Molecular Characterization of Animal Strains of Hepatitis E Virus (HEV): Avian HEV and Swine HEV

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(Abstract)

Hepatitis E virus (HEV), the causative agent of hepatitis E, is an important public health concern in many developing countries. It mainly infects young adults and has a mortality of up to 25% in pregnant women. Although hepatitis E is only sporadic in industrialized countries including the United States, a relative high seroprevalence rate has been reported in healthy individuals. Evidence suggests that there exist animal reservoirs for HEV and HEV transmission is zoonotic. Animal strains of HEV, swine HEV and avian HEV have been identified from a pig and a chicken, respectively, in the United States. Studies showed that swine HEV and avian HEV are genetically and antigenically related to human HEV, and that pigs and chickens are useful animal models to study HEV replication, pathogenesis and cross-species infection. The objectives of this dissertation were to genetically characterize both avian HEV and swine HEV, to determine their serological and molecular epidemiology in the United States, to assess the ability of avian HEV cross-species infection in non-human primates, to determine the full-length genomic sequence and genome organization, and to construct an infectious cDNA clone of avian HEV.

The prevalence of swine HEV infections in US swine herds and the heterogeneity of swine HEV isolates from different geographic regions of the United States were determined. We found that 35% pigs and 54% swine herds were positive for swine HEV RNA. Partial capsid gene region of twenty-seven US swine HEV isolates was sequenced and was showed to share 88%-100% nucleotide sequence identity to each other and 89-98% identity with the prototype US swine HEV, but only <79% identity with Taiwanese swine HEV isolates and most known human strains of HEV worldwide. All US swine HEV isolates belong to the same genotype 3 with the prototype US swine HEV and the two US strains of human HEV.

Similarly, the prevalence of avian HEV infections in US chicken flocks and the heterogeneity of avian HEV isolates were also determined. Helicase gene region of eleven field isolates of avian HEV from chickens with hepatitis-splenomegaly (HS) syndrome was sequenced and was found to share 78-100% nucleotide sequence identities with each other, 79-88% identities with the prototype avian HEV, 76-80% identities with Australian chicken big liver and spleen disease virus (BLSV), and 56-61% identities with other known strains of mammalian HEV. A relative high prevalence of anti-avian HEV antibodies was found in apparently healthy chicken flocks in 5 states. Like swine HEV, the seropositivity of avian HEV in adult chickens was higher than that in young chickens.

To genetically characterize the avian HEV genome, we determined the full-length genomic sequence of avian HEV, which is 6,654 bp in length excluding the poly (A) tail, and 600 bp shorter than that of mammalian HEVs. Avian HEV has similar genomic organization with human and swine HEVs, but shared only about 50% nucleotide sequence identity with mammalian HEVs in the complete genome. Significant genetic variations such as deletions and insertions, particularly in the ORF1 of avian HEV, were observed, but motifs in the putative functional domains of the ORF1 were relatively conserved between avian HEV and mammalian HEVs. Phylogenetic analyses based on the full-length genomic sequence revealed that avian HEV represents a branch distinct from human and swine HEVs.

Since swine HEV infects non-human primates and possibly humans, the ability of avian HEV cross-species infection in non-human primates was also assessed. However, unlike swine HEV, avian HEV failed to infect two rhesus monkeys under experimental conditions.

With the availability of the complete genome sequence of avian HEV, we constructed three full-length cDNA clones of avian HEV and tested their infectivity by *in vitro* transfection of the LMH chicken liver cells and by *in vivo* intrahepatic inoculation of specific-pathogen-free (SPF) chickens. The results showed that all 3 cDNA clones of avian HEV were infectious both *in vitro* and *in vivo*, as the capped RNA transcripts from each of the clones were replication-competent in transfected LMH cells and developed active infection in inoculated SPF chickens.

In summary, avian HEV and swine HEV infections are enzootic in chicken flocks and in swine herds in the United States, respectively. Like human HEV, swine HEV and

avian HEV isolates from different geographic regions are also genetically heterogenic. Complete genomic sequence analyses showed that avian HEV is related to, but distinct from, human and swine HEVs. Unlike swine HEV, avian HEV is probably not transmissible to non-human primates. Infectious cDNA clones of avian HEV have been successfully constructed. The availability of the infectious clones for a chicken strain of HEV now affords us an opportunity to study the mechanisms of HEV replication, pathogenesis and cross-species infection.

DEDICATION

I would like to dedicate this work to my parents, my sisters and my husband in thanks for all of their love and support.

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General Introduction

Hepatitis E is an enterically transmitted non-A, non-B acute hepatitis. The mortality rate can reach up to 25% in infected pregnant women (16). The causative agent of hepatitis E is the hepatitis E virus (HEV). HEV is presumably transmitted via the fecal-oral route through contaminated water, and mainly infects young adults (1, 2, 4, 5, 9). HEV infection is a major pubic health concern in many developing countries of Asia, Africa and in Mexico due to poor sanitation conditions (1, 9). Although only sporadic cases have been reported in industrialized countries including the United States, a relatively high proportion of healthy population are seropositive for antibodies to HEV (3, 6, 7, 11, 15, 17, 23, 24).

Recent evidence indicate that there exist animal reservoirs for HEV and that hepatitis E is a zoonosis (18, 19). The first animal strain of HEV, swine HEV, was isolated and characterized from pigs in the United States in 1997 by Meng et al. (21). Swine HEV is antigenically and genetically related to human HEV, and shares high nucleotide sequence identity (92%) with two US human HEV strains (10, 20). Interspecies transmission studies showed that swine HEV can experimentally infect non-human primates, and conversely the US-2 strain of human HEV can infect pigs (12, 20). It has also been reported that there is an increased risk of HEV infection in swine veterinarians and other pig handlers than in normal population (8, 22, 25). Avian HEV, the second animal HEV strain, was isolated and characterized from a chicken with hepatitis-splenomegly (HS) syndrome in 2001 by Haqshenas et al. (14). Avian HEV is genetically and antigenically related to human HEV as swine HEV (13, 14).

The obstacle for HEV study is the lack of an efficient cell culture and a practical animal model. The identification of animal strains of HEV, swine HEV and avian HEV, affords us an opportunity to use pigs and chickens as animal models to study HEV. Therefore, it is important to genetically characterize the two animal strains of HEV and to construct infectious cDNA clones of the animal HEV strains for HEV study of replication, pathogenesis and cross-species infection.

References

1. **Aggarwal, R., and K. Krawczynski.** 2000. Hepatitis E: an overview and recent advances in clinical and laboratory research. J Gastroenterol Hepatol **15:**9-20.
2. **Aggarwal, R., and S. R. Naik.** 1994. Hepatitis E: intrafamilial transmission versus waterborne spread. J Hepatol **21:**718-23.
3. **Balayan, M. S.** 1997. Epidemiology of hepatitis E virus infection. J Viral Hepat **4:**155-65.
4. **Balayan, M. S., A. G. Andjaparidze, S. S. Savinskaya, E. S. Ketiladze, D. M. Braginsky, A. P. Savinov, and V. F. Poleschuk.** 1983. Evidence for a virus in non-A, non-B hepatitis transmitted via the fecal-oral route. Intervirology **20:**23-31.
5. **Belabbes, E. H., A. Bouguermouh, A. Benatallah, and G. Illoul.** 1985. Epidemic non-A, non-B viral hepatitis in Algeria: strong evidence for its spreading by water. J Med Virol **16:**257-63.
6. **Choi, I. S., H. J. Kwon, N. R. Shin, and H. S. Yoo.** 2003. Identification of swine hepatitis E virus (HEV) and prevalence of anti-HEV antibodies in swine and human populations in Korea. J Clin Microbiol **41:**3602-8.
7. **Clemente-Casares, P., S. Pina, M. Buti, R. Jardi, M. MartIn, S. Bofill-Mas, and R. Girones.** 2003. Hepatitis E virus epidemiology in industrialized countries. Emerg Infect Dis **9:**448-54.
8. **Drobeniuc, J., M. O. Favorov, C. N. Shapiro, B. P. Bell, E. E. Mast, A. Dadu, D. Culver, P. Iarovoi, B. H. Robertson, and H. S. Margolis.** 2001. Hepatitis E virus antibody prevalence among persons who work with swine. J Infect Dis **184:**1594-7.
9. **Emerson, S. U., and R. H. Purcell.** 2003. Hepatitis E virus. Rev Med Virol **13:**145-54.
10. **Erker, J. C., S. M. Desai, G. G. Schlauder, G. J. Dawson, and I. K. Mushahwar.** 1999. A hepatitis E virus variant from the United States: molecular characterization and transmission in cynomolgus macaques. J Gen Virol **80 (Pt 3):**681-90.

