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Identification of Genotype 3 Hepatitis E Virus (HEV) in Serum and Fecal Samples from Pigs in Thailand and Mexico, Where Genotype 1 and 2 HEV Strains Are Prevalent in the Respective Human Populations

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Hepatitis E virus (HEV), the causative agent of hepatitis E, is an important public health concern in many developing countries. Increasing evidence indicates that hepatitis E is a zoonotic disease. There exist four major genotypes of HEV, and HEV isolates identified in samples from pigs belong to either genotype 3 or 4. Genotype 1 and 2 HEVs are found exclusively in humans. To determine whether genotype 1 and 2 HEVs also exist in pigs, a universal reverse transcription-PCR assay that is capable of detecting all four HEV genotypes was used to test for the presence of HEV RNA in 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 was epidemic. In Thailand, swine HEV RNA was detected in sera from 10/26 pigs of 2 to 4 months of age but not in sera from 50 pigs of other ages. In Mexico, swine HEV RNA was detected in 8/125 sera and 28/92 fecal samples from 2- to 4-month-old pigs. Antibodies to swine HEV were also detected in about 81% of the Mexican pigs. A total of 44 swine HEV isolates were sequenced for the open reading frame 2 gene region. Sequence analyses revealed that all swine HEV isolates identified in samples from pigs in Thailand and Mexico belong to genotype 3. Phylogenetic analyses revealed that minor branches associated with geographic origin exist among the swine HEV isolates. The results indicated that genotype 1 or 2 swine HEV does not exist in pigs from countries where the respective human HEV genotype 1 or 2 is prevalent. It is likely that only genotype 3 and 4 HEV strains have zoonotic potential.

Hepatitis E is an important public health concern in Mexico and many developing countries in Asia and Africa (2, 19). Sporadic cases of acute hepatitis E have also been reported in many industrialized countries, including the United States (4, 20, 22, 25, 26). The disease is transmitted primarily by the fecal-oral route through contaminated water. The overall mortality rate is generally low (<1%), but it can be as high as 20 to 25% among pregnant women (11). Hepatitis E virus (HEV), the causative agent of hepatitis E, is currently classified as the sole member of the genus *Hepevirus* (3). The genome of HEV is approximately 7.2 kb and contains three open reading frames (ORF) and a short noncoding region at both the 5′ and 3′ ends (8).

The first animal strain of HEV, designated swine HEV, to be isolated and characterized was obtained from a pig in the United States (14). Subsequently, many HEV isolates from swine in over a dozen countries have been identified (19). There exist at least four major genotypes of HEV (2, 19): type 1 (Asian strains), type 2 (a single Mexican strain), type 3 (strains from sporadic cases in industrialized countries), and type 4 (strains from sporadic cases in China, Japan, and Taiwan). Swine HEV isolates identified thus far belong to either genotype 3 or 4 (19). Cross-species infection with genotype 3 HEV has been experimentally demonstrated: a genotype 3 swine HEV infected nonhuman primates (15), and conversely, a genotype 3 human HEV (US-2 strain) infected pigs (5, 15). Genotype 3 and 4 strains of human HEV responsible for sporadic cases of acute hepatitis E in humans were found to be genetically closely related to, and in some cases identical to, genotype 3 and 4 strains of swine HEV, respectively (4, 9, 12, 19, 20, 22, 25, 26), indicating that genotype 3 and 4 HEVs can infect across species and that pigs are animal reservoirs (19). It is still unclear, however, whether genotype 1 and 2 HEVs can also infect across species or whether they exist in pigs at all. The objective of this study was to determine the prevalences and genotypes of swine HEVs from pigs in Mexico, where the only strain of genotype 2 human HEV was isolated, and in Thailand, where genotype 1 human HEV is thought to be endemic.

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MATERIALS AND METHODS

Sample collection. Seventy-six serum samples were collected from pigs of various ages from Aujeszky's disease-free farms in Nakorn-Pathom, Thailand (17). A total of 215 samples, including 90 pairs of serum and fecal samples and

35 serum samples without corresponding fecal samples, were taken from pigs of 2 to 4 months of age from the states of Sonora, Sinaloa, and Puebla in Mexico.

ELISA. The enzyme-linked immunosorbent assay (ELISA) procedure was performed essentially as previously described (17). Briefly, a truncated recombinant human HEV (Sar-55 strain) capsid protein was used to coat 96-well plates for an ELISA to detect immunoglobulin G (IgG) anti-HEV in pigs. The Sar-55 human HEV capsid antigen cross-reacts well with swine HEV (16, 17). Horseradish peroxidase-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. A hyperimmune anti-HEV swine serum (14) was included as a positive control and serum from a specific-pathogen-free pig was included as a negative control.

