitis E (Worm et al 2002). It is theorized that hepatitis E is a much older disease than what was previously thought and that only until recently it became geographically secluded.

Clinical manifestations:

The symptoms and laboratory findings of the clinically overt form of hepatitis E with an acute, icteric and self-limiting course are similar to those of other types of acute viral hepatitis (Worm et al 2002). Symptoms of the disease include jaundice, anorexia, hepatomegaly, abdominal tenderness and pain, nausea, vomiting, and fever. Hepatitis E is slightly more severe than hepatitis A (Emerson and Purcell 2003). The overall mortality
rate is low (<1%), but it can be as high as 20%-25% in pregnant women infected in the third trimester of the pregnancy (Meng, 2003; Vishwanathan, 1957; Khuroo, 1980; Purcell, 1996; Jaiswal et al, 2001). There are no chronic sequelae for this disease (Worm et al, 2002; Khuroo et al, 1980).

**Hepatitis E Virus:**

Hepatitis E virus, the causative agent of hepatitis E, is a spherical, nonenveloped virus approximately 32-34 nm in diameter with cup-shaped depressions on the virion surface (Purcell, 1996; Meng, 2003; Ticehurst et al, 1992; Aggarwal and Krawczynski, 2000). In the early 1980s, the first virus particles were visualized by immunoelectron microscopy in feces of patients with acute non-A, non-B hepatitis (Worm et al 2002). HEV was once considered a calicivirus due to its superficial similarity in genomic organization and particle structure. The virus was removed as a member of the genus *Caliciviridae* in 2001 due to its difference in viral gene expression, codon usage, and lack of sequence identity (Purcell and Emerson 2001). HEV was recently reclassified as the sole member of the genus *Hepevirus* in a new family designated as *Hepeviridae* (Emerson and Purcell, 2003).

**HEV Genome:**

The genome of HEV is approximately 7.2 kb, and contains a single-stranded, positive sense RNA molecule (Meng, 2003). There are three open reading frames (ORFs) and a short noncoding region each at the 5’ and 3’ ends (Meng, 2003; Worm et al., 2002). ORF1 is located towards the 5’ end and encodes a 1693 amino acid product encompassing nonstructural proteins involved in viral replication and protein processing.
(Harrison, 1999). ORF2, which lies at the 3’ end of the genome, encodes the viral capsid protein, 660 amino acids in length (Harrison, 1999). ORF3 partially overlaps both ORF1 and ORF2 and contains a cysteine rich region near its amino terminus. The ORF 3 encodes for a protein 123 amino acids in length and has been shown to bind HEV-RNA and enter into a complex with the capsid protein (Aggarwal and Krawczynski, 2000; Zafrullah et al, 1997). This protein is possibly involved in assembly of viral particles (Worm et al, 2002).

**Pathogenesis:**

HEV replicates in the cytoplasm of hepatocytes and is shed in the feces (Emerson and Purcell, 2003). Extrahepatic sites of HEV replication have been reported including the small intestine and colon (Williams et al, 2001). It is not known how ingested virus reaches the liver (Emerson and Purcell, 2003). Histological changes in the liver include focal necrosis and modest inflammation (Emerson and Purcell 2001, 2003). HEV is not thought to be cytopathogenic and it is possible that disease is due to immunological reactions but this has not been proven (Emerson and Purcell 2003).

**Transmission:**

Hepatitis E, the disease, is primarily transmitted fecal-orally. The virus is spread mainly by consumption of contaminated water due to poor sanitary conditions. Outbreaks generally follow floods when water sources get contaminated by sewage or after long dry spells where the viral load increases in limited water supplies. Person-to-person transmission does not seem to be a factor in this disease (Khuroo, 1980).
However, nosocomial spread has been documented between a pregnant patient and multiple hospital staff members in South Africa (Robson et al 1992).