11. **Fix, A. D., M. Abdel-Hamid, R. H. Purcell, M. H. Shehata, F. Abdel-Aziz, N. Mikhail, H. el Sebai, M. Nafeh, M. Habib, R. R. Arthur, S. U. Emerson, and G. T. Strickland.** 2000. Prevalence of antibodies to hepatitis E in two rural Egyptian communities. Am J Trop Med Hyg **62:**519-23.

12. **Halbur, P. G., C. Kasorndorkbua, C. Gilbert, D. Guenette, M. B. Potters, R. H. Purcell, S. U. Emerson, T. E. Toth, and X. J. Meng.** 2001. Comparative pathogenesis of infection of pigs with hepatitis E viruses recovered from a pig and a human. J Clin Microbiol **39:**918-23.

13. **Haqshenas, G., F. F. Huang, M. Fenaux, D. K. Guenette, F. W. Pierson, C. T. Larsen, H. L. Shivaprasad, T. E. Toth, and X. J. Meng.** 2002. The putative capsid protein of the newly identified avian hepatitis E virus shares antigenic epitopes with that of swine and human hepatitis E viruses and chicken big liver and spleen disease virus. J Gen Virol **83:**2201-9.

14. **Haqshenas, G., H. L. Shivaprasad, P. R. Woolcock, D. H. Read, and X. J. Meng.** 2001. Genetic identification and characterization of a novel virus related to human hepatitis E virus from chickens with hepatitis-splenomegaly syndrome in the United States. J Gen Virol **82:**2449-62.

15. **Karetnyi, Y. V., M. J. Gilchrist, and S. J. Naides.** 1999. Hepatitis E virus infection prevalence among selected populations in Iowa. J Clin Virol **14:**51-5.

16. **Kumar, A., M. Beniwal, P. Kar, J. B. Sharma, and N. S. Murthy.** 2004. Hepatitis E in pregnancy. Int J Gynaecol Obstet **85:**240-4.

17. **Mast, E. E., I. K. Kuramoto, M. O. Favorov, V. R. Schoening, B. T. Burkholder, C. N. Shapiro, and P. V. Holland.** 1997. Prevalence of and risk factors for antibody to hepatitis E virus seroreactivity among blood donors in Northern California. J Infect Dis **176:**34-40.

18. **Meng, X. J.** 2000. Novel strains of hepatitis E virus identified from humans and other animal species: is hepatitis E a zoonosis? J Hepatol **33:**842-5.

19. **Meng, X. J.** 2003. Swine hepatitis E virus: cross-species infection and risk in xenotransplantation. Curr Top Microbiol Immunol **278:**185-216.

20. **Meng, X. J., P. G. Halbur, M. S. Shapiro, S. Govindarajan, J. D. Bruna, I. K. Mushahwar, R. H. Purcell, and S. U. Emerson.** 1998. Genetic and experimental

evidence for cross-species infection by swine hepatitis E virus. J Virol **72:**9714-21.

21. **Meng, X. J., R. H. Purcell, P. G. Halbur, J. R. Lehman, D. M. Webb, T. S. Tsareva, J. S. Haynes, B. J. Thacker, and S. U. Emerson.** 1997. A novel virus in swine is closely related to the human hepatitis E virus. Proc Natl Acad Sci U S A **94:**9860-5.
22. **Meng, X. J., B. Wiseman, F. Elvinger, D. K. Guenette, T. E. Toth, R. E. Engle, S. U. Emerson, and R. H. Purcell.** 2002. Prevalence of antibodies to hepatitis E virus in veterinarians working with swine and in normal blood donors in the United States and other countries. J Clin Microbiol **40:**117-22.
23. **Thomas, D. L., R. W. Mahley, S. Badur, K. E. Palaoglu, and T. C. Quinn.** 1993. Epidemiology of hepatitis E virus infection in Turkey. Lancet **341:**1561-2.
24. **Thomas, D. L., P. O. Yarbough, D. Vlahov, S. A. Tsarev, K. E. Nelson, A. J. Saah, and R. H. Purcell.** 1997. Seroreactivity to hepatitis E virus in areas where the disease is not endemic. J Clin Microbiol **35:**1244-7.
25. **Withers, M. R., M. T. Correa, M. Morrow, M. E. Stebbins, J. Seriwatana, W. D. Webster, M. B. Boak, and D. W. Vaughn.** 2002. Antibody levels to hepatitis E virus in North Carolina swine workers, non-swine workers, swine, and murids. Am J Trop Med Hyg **66:**384-8.

Chapter 1

Literature Review

Hepatitis E disease

Hepatitis E, an enterically transmitted non-A, non-B hepatitis, was first recognized in 1980s by retrospective serological studies of water-borne epidemics of hepatitis in Indian (51, 108). Hepatitis E is an acute disease characterized by jaundice, malaise, anorexia, vomiting, abdominal pain, and fever (1, 30, 57). The disease mainly affects young adults and has a relatively low mortality rate of about 1% in the general population. However, the mortality can reach up to 25% in infected pregnant women (58).

Hepatitis E virus (HEV)

The existence of a viral etiological agent for hepatitis E was first demonstrated by Balayan et al. in 1983 (12). Spherical 27-30 nm virus-like particles were visualized by immune electron microscopy in feces from a volunteer who was infected with stool from a non-A, non-B hepatitis patient (12). In 1990, Reyes et al. cloned and determined the partial sequence of the virus responsible for a non-A, non-B hepatitis outbreak and named the new virus hepatitis E virus (HEV) (80). The first complete genomic sequence of HEV was determined by Tam et al. in 1991 (93).

Morphology and genome organization

HEV is a spherical, non-eveloped virus of about 30 nm in size (1, 12, 57). The viral genome is a single-stranded, positive-sense RNA molecule of about 7.2 kb in length. It consists of a short 5' non-coding region (NCR) and a 3' NCR, and three partially overlapping open reading frames (ORFs) (93). ORF1 encodes the nonstructural proteins. Putative functional motifs and domains such as methyltransferase, papain-like cysteine protease (PCP), helicase, and RNA-dependent RNA polymerase (RdRp) have been identified in ORF1 (55). ORF2 encodes a putative capsid protein, and ORF3 encodes a small phosphorated protein that is associated with cytoskeleton (114).

Based on the similar morphological, physiochemical and structural characteristics to caliciviruses, HEV was initially classified in the family *Caliciviridae*. However, sequence analyses indicated that the genome organization of HEV is different from that of caliciviruses, and thus HEV was recently declassified and placed in a new genus of *Hepevirus* (28).