Primer design. The following degenerate primers capable of detecting HEV strains with significant sequence variations were designed based on a multiple sequence alignment of the ORF2 genes from 18 different known strains of human HEV and the prototype strain of swine HEV as previously described (9): external primer set 3156N [forward primer; nucleotide position numbers (relative to swine HEV) 5711 to 5732; 5′-AATTATGCC(T)CAGTAC(T)CGG(A)GTTG-3′] and 3157N [reverse primer; position numbers 6419 to 6441; 5′-CCCTTA(G)TCC(T)TGCTGA(C)GCATTCTC-3′] and internal primer set 3158N [forward primer; position numbers 5996 to 6017; 5′-GTT(A)ATGCTT(C)TGCATA(T)CATGGCT-3′] and 3159N [reverse primer; position numbers 6322 to 6343; 5′-AGCCGACGAAATCAATTCTGTC-3′]. Letters in parentheses indicate degenerate bases. The expected product of the nested universal reverse transcription-PCR (RT-PCR) was 348 bp.

RT-PCR. The development and validation of the universal RT-PCR assay that is capable of detecting divergent strains of HEV have been previously reported (9). To evaluate whether the universal RT-PCR assay could detect HEV strains from all four known genotypes of HEV, total RNAs were extracted from the Pakistani Sar-55 strain of human HEV (genotype 1), the Mexican HEV strain (genotype 2), the US-2 human HEV strain (genotype 3), and the Taiwan TW6196E strain of human HEV (genotype 4). The parameters for the RT-PCR assay were essentially the same as those previously described (9).

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 directly sequenced for both strands with PCR primers at the Virginia Bioinformatics Institute (Blacksburg, Virginia). Sequence analyses were conducted using the MacVector computer program (Oxford Molecular Inc., San Diego, CA). The swine HEV sequences were subsequently compared to selected known human and swine HEV sequences available in the GenBank database, including those from 11 human HEV strains (accession numbers and geographic origins are given in parentheses), Burma (M73218), Egypt (AF051351), Madras (X99441; India), Morocco (AF065061), Mexico (M74506), Sar-55 (M80581; Pakistan), T1 (AJ272108; China), Tw261E (AF296160; Taiwan), JA11 (AB082567; Japan), JA1 (AB097812; Japan), and US-2 (AF060669), and 8 swine HEV strains, including the United States prototype strain (AF082843) and strains from Spain (AF195063), The Netherlands (AF332629), Korea (AF516179), Japan (AB073910), Canada (AY115488), and Taiwan (strains Tw32sw [AF117280] and J13 [AB097811]).

Phylogenetic analysis. A phylogenetic tree was produced using the PAUP program (David Swofford, Smithsonian Institute, Washington, D.C.; distributed by Sinauer Associates Inc., Sunderland, MA). The bootstrap method with 1,000 replications was used to generate a consensus tree. Avian HEV (6, 10) was included as an out-group.

Nucleotide sequence accession numbers. The 44 swine HEV sequences determined in this study were deposited in the GenBank database with accession numbers AY858892 through AY858937.

RESULTS

The universal RT-PCR assay is capable of detecting all four known genotypes of HEV. Although the universal RT-PCR assay has been shown to be capable of detecting divergent strains of HEV (9), it was not known whether it could detect strains from all four known genotypes. Therefore, in this study, representative strains of HEV from each of the four known genotypes, including the Pakistani Sar-55 strain of human HEV (genotype 1), the Mexican human HEV strain (genotype 2), the US-2 human HEV strain (genotype 3), and the Taiwan TW6196E human HEV strain (genotype 4), were tested with the assay. Although these four HEV strains differed in their ORF2 gene nucleotide sequences by more than 20% (9), total RNAs extracted from the four HEV strains all produced positive results by the universal RT-PCR assay (data not shown). The amplified products were sequenced and confirmed to originate from the respective reference HEV strains. Thus, the universal RT-PCR appears to be capable of detecting HEV strains from all four known genotypes.