**Epidemiology:**

Hepatitis E is an important public health disease in many developing countries. The anti-HEV antibody prevalence in healthy human populations averaging around 25% in areas where hepatitis E is known to be endemic but it has reached as high as 60% in Egypt (Worm et al, 2002; Arankalle et al, 1995; Meng, 2003). More recently, it has been proven to be endemic in many industrialized countries where it is responsible for the occurrence of sporadic, acute cases of hepatitis E (Worm et al, 2002). In the United States, Thomas et al (1997) reported that 21% of blood donors, 16% of homosexual men, and 23% of drug users from Baltimore, Maryland, have anti-HEV antibodies (Meng et al, 2000). Many other industrialized countries (but not limited to those listed) have found similar results including the Netherlands (Zaaijer et al, 1995), Italy (Zanetti et al, 1994), Sweden (Johansson et al, 1995), Germany (Langer et al, 1996), Finland (Mushahwar et al, 1997), Spain (Mushahwar et al, 1997), England (Mushahwar et al, 1997), and Taiwan (Hsieh et al, 1998). A low incidence of hepatitis E in children with correlating anti-HEV IgG levels of 0-9% has been reported in areas where HEV is endemic which is dissimilar to other hepatitis viruses (Worm et al 2002, Skidmore 1999, Arankalle et al 1995). Therefore, the age group with the highest prevalence is young adults ranging 15-40 years.

**Zoonosis and Animal Models:**

Seroepidemiological studies have shown that the geographic distribution of HEV is worldwide (See Fig. 3) and raised concerns that HEV has zoonotic potential. In
Taiwan where HEV is not thought to be endemic, Hsieh et al. (1999) found that about 37% of Taiwanese pig handlers were positive for anti-HEV antibodies compared to 8% in control subjects (Meng 2004, Hsieh et al. 1999). In addition, swine veterinarians and normal blood donors in the United States were tested for IgG anti-HEV. It was found that individuals that handled pigs were 1.46-1.51 times more likely to have antibodies than normal blood donors. In Moldova, 51% of swine handlers were seropositive compared to 25% of control subjects (Drobeniuc et al. 2001). There has also been a connection to occupational history. Those individuals that work in barns, with births, needle pricks, and a history of drinking raw milk also are at greater risk (Meng, 2003).

HEV has been demonstrated to cross species. HEV recovered from individuals infected during epidemics has also been shown to infect non-human primates (cynomolgus, rhesus, and macaque monkeys) (Harrison 1999). Balayan et al. had their first breakthrough when he experimentally transmitted the disease to himself and to cynomolgus monkeys (Purcell and Emerson 2001, Balayan et al. 1983). Later attempts to infect cynomolgus monkeys, rhesus macaques, tamarins, and chimpanzees resulted in mixed success but eventually, a number of different species of nonhuman primates were infected successfully (Purcell and Emerson 2001).

Later on in the 1990s, the group of animals capable of being infected or positive for anti-HEV antibodies was extended to swine, cattle, sheep & goats, lambs, rodents, and birds (See Fig. 4). In the United States, more than 80% of swine older than three months of age has anti-HEV antibodies (Meng et al., 1997). Three species of wild rats have been detected to contain anti-HEV antibodies with the percentages ranging from 44-
90% in the USA (Kabrane-Lazizi et al, 1999). In Vietnam, anti-HEV has been detected in 44% of chickens, 36% of pigs, 27% of dogs, and 9% of rats. In Turkmenistan, about 42 to 67% of the sheep and goats have been found to be positive for anti-HEV antibodies. Twenty-nine to 62% of the cattle from Somali, Tajikistan, and Turkmenistan and 12% of the cows from Ukraine have also been found to have anti-HEV antibodies.

**Swine Hepatitis E Virus:**

The first evidence of HEV infection in swine came from a study by Balayan et al. (1990) who reported an experimental infection of domestic swine with an Asian strain of human HEV. The first animal strain of HEV, swine HEV, was isolated and characterized from a pig in the United States (Meng et al, 1997). Subsequently, isolates of HEV have been identified in swine from many endemic and nonendemic countries (Hsieh et al., 1999; Wu et al., 2000, 2002, Garkavenko et al., 2001, Yoo et al., 2001, Okamoto et al., 2001 and Van der Poel et al., 2001).