Transmission and epidemiology

The fecal-oral route is believed to be the main transmission mode of HEV. Most reported outbreaks of HEV are related to the consumption of fecally contaminated drink water (1, 12, 16, 51). Unlike other enterically transmitted viral hepatitis, person-to-person transmission is not common for HEV (2, 52). Vertical transmission of HEV infection from mother to infant, although rare, has been reported. Babies borne to HEV-RNA positive mother had evidence of hepatitis E infection (53, 59, 89). Transmission via blood-transfusions has been also reported (54, 65). Recent studies indicated that HEV could be zoonotically transmitted by contacting infected animals or by consuming uncooked shellfish or infected animal meat products (60, 66, 94, 111).

As a fecal-orally transmitted disease, hepatitis E is endemic in many developing countries with poor sanitation conditions (1, 30, 57). In industrialized countries, HEV infection is considered rare and only sporadic hepatitis E cases have been reported. But recently, some of the reported cases have no known risk factor (84-86), indicating that HEV infection in industrialized countries is more prevalent than it was originally thought.

Pathogenesis

Due to the lack of an efficient cell culture or a practical animal model, little is known about HEV pathogenesis. As a fecal-orally transmitted virus, it is believed that HEV initially replicates in the intestinal tract and then enters the liver via primary viremia. It has been shown that HEV replicates not only in the liver but in the extrahepatic tissue as well (10, 22, 97, 106). In general, HEV is shed in feces for 3-4 weeks, but viremia is transient and cleared before the humoral immune response (20, 25, 63, 99). Anti-HEV IgM antibodies appear first, followed by long-lasting anti-HEV IgG antibodies(17, 19, 25, 63). The elevation of liver enzymes, such as the alanine

aminotransferase (ALT), and the presence of liver pathological lesions generally coincide with seroconversion, suggesting that liver damage may be immune-mediated rather than a direct cytopathic effect by the virus (90).

Fulminant hepatitis has been reported in infected pregnant women, especially during the third trimester of pregnancy (58). However, the mechanism for this manifestation is still unknown as fulminant hepatitis was not reproduced in pregnant rhesus monkeys (6, 101).

Genotypes and seroprevalence

Sequence and phylogenetic analyses indicated that HEVs are heterogeneic, and there exist at least four major genotypes (44, 67, 70, 87, 93, 105): the genotype 1 (Asian and African HEV strains), the genotype 2 (the single Mexican HEV strain), the genotype 3 (human and swine HEV strains from industrialized countries), and the genotype 4 (human and swine HEV strains from Asia).

HEV strains in different genotypes share common antigenic epitopes in the capsid gene, indicating that HEV has a single serotype (4). In endemic countries, seroprevalence of HEV is relatively low and age specific (9, 11). The prevalence of anti-HEV antibodies is less frequent in young children than in adults (9). Compared to the relative low prevalence level of anti-HEV in endemic areas, a relative high seroprevalence rate has been reported in healthy populations in many industrialized countries (26, 34, 49, 72, 95). For examples, about 21% of the blood donors from Baltimore, MD, USA (96), and 1.9-14.1% of healthy individuals from different geographic regions of Japan were positive for anti-HEV antibodies (35). Zoonotic infection with an animal strain of HEV, which doesn't cause clinical disease in humans, could explain for the observed seroprevalence of HEV in healthy populations.

Molecular characteristics

The ORF1 of HEV encodes a large nonstructural protein with putative functional motifs and domains such as methyltransferase, PCP, helicase and RdRp (55). Studies showed that *in vitro* and *in vivo* expression of the HEV ORF1 produced a polyprotein (5, 83). Ropp et al. reported that two potential processed products from the polyprotein were

detected by elongated incubation *in vivo*, but the predicted PCP was not responsible for the proteolytic cleavage (83). The presence of methyltransferase motifs in ORF1 suggests that HEV may have a capped RNA genome. Capping activity of HEV 5' NCR has been detected and was showed to be essential for infectivity *in vivo* and replication *in vitro* by using a HEV infectious cDNA clone and a HEV replicon expressing green fluorescent protein, respectively (5, 29, 32, 47, 116). Similar to Rubella virus, a virus distantly related to HEV, the GDD motif in RdRp was reported to be important for HEV replication and the *cis*-acting elements at the 3' NCR required for viral viability by RdRp binding (3, 21, 32, 104). Two predicted stem-loop (SL) structures at the 3' NCR and the polyA tract were showed to be necessary for RdRp binding during HEV genome replication (3, 32).

The ORF2 encodes the putative capsid protein with a signal peptide sequence and three potential glycosylation sites (45, 93, 113). Most of the expressed proteins in insect cells are truncated and lack the signal sequence (62, 82, 115), and yet these truncated proteins still form HEV-like particles (VLPs) (61, 62). The biological functions of the signal sequence and glycosylation in ORF2 are not known.

The ORF3 encodes a cytoskeleton-associated phosphoprotein (114), which binds with SH3-containing signal transduction molecules of eukaryotic cells or interacts with non-glycosylated ORF2 (56, 102), indicating its potential roles in cell signal regulating or HEV structural assembly.

Control and prevention

Since HEV transmission is mainly through contaminated water, good hygiene practice could effectively prevent HEV infection. Uncooked shellfish, raw meats and contact with infected animals should be also voided.

A vaccine for HEV is not yet available. Protection from HEV infection was observed in primates by passive immunoprophylaxis (100). An attenuated or killed vaccine could not be developed due to the lack of a cell culture system for propagation. Recombinant capsid proteins expressed in *E. coli.* or in insect cells as recombinant vaccines have been shown to provide protection against disease after HEV challenge, but not against virus shedding in feces (31).

Swine HEV

Discovery and characterization

Experimental infection of domestic pigs with an Asian strain of human HEV was reported by Balayan et al. in 1990 (13). Clayson subsequently reported the detection of HEV-RNA in sera or feces from pigs in Nepal (24). But the virus infecting pigs in those studies was not characterized. In 1997, Meng et al. isolated and characterized the first animal strain of HEV, designated swine HEV, from a pig in the United State (70).

The complete genomic sequence of swine HEV has been determined (40, 69). The genomic organization of swine HEV is very similar to human HEV. Putative functional domains and motifs such as methyltransferase, PCP, hypervariable region, helicase and RdRp were also identified in the ORF1. ORF2 encodes the putative capsid protein, and ORF3 encodes a small protein.

Epidemiology

Seroepidemiological studies demonstrated that swine HEV is ubiquitous in pigs not only from HEV endemic regions but also from industrialized countries. Swine HEV has been detected in the United States, Canada, Taiwan, Japan, Korea, Australia, New Zealand and many European and Asian countries (7, 8, 14, 15, 18, 23, 24, 36, 42, 70, 74, 77, 78, 91, 92, 110, 112). The infection generally occurs in pigs at 2-4 months of age (71, 110). The ubiquitous nature of swine HEV infection in pigs suggests that swine are animal reservoirs for HEV.

Sequence comparison showed that swine HEV isolates are heterogeneic in different geographic regions. For examples, swine HEV isolated from Taiwan are genetically closely related with each other sharing 84-100% nucleotide sequence identities, but distinct from the prototype US swine HEV with only about 74-77% nucleotide sequence identities (42, 43, 109, 110). Similarly, Indian swine HEV isolates shares 96-98% nucleotide sequence identities with each other, 84-88% nucleotide sequence identities with Taiwanese swine HEV, but only 73-78% nucleotide sequence identities with the US swine HEV (7, 8, 42, 43). The swine HEV isolates in Japan are more heterogenic: 137 Japanese swine HEV isolates were grouped into four distinct clusters (91). One cluster falls into genotype 4 with Taiwanese and Indian swine HEVs,

and the other three clusters belong to genotype 3 with isolates from the United States and European countries.