TABLE 1. Detection of swine HEV RNA by RT-PCR in samples from pigs of different ages in Thailand and from pigs of 2 to 4 months of age in Mexico

Origin of pigs	Age (mo)	Farm(s)	No. of samples positive/no. tested (%)	
			Serum	Feces
Mexico				
Sonora	2–4	Sonora 1	2/10 (20)	8/10 (80)
	2–4	Sonora 2	0/10 (0)	4/10 (40)
	2–4	Sonora 3	0/10 (0)	0/10 (0)
	2–4	Sonora 4	5/10 (50)	9/10 (90)
	2–4	Sonora 5	0/10 (0)	0/10 (0)
	2–4	Sonora 6	0/10 (0)	0/10 (0)
Sinaloa	2–4	Granja 1	0/10 (0)	0/10 (0)
	2–4	Granja 2	0/10 (0)	2/10 (20)
	2–4	Granja 3	1/10 (0)	5/10 (50)
Puebla	2–4	Group 2	0/35 (0)	N/A[a]
Subtotal			8/125 (6)	28/90 (31)
Thailand	1	A	0/10 (0)	N/A
	2	A	6/10 (60)	N/A
	3	A	1/6 (17)	N/A
	4	A	3/10 (30)	N/A
	6	B	0/10 (0)	N/A
	Adult	C	0/10 (0)	N/A
	Sows	B, D	0/20 (0)	N/A
Subtotal			10/76 (13)	

[a] N/A, samples not available.

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

A total of 125 pigs of 2 to 4 months of age from Mexico were tested for the presence of swine HEV RNA. Paired serum and fecal samples were obtained from 90 pigs from the states of Sonora and Sinaloa, and only serum samples were obtained from the remaining 35 pigs from Puebla. A total of 8 sera (6%) were found positive for swine HEV RNA (Table 1). Seven of the swine sera collected from four farms in the northern part of Sonora (Sonora farms 1 through 4) were positive for swine HEV RNA (Table 1). However, none of the 20 sera from farms in central Sonora (Sonora farms 5 and 6) were positive. One of the 30 sera collected from pigs in Culiacan, Sinaloa (Granja farm 3), was positive. The 35 sera collected from pigs in Puebla were all negative for swine HEV RNA (Table 1).

Twenty-one (53%) of the 40 fecal samples from Sonora farms 1 through 4 and 7 of the 30 fecal samples from Sinaloa (Granja farms 1 through 3) were positive for swine HEV RNA.

TABLE 2. Seroprevalence of swine HEV antibody in Mexican pigs of 2 to 4 months of age

Farm	Total no. tested	No. positive (%)
Sonora 1	10	10 (100)
Sonora 2	10	10 (100)
Sonora 3	10	10 (100)
Sonora 4	10	10 (100)
Sonora 5	10	4 (40)
Sonora 6	10	5 (50)
Granja 1	10	3 (30)
Granja 2	10	7 (70)
Granja 3	10	10 (100)
Group 2	35	31 (88)
Total	125	100 (80)

However, all 20 fecal samples originating from central Sonora (Sonora farms 5 and 6) were negative (Table 1).

Seroprevalence of swine HEV antibodies in Mexican pigs. The seroprevalence of swine HEV in Thailand has been previously reported (17). Thus, only Mexican swine sera were tested for IgG anti-HEV. Of the 125 pigs tested, 100 (80%) were positive. The prevalence rates varied from farm to farm, ranging from 30 to 100% (Table 2).

Swine HEV isolates from pigs in Mexico and Thailand belong to genotype 3. The ORF2 capsid gene regions from a total of 44 swine HEV isolates from Mexico and Thailand were sequenced. The resulting 304-bp sequence from each of the 44 swine HEV isolates was compared to the other sequences and to those from five selected human and swine HEV isolates representing the four known genotypes: genotype 1 (Sar-55 strain), genotype 2 (Mexico strain), genotype 3 (swine HEV and US-2 strains), and genotype 4 (T1 strain). Swine HEV isolates from Thailand showed 97 to 100% nucleotide sequence identity to one another and 90 to 93% identity to genotype 3 swine and human HEVs but only 74 to 77% identity to genotype 1, 2, and 4 HEVs (data not shown). The Mexican swine HEV isolates showed 90 to 99% nucleotide sequence identity to one another, 88 to 92% identity to genotype 3 swine and human HEVs, and only 74 to 78% identity to genotype 1, 2, and 4 HEVs (data not shown). Both the Mexican and Thai swine HEV isolates are more closely related to the genotype 3 HEV strains than to strains from other genotypes, indicating that they all belong to genotype 3.

Phylogenetic analyses revealed geographic clustering of swine HEV isolates. Phylogenetic analysis confirmed that the swine HEV isolates from pigs in Mexico and Thailand all belong to genotype 3. However, geographic clustering was evident, as all Thai swine HEV isolates form a distinct minor branch (Fig. 1). Among the Mexican swine HEV isolates, several minor branches associated with farm origins were formed, indicating the heterogeneic nature of swine HEV isolates from different Mexican farms (Fig. 1).