There are at least four major genotypes of HEV: type 1 (Asian and African strains including Burma, India, and Pakistan), type 2 (a single Mexican strain), type 3 (strains from sporadic cases in industrialized countries, and swine HEV), and type 4 (China, Japan, and Taiwan human and swine HEV strains). Swine HEV isolates identified thus far belong to either genotype 3 or genotype 4. Cross-species infection with genotype 3 swine HEV has been experimentally demonstrated; Prototype genotype 3 swine HEV has been used to infect non-human primates. Genotype 3 human HEV (US2 strain) has been used to infect pigs (Meng et al 2004). More recently, genotypes 3 and 4 human HEV strains responsible for sporadic cases of hepatitis E were found to be
genetically closely related to genotypes 3 and 4 swine HEV in the same geographic regions, suggesting that swine are reservoirs for genotypes 3 and 4 HEV. However, it is not known if genotypes 1 or 2 can also infect across species or if they occur in pigs at all. The objectives of this study were to determine the prevalence and genotype of swine HEV from pigs in Mexico (where genotype 2 human HEV is prevalent), and in Thailand (where genotype 1 human HEV is endemic).
Identification of genotype 3 hepatitis E virus (HEV) isolates from pigs in Thailand and Mexico where genotype 1 or 2 HEV strains are epidemics in humans

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Running title: Swine HEV in Mexico and Thailand

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Abstract: Hepatitis E virus (HEV), the causative agent of hepatitis E, is endemic in many developing countries. However, sporadic cases of acute hepatitis E have also been reported in industrialized countries including the United States. Increasing evidence suggest that hepatitis E is a zoonosis. Swine HEV was discovered in 1997 from a pig in the United States, and has the ability to cross species barrier and infect humans. There are four major genotypes of HEV worldwide, and swine HEV strains identified to date in different countries belongs to either genotypes 3 or 4. Thus far, genotypes 1 (Asian strains) and 2 (a single Mexican strain) HEVs are exclusively found in humans. To determine if genotypes 1 and 2 HEV also exist in pigs, a universal RT-PCR was standardized to detect all 4 different genotypes of HEV and used in this study. Serum and/or fecal samples from pigs in Thailand where genotype 1 human HEV is prevalent and from pigs in Mexico where genotype 2 human HEV epidemics were reported were tested by RT-PCR for swine HEV infection. Swine HEV RNA was detected in 10 of 26 serum samples from pigs of 2-4 months of age but in 0 of 50 pigs of 1-month-old, 6-month-old, adult or sows from Thailand. In Mexico, swine HEV RNA was detected in 8 of 125 sera and 28 of 92 fecal samples of 2-4-month-old pigs. Antibodies to swine HEV were detected in 101 of 125 (80.8%) Mexican pigs. A total of 44 swine HEV isolates were amplified and sequenced for the ORF2 capsid gene region. Sequence analyses revealed that all the swine HEV isolates identified from pigs in Thailand and Mexico belong to genotype 3. Overall, the Mexican swine HEV isolates shared 89-100% sequence identity to each other, and about 89-92% identity to the prototype genotype 3 US swine HEV. The Thailand swine HEV isolates displayed 97-100% nucleotide
sequence identity to each other, and 90-91% identity to the prototype genotype 3 swine HEV. Phylogenic analyses revealed that minor branches associated with geographic origin exist among Mexican swine HEV isolates. The results from this study indicated that genotype 1 or 2 swine HEV does not exist in pig from countries where human HEV genotypes 1 and 2 are prevalent, and thus it is likely that only genotypes 3 and 4 HEV strains have zoonotic transmission potential.
Introduction

Hepatitis E is an important public health disease in many developing countries on almost all continents including Asia, Africa and Mexico. Sporadic cases of acute hepatitis E have also been reported in many industrialized countries including the United States. The disease is primarily transmitted by fecal-oral route through contaminated water. The overall mortality rate is generally low (<1%), but it can be as high as 20-25% in pregnant women (Meng, 2003; Vishwanathan, 1957; Khuroo, 1980; Purcell, 1996; Jaiswal et al., 2001).