All swine HEV isolates identified thus far belong to either genotype 3 or genotype 4. Genotype 1 or 2 swine HEV has not been identified. Most swine isolates from the United States, Canada, Australia and other industrialized countries of Asia and Europe belong to genotype 3. Swine HEV isolates from HEV endemic regions such as India and several industrialized Asian countries such as Japan and Taiwan are segregated into the genotype 4.

Pathogenesis

The pathogenesis of swine HEV in pigs is similar to that of human HEV. Feces from infected pigs seem to be responsible for swine HEV spread, even though experimental infection of SPF pigs via the oral route is difficult (38, 68). It has been shown that an uninfected pig housed in the same room with a swine HEV-inoculated pig became infected (69), indicating that the infection of the contact control pig may come from feces-contaminated feed or water, or through direct contact with the infected pigs. Williams et al. reported that replicative, negative-strand HEV RNA was detected in the small intestines, lymph nodes, colons, and livers of pigs experimentally infected with the prototype swine HEV (106). In another study, swine HEV was also found in different tissues of pigs naturally infected with swine HEV, including primary hepatocytes and bile ducts, as well as small and large intestines, lymph nodes, tonsils, spleens, and kidneys (22, 37). However, unlike HEV infection in human, natural swine HEV infection in pigs does not cause clinical disease and the infection is subclinical (38, 68). The effect of swine HEV infection on pregnant gilts was also determined by Kasorndorkbua et al. (50). However no clinical disease was observed in the gilts infected with swine HEV or their offsprings.

Potential zoonotic transmission

Evidence suggests that HEV could be zoonotically transmitted from pigs to humans or vice versa. Experimental cross-species infections showed that a US strain of human HEV infected pigs, and the prototype swine HEV infected non-human primates

(38, 69). Increased risk of zoonotic HEV infection was reported in swine veterinarians and other pig workers, especially in countries where HEV is considered rare, such as Taiwan, USA and Moldova (27, 42, 71, 107). Recently hepatitis E cases with unknown epidemiological risks have been reported in industrialized countries. Sequence analyses revealed that the HEV strains from different geographic regions are heterogeneic but they are more closely related to swine HEV isolates from the same geographic areas. For examples, mammalian HEV strains from the United States and European countries are all clustered in genotype 3. However, The US human HEV strains are more closely related to the US swine HEV (33, 48, 84). Similarly, Spanish human HEV strains are more closely related to a Spanish HEV of swine origin (78, 79). A human HEV and swine HEV from United Kingdom have identical amino acid sequence (14). A Japanese indigenous HEV strain recovered from a patient in Hokkaido in 1997 shares 100 % sequence identity with a swine HEV isolated from a pig in Hokkaido in 2002 (73). However, the Hokkaido human HEV strains and swine HEV isolates from other regions of Japan are separated into different genotypes (73). More recently direct evidence of zoonotic HEV transmission was reported in hepatitis E patients who consumed raw meats from pig livers or wild boar (66, 111). Taken together, these data strongly suggested that pigs are animal reservoirs and zoonotic transmission of HEV between swine and human exists.

Avian HEV

Antibodies to human HEV were detected in 44% of chickens in Vietnam (98), suggesting that the chickens had been infected with HEV (or a related agent). In 1999, Payne et al. first reported the identification of a virus from chickens with big liver and spleen disease (BLS) in Australia (76), designated BLSV. Based on a 523-bp sequence, BLSV was found to be genetically related to human HEV with about 62% nucleotide sequence identity (76).

Hepatitis–splenomegaly (HS) syndrome is an emerging disease in chickens in North America since 1991 (81). HS syndrome is characterized by increased mortality in broiler breeder hens and laying hens of between 30 and 72 weeks of age (81). Affected chickens usually demonstrate ovarian regression, red fluid in the abdomen, enlarged

spleens, and a swollen and friable liver which is mottled tan and red with multifocal miliary pale areas and hemorrhage (81). Microscopically, liver lesions vary from multifocal patches to areas of extensive hepatic necrosis and hemorrhage (81). A viral etiology for HS syndrome has been suspected, but attempts to identify the virus in either cell culture or embryonated eggs were unsuccessful (88). In 2001, Haqshenas et al. identified a virus from a chicken with HS syndrome in the United States (41). Based on the genetic relatedness of this novel chicken virus with HEV, it is designated avian HEV to distinguish from human HEV and swine HEV. Electron microscopic examination revealed that avian HEV is a non-enveloped virus particle of 30–35 nm in diameter, with similar size and morphology to human HEVs (41). Avian HEV shared about 80% nucleotide sequence identity with BLSV in a short stretch of sequence available for BLSV, and about 57-61% nucleotide sequence identity with mammalian HEVs (41). Avian HEV was also found to share common antigenic epitopes in its putative capsid protein with human and swine HEV as well as with BLSV (39). Therefore, avian HEV is not only genetically but also antigenically related to human and swine HEV. Since avian HEV is a newly identified virus, biological, experimental and genetic characterizations are warranted.

HEV in other animal species

It is indicated that many animal species are likely infected with HEV or a related agent, and that there potential exist a wide range of animal reservoirs for HEV. In addition to pigs and chickens, anti-HEV antibodies have also been detected in many other domestic and wild animals, including rodents, sheep, goats, cattle, dogs and cats in both endemic and non-endemic regions (46, 60, 75). Experimental infections of HEV have been reported in laboratory rats and lambs (64, 103). More recently, a wild deer was found infected with HEV and digestion of the infected deer meat caused zoonotic transmission in humans (94). However, thus far the virus responsible for the seroconversion in animals has only been identified from pig and chickens.

References

1. **Aggarwal, R., and K. Krawczynski.** 2000. Hepatitis E: an overview and recent advances in clinical and laboratory research. J Gastroenterol Hepatol **15:**9-20.
2. **Aggarwal, R., and S. R. Naik.** 1994. Hepatitis E: intrafamilial transmission versus waterborne spread. J Hepatol **21:**718-23.
3. **Agrawal, S., D. Gupta, and S. K. Panda.** 2001. The 3' end of hepatitis E virus (HEV) genome binds specifically to the viral RNA-dependent RNA polymerase (RdRp). Virology **282:**87-101.
4. **Anderson, D. A., F. Li, M. Riddell, T. Howard, H. F. Seow, J. Torresi, G. Perry, D. Sumarsidi, S. M. Shrestha, and I. L. Shrestha.** 1999. ELISA for IgG-class antibody to hepatitis E virus based on a highly conserved, conformational epitope expressed in Escherichia coli. J Virol Methods **81:**131-42.
5. **Ansari, I. H., S. K. Nanda, H. Durgapal, S. Agrawal, S. K. Mohanty, D. Gupta, S. Jameel, and S. K. Panda.** 2000. Cloning, sequencing, and expression of the hepatitis E virus (HEV) nonstructural open reading frame 1 (ORF1). J Med Virol **60:**275-83.
6. **Arankalle, V. A., M. S. Chadha, K. Banerjee, M. A. Srinivasan, and L. P. Chobe.** 1993. Hepatitis E virus infection in pregnant rhesus monkeys. Indian J Med Res **97:**4-8.
7. **Arankalle, V. A., L. P. Chobe, M. V. Joshi, M. S. Chadha, B. Kundu, and A. M. Walimbe.** 2002. Human and swine hepatitis E viruses from Western India belong to different genotypes. J Hepatol **36:**417-25.
8. **Arankalle, V. A., L. P. Chobe, A. M. Walimbe, P. N. Yergolkar, and G. P. Jacob.** 2003. Swine HEV infection in south India and phylogenetic analysis (1985-1999). J Med Virol **69:**391-6.
9. **Arankalle, V. A., S. A. Tsarev, M. S. Chadha, D. W. Alling, S. U. Emerson, K. Banerjee, and R. H. Purcell.** 1995. Age-specific prevalence of antibodies to hepatitis A and E viruses in Pune, India, 1982 and 1992. J Infect Dis **171:**447-50.