DISCUSSION

Accumulated evidence indicated that hepatitis E is a zoonotic disease and that there exist animal reservoirs for HEV (2, 18, 19). In addition to swine HEV, strains of HEV antigenically and genetically related to human HEV have been identified in samples from chickens and designated avian HEV (6, 7). Avian HEV from a chicken has been shown to infect turkeys (21) but not rhesus monkeys (10). In contrast, swine HEV readily infected both rhesus monkeys and a chimpanzee (15). It has been shown that pig handlers are at increased risk of zoonotic HEV infection (1, 18). Clinical cases of acute hepatitis E were epidemiologically linked to consumption of undercooked pig livers (26). Most importantly, the virus isolates recovered from human patients were genetically indistinguishable from, or in some cases identical to, swine HEV isolates recovered from packaged pig livers sold in local grocery stores (26). More recently, a cluster of acute hepatitis E cases was reported in four patients in Japan who consumed uncooked deer meats (23), and the virus recovered from the patients is indistinguishable from the virus recovered from the leftover deer meats (23), thus providing more direct evidence for zoonotic HEV transmission. Among strains of the four known genotypes of HEV, genotype 3 and 4 strains are believed to infect across species between humans and swine (and maybe other species) and cause sporadic cases of acute hepatitis E (2, 19). In contrast, genotype 1 and 2 HEV strains have been identified only in samples from humans, and thus it was hypothesized that genotype 1 and 2 HEV strains may not be zoonotic. The objective of this study was to determine the genotype of HEV circulating in pigs from countries in which human HEV genotype 1 or 2 is prevalent.

Thus far, only a single strain of genotype 2 human HEV (Mexico-14 strain) has been identified, and it was from an outbreak in Mexico (8). Genotype 2 HEV has not yet been isolated from any other regions of the world. Since genotype 2 HEV was epidemic among humans in Mexico, and since genotype 3 and 4 strains were identified in samples from pigs in other countries, it was important to determine the swine HEV genotype circulating in Mexican pigs. Our results indicated that about 80% of the Mexican pigs of 2 to 4 months of age were seropositive for HEV, suggesting that swine HEV infection in Mexican pigs is widespread. It was previously shown that swine HEV viremia and fecal virus shedding generally occur in pigs of 2 to 4 months of age (9); thus, we tested only pigs in this age group from Mexico. We found that about 6% of sera and 31% of fecal samples were positive for swine HEV RNA, which is consistent with reports from other countries (19, 22). Sequence and phylogenetic analyses of swine HEV isolates from pigs in Mexico revealed that all Mexican swine HEV isolates belong to genotype 3. Minor branches in the phylogenetic tree exist among swine HEV isolates from different farms, indicating heterogeneity of swine HEV isolates from farm to farm.

It is believed that genotype 1 human HEV is prevalent in many Asian countries, including Thailand (2, 12, 19). Although it is known that pigs in Thailand are infected with HEV (17), the virus infecting Thai pigs has not yet been genetically characterized. A genotype 3 strain of human HEV has been isolated from a patient who returned from a visit to Thailand (12). In the present study, we tested serum samples from 76 pigs of various ages in Thailand for the presence of swine HEV RNA. About 39% of the pigs of 2 to 4 months of age were positive, whereas 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 to 4 months of age. Sequence