Hepatitis E virus (HEV), the causative agent of hepatitis E, is a spherical, nonenveloped virus approximately 32-34 nm in diameter with cup-shaped depressions on the surface (Purcell, 1996; Meng, 2003; Ticehurst et al., 1992; Aggarwal and Krawczynski, 2000). HEV is currently classified as the sole member of the genus hepevirus in the family of Hepeviridae (Emerson and Purcell, 2003). The genome of HEV is approximately 7.2kb, and contains a single-stranded, positive sense RNA molecule (Meng, 2003). There are three open reading frames and a short noncoding region each at the 5’ and 3’ ends (Meng, 2003; Worm et al., 2002). ORF1 is located towards the 5’ end and encodes nonstructural proteins (Harrison, 1999). ORF2, which lies at the 3’ end of the genome, encodes the viral capsid protein (Harrison, 1999). ORF3 partially overlaps both ORF1 and ORF2. The ORF3 protein has a cysteine rich region near its amino terminus and has been shown to bind viral RNA and enter into a complex with the capsid protein (Aggarwal and Krawczynski, 2000; Zafrullah et al. 1997).
The first evidence of HEV infection in swine came from a study by Balayan et al. (1990) who reported an experimental infection of domestic swine with an Asian strain of human HEV. The first animal strain of HEV, designated swine HEV, was isolated and characterized from a pig in the United States (Meng et al, 1997). Subsequently, many swine HEV isolates have been identified in swine from over a dozen endemic and nonendemic countries (Hsieh et al., 1999; Wu et al., 2000 & 2002; Garkavenko et al., 2001; Yoo et al., 2001; Okamoto et al., 2001; Van der Poel et al., 2001).

There exist at least four major genotypes of HEV: type 1 (mostly Asian strains including Burma, China, India, and Pakistan), type 2 (a single Mexican strain), type 3 (strains from sporadic cases in industrialized countries), and type 4 (strains from sporadic cases in Japan, China and Taiwan). Swine HEV isolates identified thus far belong to either genotype 3 or 4. Cross-species infection of genotype 3 HEV has been experimentally demonstrated: genotype 3 swine HEV (prototype strain) infection in non-human primates and genotype 3 of human HEV (US2 strain) infection in pigs (Meng et al, 1998). Genotype 3 and 4 human HEV strains responsible for sporadic cases of acute hepatitis E were found to be genetically closely related to genotype 3 and 4 swine HEV strains in the same geographic regions, suggesting that these HEV strains can infect across species and that swine are potential reservoirs. It is still unclear if genotypes 1 or 2 HEV can also infect across species or if they exist in pigs at all. The objectives of this study were to determine the prevalence and genotype of swine HEV from pigs in Mexico where the only strain of genotype 2 human HEV has been isolated, and in Thailand where genotype 1 human HEV is endemic.
Methods and Materials

Sample collection. Seventy-six serum samples were collected from pigs of various ages in Aujeszky’s disease-free farms from Nakorn-Pathom, Thailand (Meng et al, 1999). A total of 215 samples (125 serum and 90 corresponding fecal) were taken from pigs 2-4 months-of-age from Sonora, Sinaloa, and Puebla states of Mexico.

ELISA. A truncated recombinant human HEV (Sar-55 strain) protein was used to coat the 96 well plates (Linbro/Titertek, Bucks, UK) for an ELISA to detect IgG anti-HEV in pigs as previously described (Haqshenas et al 2002). HRP-conjugated goat anti-swine IgG (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was used as the secondary antibody. Each serum sample was diluted 1:100 in blocking buffer (0.5% gelatin, 0.03 M NaCl, 10% fetal bovine serum), and tested in duplicate. Hyperimmune anti-HEV swine serum (Meng et al, 1997) was included as a positive control and serum from a SPF pig was used for a negative control.

Primer design. Primers capable of detecting HEV strains with significant sequence variations were designed based on a multiple sequence alignment of the ORF2 genes of 18 different known strains of human HEV and the prototype U.S. strain of swine HEV (Huang et al, 2002). Two sets of degenerate HEV primers from the ORF2 gene region were designed for a universal nested RT-PCR assay: external primer set 3156N [forward, 5'-AATTATGCC(T)CAGTAC(T)CGG(A)GTTG-3'] and 3157N [reverse, 5'-
CCCTTA(G)TCC(T)TGCTGA(C)GCATTCTC-3'], and internal primer set 3158N [forward, 5'-GTT(A)ATGCTT(C)TGCATA(T)CATGGCT-3'] and 3159N [reverse, 5'-AGCCGACGAAATCAATTCTGTC-3']. The expected product of the universal nested RT-PCR was 348 bp.