10. **Asher, L. V., B. L. Innis, M. P. Shrestha, J. Ticehurst, and W. B. Baze.** 1990. Virus-like particles in the liver of a patient with fulminant hepatitis and antibody to hepatitis E virus. J Med Virol **31:**229-33.

11. **Balayan, M. S.** 1997. Epidemiology of hepatitis E virus infection. J Viral Hepat **4:**155-65.

12. **Balayan, M. S., A. G. Andjaparidze, S. S. Savinskaya, E. S. Ketiladze, D. M. Braginsky, A. P. Savinov, and V. F. Poleschuk.** 1983. Evidence for a virus in non-A, non-B hepatitis transmitted via the fecal-oral route. Intervirology **20:**23-31.

13. **Balayan, M. S., R. K. Usmanov, N. A. Zamyatina, D. I. Djumalieva, and F. R. Karas.** 1990. Brief report: experimental hepatitis E infection in domestic pigs. J Med Virol **32:**58-9.

14. **Banks, M., R. Bendall, S. Grierson, G. Heath, J. Mitchell, and H. Dalton.** 2004. Human and porcine hepatitis E virus strains, United Kingdom. Emerg Infect Dis **10:**953-5.

15. **Banks, M., G. S. Heath, S. S. Grierson, D. P. King, A. Gresham, R. Girones, F. Widen, and T. J. Harrison.** 2004. Evidence for the presence of hepatitis E virus in pigs in the United Kingdom. Vet Rec **154:**223-7.

16. **Belabbes, E. H., A. Bouguermouh, A. Benatallah, and G. Illoul.** 1985. Epidemic non-A, non-B viral hepatitis in Algeria: strong evidence for its spreading by water. J Med Virol **16:**257-63.

17. **Bryan, J. P., S. A. Tsarev, M. Iqbal, J. Ticehurst, S. Emerson, A. Ahmed, J. Duncan, A. R. Rafiqui, I. A. Malik, and R. H. Purcell.** 1994. Epidemic hepatitis E in Pakistan: patterns of serologic response and evidence that antibody to hepatitis E virus protects against disease. J Infect Dis **170:**517-21.

18. **Chandler, J. D., M. A. Riddell, F. Li, R. J. Love, and D. A. Anderson.** 1999. Serological evidence for swine hepatitis E virus infection in Australian pig herds. Vet Microbiol **68:**95-105.

19. **Chauhan, A., J. B. Dilawari, R. Sharma, M. Mukesh, and S. R. Saroa.** 1998. Role of long-persisting human hepatitis E virus antibodies in protection. Vaccine **16:**755-6.

20. **Chauhan, A., S. Jameel, J. B. Dilawari, Y. K. Chawla, U. Kaur, and N. K. Ganguly.** 1993. Hepatitis E virus transmission to a volunteer. Lancet **341:**149-50.

21. **Chen, M. H., and T. K. Frey.** 1999. Mutagenic analysis of the 3' cis-acting elements of the rubella virus genome. J Virol **73:**3386-403.

22. **Choi, C., and C. Chae.** 2003. Localization of swine hepatitis E virus in liver and extrahepatic tissues from naturally infected pigs by in situ hybridization. J Hepatol **38:**827-32.

23. **Choi, I. S., H. J. Kwon, N. R. Shin, and H. S. Yoo.** 2003. Identification of swine hepatitis E virus (HEV) and prevalence of anti-HEV antibodies in swine and human populations in Korea. J Clin Microbiol **41:**3602-8.

24. **Clayson, E. T., B. L. Innis, K. S. Myint, S. Narupiti, D. W. Vaughn, S. Giri, P. Ranabhat, and M. P. Shrestha.** 1995. Detection of hepatitis E virus infections among domestic swine in the Kathmandu Valley of Nepal. Am J Trop Med Hyg **53:**228-32.

25. **Clayson, E. T., K. S. Myint, R. Snitbhan, D. W. Vaughn, B. L. Innis, L. Chan, P. Cheung, and M. P. Shrestha.** 1995. Viremia, fecal shedding, and IgM and IgG responses in patients with hepatitis E. J Infect Dis **172:**927-33.

26. **Clemente-Casares, P., S. Pina, M. Buti, R. Jardi, M. MartIn, S. Bofill-Mas, and R. Girones.** 2003. Hepatitis E virus epidemiology in industrialized countries. Emerg Infect Dis **9:**448-54.

27. **Drobeniuc, J., M. O. Favorov, C. N. Shapiro, B. P. Bell, E. E. Mast, A. Dadu, D. Culver, P. Iarovoi, B. H. Robertson, and H. S. Margolis.** 2001. Hepatitis E virus antibody prevalence among persons who work with swine. J Infect Dis **184:**1594-7.

28. **Emerson, S. U., D. Anderson, A. Arankalle, X. J. Meng, M. Purdy, G. G. Schlauder, and S. A. Tsarev.** 2004. *Hepevirus*. C.M. Fauquet, M.A. Mayo, J. Maniloff, U. Desselberger, and L.A. Ball. Elsevier/Academic Press. p. 851-855.

29. **Emerson, S. U., H. Nguyen, J. Graff, D. A. Stephany, A. Brockington, and R. H. Purcell.** 2004. In vitro replication of hepatitis E virus (HEV) genomes and of an HEV replicon expressing green fluorescent protein. J Virol **78:**4838-46.

30. **Emerson, S. U., and R. H. Purcell.** 2003. Hepatitis E virus. Rev Med Virol **13:**145-54.

31. **Emerson, S. U., and R. H. Purcell.** 2001. Recombinant vaccines for hepatitis E. Trends Mol Med **7:**462-6.

32. **Emerson, S. U., M. Zhang, X. J. Meng, H. Nguyen, M. St Claire, S. Govindarajan, Y. K. Huang, and R. H. Purcell.** 2001. Recombinant hepatitis E virus genomes infectious for primates: importance of capping and discovery of a cis-reactive element. Proc Natl Acad Sci U S A **98:**15270-5.

33. **Erker, J. C., S. M. Desai, G. G. Schlauder, G. J. Dawson, and I. K. Mushahwar.** 1999. A hepatitis E virus variant from the United States: molecular characterization and transmission in cynomolgus macaques. J Gen Virol **80 (Pt 3):**681-90.

34. **Fix, A. D., M. Abdel-Hamid, R. H. Purcell, M. H. Shehata, F. Abdel-Aziz, N. Mikhail, H. el Sebai, M. Nafeh, M. Habib, R. R. Arthur, S. U. Emerson, and G. T. Strickland.** 2000. Prevalence of antibodies to hepatitis E in two rural Egyptian communities. Am J Trop Med Hyg **62:**519-23.

35. **Fukuda, S., J. Sunaga, N. Saito, K. Fujimura, Y. Itoh, M. Sasaki, F. Tsuda, M. Takahashi, T. Nishizawa, and H. Okamoto.** 2004. Prevalence of antibodies to hepatitis E virus among Japanese blood donors: identification of three blood donors infected with a genotype 3 hepatitis E virus. J Med Virol **73:**554-61.

36. **Garkavenko, O., A. Obriadina, J. Meng, D. A. Anderson, H. J. Benard, B. A. Schroeder, Y. E. Khudyakov, H. A. Fields, and M. C. Croxson.** 2001. Detection and characterisation of swine hepatitis E virus in New Zealand. J Med Virol **65:**525-9.