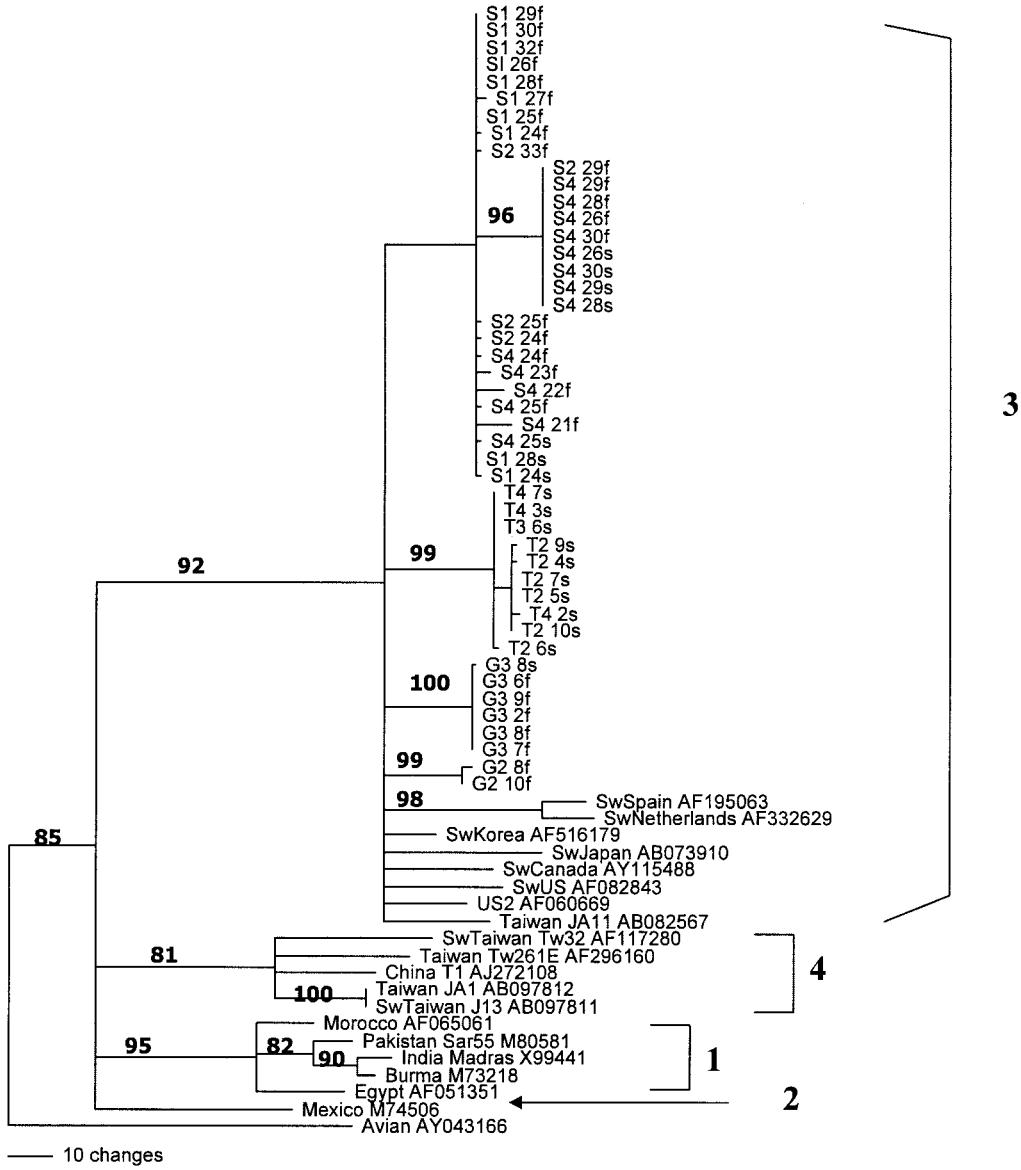

FIG. 1. A phylogenetic tree based on the ORF2 gene regions of 44 swine HEV isolates from pigs in Mexico and Thailand and sequences from selected human and swine HEV isolates representing each of the four genotypes. Avian HEV was included as an out-group. The tree was constructed with the bootstrap search option with 1,000 replications by using the PAUP program. Bootstrap values for major branches are shown. All 44 swine HEV isolates identified in samples from pigs in Mexico (designated S for Sonora or G for Granja) and in Thailand (designated T for Thailand) belong to genotype 3. The small letters (s and f) at the end of each isolate number indicate the source of the virus: s, serum; f, feces.

and phylogenetic analyses of the Thai swine HEV isolates revealed that, like Mexican swine HEV isolates, they all belong to genotype 3.

Under experimental conditions, attempts to infect pigs with a genotype 1 strain (Sar-55) and the genotype 2 strain (Mexico-14) of human HEV were unsuccessful (16), even though pigs could be readily infected with a genotype 3 human HEV (US-2 strain) (5, 15). The lack of detection of HEV genotypes 1 and 2 in samples from pigs in countries where HEV genotype 1 or 2 is prevalent in the respective human populations provided credence to the hypothesis that, unlike genotype 3 and 4 strains, genotype 1 and 2 HEVs are restricted to humans and are not zoonotic. Genotype 3 HEV 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. In many industrialized countries, including the United States, anti-HEV antibodies have also been detected in a significant proportion of healthy people (13, 18, 24), indicating prior exposure to HEV. It will now be important to investigate whether or not sporadic cases of human hepatitis E exist in Mexico and, if so, which genotype of HEV is responsible for them.

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