**Reverse Transcription Polymerase Chain Reaction (RT-PCR).** A universal RT-PCR assay that is capable of detecting genetically divergent strains of HEV has been previously developed (Huang et al, 2002). To evaluate if the universal RT-PCR assay with degenerate HEV primers could detect HEV strains from the 4 known HEV genotypes, total RNAs were extracted from the Pakistani Sar-55 HEV (genotype 1), the Mexican HEV strain (genotype 2), the US-2 human HEV strain (genotype 3), and the Taiwan TW6196E human HEV strain (genotype 4). The parameters for the RT-PCR assay were performed as previously described (Huang et al, 2002).

**DNA sequencing and sequence analyses.** Amplified PCR products were separated in a 0.8% agarose gel. The expected band was excised from the gel and purified by the glassmilk procedure with a Geneclean III kit (Bio101, Inc. Carlsbad, CA). The purified products were sequenced directly with PCR primers for both strands at the Virginia Bioinformatics Institute, Virginia Polytechnic Institute and State University, Blacksburg, Virginia. Sequence analyses were conducted using the MacVector computer program (Oxford Molecular Inc. San Diego, CA). The swine HEV sequences for this study were compared genetically and phylogenetically to other selected known human and swine
HEV sequences in the GenBank database including 11 human HEV strains: Burma (M73218), Egypt (AF051351), Madras (X99441, India), Morocco (AF065061, Africa), Mexico (M74506), Sar55 (M80581, Pakistan), T1 (AJ272108, China), Tw261E (AF296160, Taiwan), JA11 (AB082567, Taiwan), JA1 (AB097812, Taiwan), and US2 (AF060669); and 8 swine HEV strains: Spain (AF195063), Netherlands (AF332629), Korea (AF516179), Japan (AB073910), Canada (AY115488), US prototype (AF082843), Taiwan (Tw32sw AF117280), and Taiwan (J13 AB097811).

**Phylogenetic analysis.** A phylogenetic tree was produced using the PAUP program (David L. Swofford, Smithsonian Institute, Washington, D.C.; distributed by Sinauer Associates Inc., Sunderland, Massachusetts.). The bootstrap method with 1000 replicants was used to generate a consensus tree. Avian HEV (AY043166) was used as the outgroup.

**Results**

**Standardization of a universal RT-PCR that can detect 4 known genotypes of HEV.** To evaluate if the universal RT-PCR assay with degenerate primers is capable of detecting genetically divergent strains of HEV, we first tested the assay using 4 different genotypes of HEV: Pakistani Sar-55 human HEV strain (genotype 1), Mexican human HEV strain (genotype 2), US-2 human HEV strain (genotype 3), and Taiwan TW6196E human HEV strain (genotype 4). Although these 4 divergent HEV strains differed in their ORF2 gene nucleotide sequences by more than 20%, total RNAs extracted from the 4 HEV strains all tested positive by the universal RT-PCR assay (Fig. 1). The amplified
products were sequenced and confirmed to originate from the respective reference HEV strains. Therefore, the universal assay is capable of detecting HEV strains from any of the 4 known genotypes.

Prevalence of swine HEV from pigs in Mexico and Thailand. Ten of the 76 serum samples from pigs of various ages originating from Thailand were positive for swine HEV RNA. All positive samples were from pigs of 2-4 months of age (Table 1). One-month old, 6-month old, and adult pigs were negative.

A total of 125 2-4 month old pigs from Mexico were tested for the presence of swine HEV RNA. Of these, 90 pigs from the states of Sonora and Sinaloa had paired serum and fecal samples, and the remaining 35 pigs from Puebla had only serum samples. A total of 8 (6%) serum samples were found positive for swine HEV RNA (Table 2). Seven of the swine samples collected from four small farms in the northern part of Sonora (Sonora 1-4) were positive for swine HEV RNA. None of the 20 sera from Granja Sem#9 (1-10), LA (1-10), or central Sonoroa (Municipio de Cajeme) were positive for swine HEV RNA. One of the 30 sera collected from pigs in Culiacon, Sinaloa (Granja 1-3) was positive. The 35 serum samples collected from pigs in Puebla were all negative for swine HEV RNA.