37. **Ha, S. K., and C. Chae.** 2004. Immunohistochemistry for the detection of swine hepatitis E virus in the liver. J Viral Hepat **11:**263-7.

38. **Halbur, P. G., C. Kasorndorkbua, C. Gilbert, D. Guenette, M. B. Potters, R. H. Purcell, S. U. Emerson, T. E. Toth, and X. J. Meng.** 2001. Comparative pathogenesis of infection of pigs with hepatitis E viruses recovered from a pig and a human. J Clin Microbiol **39:**918-23.

39. **Haqshenas, G., F. F. Huang, M. Fenaux, D. K. Guenette, F. W. Pierson, C. T. Larsen, H. L. Shivaprasad, T. E. Toth, and X. J. Meng.** 2002. The putative capsid protein of the newly identified avian hepatitis E virus shares antigenic epitopes with that of swine and human hepatitis E viruses and chicken big liver and spleen disease virus. J Gen Virol **83:**2201-9.

40. **Haqshenas, G., and X. J. Meng.** 2001. Determination of the nucleotide sequences at the extreme 5' and 3' ends of swine hepatitis E virus genome. Arch Virol **146:**2461-7.

41. **Haqshenas, G., H. L. Shivaprasad, P. R. Woolcock, D. H. Read, and X. J. Meng.** 2001. Genetic identification and characterization of a novel virus related to human hepatitis E virus from chickens with hepatitis-splenomegaly syndrome in the United States. J Gen Virol **82:**2449-62.

42. **Hsieh, S. Y., X. J. Meng, Y. H. Wu, S. T. Liu, A. W. Tam, D. Y. Lin, and Y. F. Liaw.** 1999. Identity of a novel swine hepatitis E virus in Taiwan forming a monophyletic group with Taiwan isolates of human hepatitis E virus. J Clin Microbiol **37:**3828-34.

43. **Hsieh, S. Y., P. Y. Yang, Y. P. Ho, C. M. Chu, and Y. F. Liaw.** 1998. Identification of a novel strain of hepatitis E virus responsible for sporadic acute hepatitis in Taiwan. J Med Virol **55:**300-4.

44. **Huang, C. C., D. Nguyen, J. Fernandez, K. Y. Yun, K. E. Fry, D. W. Bradley, A. W. Tam, and G. R. Reyes.** 1992. Molecular cloning and sequencing of the Mexico isolate of hepatitis E virus (HEV). Virology **191:**550-8.

45. **Jameel, S., M. Zafrullah, M. H. Ozdener, and S. K. Panda.** 1996. Expression in animal cells and characterization of the hepatitis E virus structural proteins. J Virol **70:**207-16.

46. **Kabrane-Lazizi, Y., J. B. Fine, J. Elm, G. E. Glass, H. Higa, A. Diwan, C. J. Gibbs, Jr., X. J. Meng, S. U. Emerson, and R. H. Purcell.** 1999. Evidence for widespread infection of wild rats with hepatitis E virus in the United States. Am J Trop Med Hyg **61:**331-5.

47. **Kabrane-Lazizi, Y., X. J. Meng, R. H. Purcell, and S. U. Emerson.** 1999. Evidence that the genomic RNA of hepatitis E virus is capped. J Virol **73:**8848-50.

48. **Kabrane-Lazizi, Y., M. Zhang, R. H. Purcell, K. D. Miller, R. T. Davey, and S. U. Emerson.** 2001. Acute hepatitis caused by a novel strain of hepatitis E virus most closely related to United States strains. J Gen Virol **82:**1687-93.

49. **Kamel, M. A., H. Troonen, H. P. Kapprell, A. el-Ayady, and F. D. Miller.** 1995. Seroepidemiology of hepatitis E virus in the Egyptian Nile Delta. J Med Virol **47:**399-403.

50. **Kasorndorkbua, C., B. J. Thacker, P. G. Halbur, D. K. Guenette, R. M. Buitenwerf, R. L. Royer, and X. J. Meng.** 2003. Experimental infection of pregnant gilts with swine hepatitis E virus. Can J Vet Res **67:**303-6.

51. **Khuroo, M. S.** 1980. Study of an epidemic of non-A, non-B hepatitis. Possibility of another human hepatitis virus distinct from post-transfusion non-A, non-B type. Am J Med **68:**818-24.

52. **Khuroo, M. S., and M. Y. Dar.** 1992. Hepatitis E: evidence for person-to-person transmission and inability of low dose immune serum globulin from an Indian source to prevent it. Indian J Gastroenterol **11:**113-6.

53. **Khuroo, M. S., S. Kamili, and S. Jameel.** 1995. Vertical transmission of hepatitis E virus. Lancet **345:**1025-6.

54. **Khuroo, M. S., S. Kamili, and G. N. Yattoo.** 2004. Hepatitis E virus infection may be transmitted through blood transfusions in an endemic area. J Gastroenterol Hepatol **19:**778-84.

55. **Koonin, E. V., A. E. Gorbalenya, M. A. Purdy, M. N. Rozanov, G. R. Reyes, and D. W. Bradley.** 1992. Computer-assisted assignment of functional domains in the nonstructural polyprotein of hepatitis E virus: delineation of an additional group of positive-strand RNA plant and animal viruses. Proc Natl Acad Sci U S A **89:**8259-63.

56. **Korkaya, H., S. Jameel, D. Gupta, S. Tyagi, R. Kumar, M. Zafrullah, M. Mazumdar, S. K. Lal, L. Xiaofang, D. Sehgal, S. R. Das, and D. Sahal.** 2001.

The ORF3 protein of hepatitis E virus binds to Src homology 3 domains and activates MAPK. J Biol Chem **276:**42389-400.

57. **Krawczynski, K., R. Aggarwal, and S. Kamili.** 2000. Hepatitis E. Infect Dis Clin North Am **14:**669-87.
58. **Kumar, A., M. Beniwal, P. Kar, J. B. Sharma, and N. S. Murthy.** 2004. Hepatitis E in pregnancy. Int J Gynaecol Obstet **85:**240-4.
59. **Kumar, R. M., S. Uduman, S. Rana, J. K. Kochiyil, A. Usmani, and L. Thomas.** 2001. Sero-prevalence and mother-to-infant transmission of hepatitis E virus among pregnant women in the United Arab Emirates. Eur J Obstet Gynecol Reprod Biol **100:**9-15.
60. **Kuno, A., K. Ido, N. Isoda, Y. Satoh, K. Ono, S. Satoh, H. Inamori, K. Sugano, N. Kanai, T. Nishizawa, and H. Okamoto.** 2003. Sporadic acute hepatitis E of a 47-year-old man whose pet cat was positive for antibody to hepatitis E virus. Hepatol Res **26:**237-242.
61. **Li, T. C., Y. Suzaki, Y. Ami, T. N. Dhole, T. Miyamura, and N. Takeda.** 2004. Protection of cynomolgus monkeys against HEV infection by oral administration of recombinant hepatitis E virus-like particles. Vaccine **22:**370-7.
62. **Li, T. C., Y. Yamakawa, K. Suzuki, M. Tatsumi, M. A. Razak, T. Uchida, N. Takeda, and T. Miyamura.** 1997. Expression and self-assembly of empty virus-like particles of hepatitis E virus. J Virol **71:**7207-13.
63. **Longer, C. F., S. L. Denny, J. D. Caudill, T. A. Miele, L. V. Asher, K. S. Myint, C. C. Huang, W. F. Engler, J. W. LeDuc, and L. N. Binn.** 1993. Experimental hepatitis E: pathogenesis in cynomolgus macaques (Macaca fascicularis). J Infect Dis **168:**602-9.
64. **Maneerat, Y., E. T. Clayson, K. S. Myint, G. D. Young, and B. L. Innis.** 1996. Experimental infection of the laboratory rat with the hepatitis E virus. J Med Virol **48:**121-8.
65. **Matsubayashi, K., Y. Nagaoka, H. Sakata, S. Sato, K. Fukai, T. Kato, K. Takahashi, S. Mishiro, M. Imai, N. Takeda, and H. Ikeda.** 2004. Transfusion-transmitted hepatitis E caused by apparently indigenous hepatitis E virus strain in Hokkaido, Japan. Transfusion **44:**934-40.