Twenty-one of the 40 (53%) fecal samples from Sonora 1-4 were positive for swine HEV RNA. Seven of the 30 fecal samples from Granja 1-3 were also positive (Table 2). All 20 fecal samples originating from Cajeme were negative.
Seroprevalence of swine HEV antibodies in Mexican pigs. The seroprevalence of swine HEV in Thailand has been previously reported (Meng et al, 1999). Therefore, only Mexican swine sera were tested for IgG anti-HEV in this study. Of the 125 pigs tested, 100 (80%) were positive (Table 3). The prevalence rates varied from farm to farm, ranging from 30% to 100%.

Swine HEV from Mexico and Thailand belong to genotype 3. A total of 44 swine HEV isolates were sequenced for the ORF 2 capsid gene region. The resulting 304 bp sequence from each swine HEV isolate was compared to the others and to six selected human and swine HEV isolates that represent the 4 genotypes. Swine HEV isolates from Thailand shared 97-100% sequence identity to each other and are more closely related to genotype 3 HEV than to other genotypes (Table 4). The Mexican swine HEV isolates from Sonora 1-4 shared 94-100% nucleotide sequence identity to each other. Isolates from Granja 1-3 shared 90-100% sequence identity. Sonora and Sinaloa groups shared 90-92% nucleotide sequence identity. All Mexican swine HEV isolates were most closely related to genotype 3 HEV possessing 90-92% sequence identity (Table 5).

Phylogenetic analyses revealed geographic clustering of swine HEV isolates. Phylogenetic analysis showed that swine HEV isolates from Mexico and Thailand clustered into genotype 3. Geographic clustering was also evident as all Thai swine HEV isolates form a distinct branch. Within the Mexican swine HEV isolates, several minor
branches associated with farm origins were formed, indicating heterogeneity of swine HEV isolates in different farms in Mexico (Fig. 2).

**Discussion**

Accumulated evidence suggests that hepatitis E is a zoonosis (Wang et al 2002, Meng 2000). Among the 4 recognized major genotypes of HEV, genotypes 3 and 4 HEV strains are believed to cross infect between humans and swine, and cause sporadic cases of acute hepatitis E in both developing and industrialized countries. In contrast, genotypes 1 and 2 HEV strains have not been identified in animals, and thus it was hypothesized that genotypes 1 and 2 HEV are not zoonotic. The objective of this study was to determine the genotype of HEV circulating in pigs from countries in which human genotypes 1 or 2 HEV strains are prevalent.

Thus far, only a single strain of genotype 2 human HEV has been identified, that originating from an outbreak in Mexico (Mexico-14 strain, Huang et al 1992). Genotype 2 HEV has not yet been isolated from any other part of the world. Since genotype 3 and 4 strains were identified from pigs in other countries, it was important to determine whether genotype 1 or 2 HEV strains were circulating in Mexican pigs. Our results indicated that about 80% of the Mexican pigs tested were seropositive HEV, suggesting that infection in Mexican pigs is widespread. Our previous study showed that swine HEV viremia and fecal virus shedding generally occur at 2-4 months of age (Huang et al 2002), thus we only tested pigs in this age group from Mexico. We found that about 6% of sera and 31% of fecal samples are positive for swine HEV RNA, which are consistent with the reports from other countries (Wibawa et al 2004, Takahashi et al 2003).
Sequence and phylogenetic analyses of swine HEV isolates from pigs in Mexico revealed that all swine HEV isolates from Mexican pigs belong to genotype 3. There exist minor branches in the phylogenetic tree among farms, indicating heterogeneity of the swine HEV isolates from farm to farm. Unlike the United States, pig movements across the states of Mexico are limited, and this may explain the observed genetic clustering of the swine HEV isolates among farms (Southard 1999).

It is believed that genotype 1 HEV strains are prevalent in many Asian countries including Thailand (Schlauder & Mushahwar, 2001). A genotype 3 HEV has been isolated from a patient who returned from a visit to Thailand (Kabran-Lazizi et al, 2001). Although it is known that pigs in Thailand are infected with HEV (Meng et al, 1999), the virus has not yet been genetically characterized. In this study, we tested serum samples from 76 pigs of various ages from Thailand for the presence of swine HEV RNA. About 38.5% (10/26) of pigs 2-4 months of age were positive where none of the 1-month old, 6-month old, or adult pigs were positive, further confirming that active swine HEV infection generally occurs in pigs of 2-4 months of age. Sequence analyses of the Thai swine HEV isolates revealed that they all belong to genotype 3. The swine HEV isolates from Thailand form a distinct branch in the phylogenetic tree.

Under experimental conditions, attempts to infect SPF pigs with a genotype 1 strain (Sar-55) and the genotype 2 strain (Mexican-14) of human HEV were unsuccessful (Meng et al, 1998a) even though pigs could be readily infected with genotype 3 human HEV (US-2 strain) (Meng et al, 1998b, 1999; Halbur et al 2001). The lack of detection of genotypes 1 and 2 HEV strains from pigs in countries where genotypes 1 or 2 are
prevalent in humans further validates the hypothesis that unlike genotypes 3 and 4, genotypes 1 and 2 are likely restricted in humans, and are not zoonotic. Genotype 3 strains have been detected in pigs from many countries, and are likely responsible for most of the sporadic cases of acute hepatitis E in humans. It will be important to investigate whether sporadic cases of human hepatitis E exist in Mexico, and if so, which genotype is responsible. Unfortunately, sporadic cases of acute hepatitis E is often under diagnosed (Santos et al, 2002; Wang et al, 2001; McCrudden et al, 2000).

Acknowledgments

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We would also like to thank Drs. T. Sirinarumitr and K. Urairong for providing the sera and Mr. Denis Guenette for his technical assistance.
References


General Conclusions

In conclusion, it was demonstrated that swine HEV exist in pigs from Mexico and Thailand. It was shown that the HEV strains infecting pigs in these two countries all belong to genotype 3, even though genotypes 1 (Thailand) and 2 (Mexico) HEV strains are prevalent in the human populations. Our data indicated that genotypes 3 and 4 HEV, but not genotypes 1 or 2 are likely to be zoonotic. It was shown that there exists genetic variation among swine HEV isolates from different geographic origins. The results from this study have important implications for understanding HEV zoonosis.
References


Table 1: Detection of swine HEV RNA by RT-PCR from pigs of different ages in Thailand.

<table>
<thead>
<tr>
<th>Age in months</th>
<th>No. positive/no. total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0/10 (0%)</td>
</tr>
<tr>
<td>2</td>
<td>6/10 (60%)</td>
</tr>
<tr>
<td>3</td>
<td>1/6 (17%)</td>
</tr>
<tr>
<td>4</td>
<td>3/10 (30%)</td>
</tr>
<tr>
<td>6</td>
<td>0/10 (0%)</td>
</tr>
<tr>
<td>Adult</td>
<td>0/10 (0%)</td>
</tr>
<tr>
<td>Sows</td>
<td>0/20 (0%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>10/76 (13%)</strong></td>
</tr>
</tbody>
</table>
Table 2: Detection of swine HEV RNA from pigs of 2-4 months of age in Mexico.

<table>
<thead>
<tr>
<th>State</th>
<th>Farm ID</th>
<th>No. positive / no. tested (%)</th>
<th>Serum samples</th>
<th>Fecal samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sonora</td>
<td>Sonora 1</td>
<td>2/10 (20%)</td>
<td>8/10 (80%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sonora 2</td>
<td>0/10 (0%)</td>
<td>4/10 (40%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sonora 3</td>
<td>0/10 (0%)</td>
<td>0/10 (0%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sonora 4</td>
<td>5/10 (50%)</td>
<td>9/10 (90%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Granja Sem#9</td>
<td>0/10 (0%)</td>
<td>0/10 (0%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Granja LA</td>
<td>0/10 (0%)</td>
<td>0/10 (0%)</td>
<td></td>
</tr>
<tr>
<td>Sinaloa</td>
<td>G1</td>
<td>0/10 (0%)</td>
<td>0/10 (0%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G2</td>
<td>0/10 (0%)</td>
<td>2/10 (20%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G3</td>
<td>1/10 (0%)</td>
<td>5/10 (50%)</td>
<td></td>
</tr>
<tr>
<td>Puebla</td>
<td>Group 2</td>
<td>0/35 (0%)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>8/125 (6%)</strong></td>
<td><strong>28/90 (31%)</strong></td>
<td></td>
</tr>
</tbody>
</table>
Table 3: Seroprevalence of swine HEV antibody in Mexican pigs.