66. **Matsuda, H., K. Okada, K. Takahashi, and S. Mishiro.** 2003. Severe hepatitis E virus infection after ingestion of uncooked liver from a wild boar. J Infect Dis **188:**944.

67. **Meng, J., M. Cong, X. Dai, J. Pillot, M. A. Purdy, H. A. Fields, and Y. E. Khudyakov.** 1999. Primary structure of open reading frame 2 and 3 of the hepatitis E virus isolated from Morocco. J Med Virol **57:**126-33.

68. **Meng, X. J., P. G. Halbur, J. S. Haynes, T. S. Tsareva, J. D. Bruna, R. L. Royer, R. H. Purcell, and S. U. Emerson.** 1998. Experimental infection of pigs with the newly identified swine hepatitis E virus (swine HEV), but not with human strains of HEV. Arch Virol **143:**1405-15.

69. **Meng, X. J., P. G. Halbur, M. S. Shapiro, S. Govindarajan, J. D. Bruna, I. K. Mushahwar, R. H. Purcell, and S. U. Emerson.** 1998. Genetic and experimental evidence for cross-species infection by swine hepatitis E virus. J Virol **72:**9714-21.

70. **Meng, X. J., R. H. Purcell, P. G. Halbur, J. R. Lehman, D. M. Webb, T. S. Tsareva, J. S. Haynes, B. J. Thacker, and S. U. Emerson.** 1997. A novel virus in swine is closely related to the human hepatitis E virus. Proc Natl Acad Sci U S A **94:**9860-5.

71. **Meng, X. J., B. Wiseman, F. Elvinger, D. K. Guenette, T. E. Toth, R. E. Engle, S. U. Emerson, and R. H. Purcell.** 2002. Prevalence of antibodies to hepatitis E virus in veterinarians working with swine and in normal blood donors in the United States and other countries. J Clin Microbiol **40:**117-22.

72. **Moaven, L., M. Van Asten, N. Crofts, and S. A. Locarnini.** 1995. Seroepidemiology of hepatitis E in selected Australian populations. J Med Virol **45:**326-30.

73. **Nishizawa, T., M. Takahashi, H. Mizuo, H. Miyajima, Y. Gotanda, and H. Okamoto.** 2003. Characterization of Japanese swine and human hepatitis E virus isolates of genotype IV with 99 % identity over the entire genome. J Gen Virol **84:**1245-51.

74. **Okamoto, H., M. Takahashi, T. Nishizawa, K. Fukai, U. Muramatsu, and A. Yoshikawa.** 2001. Analysis of the complete genome of indigenous swine hepatitis E virus isolated in Japan. Biochem Biophys Res Commun **289:**929-36.

75. **Okamoto, H., M. Takahashi, T. Nishizawa, R. Usui, and E. Kobayashi.** 2004. Presence of antibodies to hepatitis E virus in Japanese pet cats. Infection **32:**57-8.

76. **Payne, C. J., T. M. Ellis, S. L. Plant, A. R. Gregory, and G. E. Wilcox.** 1999. Sequence data suggests big liver and spleen disease virus (BLSV) is genetically related to hepatitis E virus. Vet Microbiol **68:**119-25.

77. **Pei, Y., and D. Yoo.** 2002. Genetic characterization and sequence heterogeneity of a canadian isolate of Swine hepatitis E virus. J Clin Microbiol **40:**4021-9.

78. **Pina, S., M. Buti, M. Cotrina, J. Piella, and R. Girones.** 2000. HEV identified in serum from humans with acute hepatitis and in sewage of animal origin in Spain. J Hepatol **33:**826-33.

79. **Pina, S., J. Jofre, S. U. Emerson, R. H. Purcell, and R. Girones.** 1998. Characterization of a strain of infectious hepatitis E virus isolated from sewage in an area where hepatitis E is not endemic. Appl Environ Microbiol **64:**4485-8.

80. **Reyes, G. R., M. A. Purdy, J. P. Kim, K. C. Luk, L. M. Young, K. E. Fry, and D. W. Bradley.** 1990. Isolation of a cDNA from the virus responsible for enterically transmitted non-A, non-B hepatitis. Science **247:**1335-9.

81. **Ritchie, S. J. R., C.** 1991. Hepatitis-splenomegaly syndrome in commercial egg-laying hens. Canadian Veterinary Journal **32:**500-501.

82. **Robinson, R. A., W. H. Burgess, S. U. Emerson, R. S. Leibowitz, S. A. Sosnovtseva, S. Tsarev, and R. H. Purcell.** 1998. Structural characterization of recombinant hepatitis E virus ORF2 proteins in baculovirus-infected insect cells. Protein Expr Purif **12:**75-84.

83. **Ropp, S. L., A. W. Tam, B. Beames, M. Purdy, and T. K. Frey.** 2000. Expression of the hepatitis E virus ORF1. Arch Virol **145:**1321-37.

84. **Schlauder, G. G., G. J. Dawson, J. C. Erker, P. Y. Kwo, M. F. Knigge, D. L. Smalley, J. E. Rosenblatt, S. M. Desai, and I. K. Mushahwar.** 1998. The sequence and phylogenetic analysis of a novel hepatitis E virus isolated from a

patient with acute hepatitis reported in the United States. J Gen Virol **79 (Pt 3):**447-56.

85. **Schlauder, G. G., S. M. Desai, A. R. Zanetti, N. C. Tassopoulos, and I. K. Mushahwar.** 1999. Novel hepatitis E virus (HEV) isolates from Europe: evidence for additional genotypes of HEV. J Med Virol **57:**243-51.

86. **Schlauder, G. G., B. Frider, S. Sookoian, G. C. Castano, and I. K. Mushahwar.** 2000. Identification of 2 novel isolates of hepatitis E virus in Argentina. J Infect Dis **182:**294-7.

87. **Schlauder, G. G., and I. K. Mushahwar.** 2001. Genetic heterogeneity of hepatitis E virus. J Med Virol **65:**282-92.

88. **Shivaprasad, H. L. W., P. R.** 1995. Presented at the Proceedings of Western Poultry Diseases Conference, Sacramento, CA, USA.

89. **Singh, S., A. Mohanty, Y. K. Joshi, D. Deka, S. Mohanty, and S. K. Panda.** 2003. Mother-to-child transmission of hepatitis E virus infection. Indian J Pediatr **70:**37-9.

90. **Soe, S., T. Uchida, K. Suzuki, K. Komatsu, J. Azumi, Y. Okuda, F. Iida, T. Shikata, T. Rikihisa, and K. Mizuno.** 1989. Enterically transmitted non-A, non-B hepatitis in cynomolgus monkeys: morphology and probable mechanism of hepatocellular necrosis. Liver **9:**135-45.

91. **Takahashi, M., T. Nishizawa, H. Miyajima, Y. Gotanda, T. Iita, F. Tsuda, and H. Okamoto.** 2003. Swine hepatitis E virus strains in Japan form four phylogenetic clusters comparable with those of Japanese isolates of human hepatitis E virus. J Gen Virol **84:**851-62.