<table>
<thead>
<tr>
<th>Farm ID</th>
<th>no. positive/ no. tested (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sonora 1</td>
<td>10/10 (100%)</td>
</tr>
<tr>
<td>Sonora 2</td>
<td>10/10 (100%)</td>
</tr>
<tr>
<td>Sonora 3</td>
<td>10/10 (100%)</td>
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<tr>
<td>Sonora 4</td>
<td>10/10 (100%)</td>
</tr>
<tr>
<td>Sem #9</td>
<td>4/10 (40%)</td>
</tr>
<tr>
<td>LA</td>
<td>5/10 (50%)</td>
</tr>
<tr>
<td>G1</td>
<td>3/10 (30%)</td>
</tr>
<tr>
<td>G2</td>
<td>7/10 (70%)</td>
</tr>
<tr>
<td>G3</td>
<td>10/10 (100%)</td>
</tr>
<tr>
<td>Group 2</td>
<td>31/35 (88%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100/125 (80%)</strong></td>
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</table>


Table 4: Pairwise nucleotide sequence comparison of the ORF 2 gene region of Thai swine HEV isolates and selected representative HEV strains of 4 genotypes.

<table>
<thead>
<tr>
<th>Isolate IDs*</th>
<th>Percent nucleotide sequence identities</th>
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<td></td>
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<tr>
<td>2-4</td>
<td>99</td>
</tr>
<tr>
<td>2-5</td>
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<td>2-6</td>
<td>98</td>
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<tr>
<td>2-7</td>
<td>99</td>
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<tr>
<td>2-9</td>
<td>98</td>
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<tr>
<td>2-10</td>
<td>98</td>
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<td>3-6</td>
<td>98</td>
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<tr>
<td>4-2</td>
<td>98</td>
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<tr>
<td>4-3</td>
<td>100</td>
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<tr>
<td>4-7</td>
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<tr>
<td>US-2</td>
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</tr>
<tr>
<td>Taiwan</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td></td>
</tr>
<tr>
<td>Swine Prototype</td>
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<tr>
<td>Sar-55</td>
<td></td>
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</tbody>
</table>

* Six known human and swine HEV isolates were included in the comparison: US-2 (genotype 3), Taiwan (genotype 4), T1 (genotype 4 from China), Swine prototype (swine prototype HEV, genotype 3), Sar-55 (genotype 1 from Pakistan), and Mexico (genotype 2).
Table 5: Pairwise comparison of Mexican swine HEV sequences with selected representative known HEV sequences in the 4 genotypes.

<table>
<thead>
<tr>
<th>Isolate IDs***</th>
<th>Percent nucleotide sequence identities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S1-25+</td>
</tr>
<tr>
<td>S1-24*</td>
<td>99</td>
</tr>
<tr>
<td>S1-25+</td>
<td>99</td>
</tr>
<tr>
<td>S1-27</td>
<td>94</td>
</tr>
<tr>
<td>S2-29#</td>
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<td>S4-21</td>
<td>99</td>
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<td>S4-22</td>
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<td>S4-24!</td>
<td>90</td>
</tr>
<tr>
<td>G2-8</td>
<td>99</td>
</tr>
<tr>
<td>G2-10</td>
<td></td>
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<tr>
<td>G3-2^</td>
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</tbody>
</table>

***See Table 4 for footnote.

Isolates denoted with symbols are collective representatives of multiple isolates with the same sequence
*S1-24, S2-24, S2-25, S2-33
+S1-25, S1-28, S1-26, S1-29, S1-30, S1-32
#S2-29, S4-26, S4-28, S4-29, S4-30
!S4-24, S4-25
^G3-2,G3-6,G3-7,G3-8,G3-9

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Figure 1: Standardization of a universal RT-PCR assay with degenerate primers by using representative strains of HEV from each of the 4 known genotypes
Fig. 2: Phylogenetic Tree of All Swine HEV Isolates and Selected Human and Swine HEV Isolates
Fig. 3: Distribution of anti-HEV antibody seroprevalence or viral RNA detection.
Fig 4: Cross-species infections and zoonotic potential of the hepatitis E virus
Fig. 5: The genomic organization of HEV.