92. **Takahashi, M., T. Nishizawa, and H. Okamoto.** 2003. Identification of a genotype III swine hepatitis E virus that was isolated from a Japanese pig born in 1990 and that is most closely related to Japanese isolates of human hepatitis E virus. J Clin Microbiol **41:**1342-3.

93. **Tam, A. W., M. M. Smith, M. E. Guerra, C. C. Huang, D. W. Bradley, K. E. Fry, and G. R. Reyes.** 1991. Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. Virology **185:**120-31.

94. **Tei, S., N. Kitajima, K. Takahashi, and S. Mishiro.** 2003. Zoonotic transmission of hepatitis E virus from deer to human beings. Lancet **362:**371-3.

95. **Thomas, D. L., R. W. Mahley, S. Badur, K. E. Palaoglu, and T. C. Quinn.** 1993. Epidemiology of hepatitis E virus infection in Turkey. Lancet **341:**1561-2.

96. **Thomas, D. L., P. O. Yarbough, D. Vlahov, S. A. Tsarev, K. E. Nelson, A. J. Saah, and R. H. Purcell.** 1997. Seroreactivity to hepatitis E virus in areas where the disease is not endemic. J Clin Microbiol **35:**1244-7.

97. **Ticehurst, J., L. L. Rhodes, Jr., K. Krawczynski, L. V. Asher, W. F. Engler, T. L. Mensing, J. D. Caudill, M. H. Sjogren, C. H. Hoke, Jr., and J. W. LeDuc.** 1992. Infection of owl monkeys (Aotus trivirgatus) and cynomolgus monkeys (Macaca fascicularis) with hepatitis E virus from Mexico. J Infect Dis **165:**835-45.

98. **Tien, N. T., H. T. Clayson, H. B. Khiem, P. K. Sac, A. L. Corwin, K. S. Myint, and D. W. Vaughn.** 1997. Detection of immunoglobulin G to the hepatitis E virus among several animal species in Vietnam. Am J Trop Med Hyg **57:**211.

99. **Tsarev, S. A., S. U. Emerson, T. S. Tsareva, P. O. Yarbough, M. Lewis, S. Govindarajan, G. R. Reyes, M. Shapiro, and R. H. Purcell.** 1993. Variation in course of hepatitis E in experimentally infected cynomolgus monkeys. J Infect Dis **167:**1302-6.

100. **Tsarev, S. A., T. S. Tsareva, S. U. Emerson, S. Govindarajan, M. Shapiro, J. L. Gerin, and R. H. Purcell.** 1994. Successful passive and active immunization of cynomolgus monkeys against hepatitis E. Proc Natl Acad Sci U S A **91:**10198-202.

101. **Tsarev, S. A., T. S. Tsareva, S. U. Emerson, M. K. Rippy, P. Zack, M. Shapiro, and R. H. Purcell.** 1995. Experimental hepatitis E in pregnant rhesus monkeys: failure to transmit hepatitis E virus (HEV) to offspring and evidence of naturally acquired antibodies to HEV. J Infect Dis **172:**31-7.

102. **Tyagi, S., H. Korkaya, M. Zafrullah, S. Jameel, and S. K. Lal.** 2002. The phosphorylated form of the ORF3 protein of hepatitis E virus interacts with its

non-glycosylated form of the major capsid protein, ORF2. J Biol Chem **277:**22759-67.

103. **Usmanov, R. K., M. S. Balaian, O. V. Dvoinikova, D. B. Alymbaeva, N. A. Zamiatina, A. Kazachkov Iu, and V. I. Belov.** 1994. [An experimental infection in lambs by the hepatitis E virus]. Vopr Virusol **39:**165-8.

104. **Wang, X., and S. Gillam.** 2001. Mutations in the GDD motif of rubella virus putative RNA-dependent RNA polymerase affect virus replication. Virology **285:**322-31.

105. **Wang, Y., H. Zhang, R. Ling, H. Li, and T. J. Harrison.** 2000. The complete sequence of hepatitis E virus genotype 4 reveals an alternative strategy for translation of open reading frames 2 and 3. J Gen Virol **81:**1675-86.

106. **Williams, T. P., C. Kasorndorkbua, P. G. Halbur, G. Haqshenas, D. K. Guenette, T. E. Toth, and X. J. Meng.** 2001. Evidence of extrahepatic sites of replication of the hepatitis E virus in a swine model. J Clin Microbiol **39:**3040-6.

107. **Withers, M. R., M. T. Correa, M. Morrow, M. E. Stebbins, J. Seriwatana, W. D. Webster, M. B. Boak, and D. W. Vaughn.** 2002. Antibody levels to hepatitis E virus in North Carolina swine workers, non-swine workers, swine, and murids. Am J Trop Med Hyg **66:**384-8.

108. **Wong, D. C., R. H. Purcell, M. A. Sreenivasan, S. R. Prasad, and K. M. Pavri.** 1980. Epidemic and endemic hepatitis in India: evidence for a non-A, non-B hepatitis virus aetiology. Lancet **2:**876-9.

109. **Wu, J. C., C. M. Chen, T. Y. Chiang, I. J. Sheen, J. Y. Chen, W. H. Tsai, Y. H. Huang, and S. D. Lee.** 2000. Clinical and epidemiological implications of swine hepatitis E virus infection. J Med Virol **60:**166-71.

110. **Wu, J. C., C. M. Chen, T. Y. Chiang, W. H. Tsai, W. J. Jeng, I. J. Sheen, C. C. Lin, and X. J. Meng.** 2002. Spread of hepatitis E virus among different-aged pigs: two-year survey in Taiwan. J Med Virol **66:**488-92.

111. **Yazaki, Y., H. Mizuo, M. Takahashi, T. Nishizawa, N. Sasaki, Y. Gotanda, and H. Okamoto.** 2003. Sporadic acute or fulminant hepatitis E in Hokkaido, Japan, may be food-borne, as suggested by the presence of hepatitis E virus in pig liver as food. J Gen Virol **84:**2351-7.

112. **Yoo, D., P. Willson, Y. Pei, M. A. Hayes, A. Deckert, C. E. Dewey, R. M. Friendship, Y. Yoon, M. Gottschalk, C. Yason, and A. Giulivi.** 2001. Prevalence of hepatitis E virus antibodies in Canadian swine herds and identification of a novel variant of swine hepatitis E virus. Clin Diagn Lab Immunol **8:**1213-9.

113. **Zafrullah, M., M. H. Ozdener, R. Kumar, S. K. Panda, and S. Jameel.** 1999. Mutational analysis of glycosylation, membrane translocation, and cell surface expression of the hepatitis E virus ORF2 protein. J Virol **73:**4074-82.

114. **Zafrullah, M., M. H. Ozdener, S. K. Panda, and S. Jameel.** 1997. The ORF3 protein of hepatitis E virus is a phosphoprotein that associates with the cytoskeleton. J Virol **71:**9045-53.

115. **Zhang, M., S. U. Emerson, H. Nguyen, R. E. Engle, S. Govindarajan, J. L. Gerin, and R. H. Purcell.** 2001. Immunogenicity and protective efficacy of a vaccine prepared from 53 kDa truncated hepatitis E virus capsid protein expressed in insect cells. Vaccine **20:**853-7.

116. **Zhang, M., R. H. Purcell, and S. U. Emerson.** 2001. Identification of the 5' terminal sequence of the SAR-55 and MEX-14 strains of hepatitis E virus and confirmation that the genome is capped. J Med Virol **65:**293-5.

Chapter 2

Heterogeneity and Seroprevalence of the Newly Identified Avian Hepatitis E Virus from Chickens in the United States

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ABSTRACT

We recently identified and characterized a novel virus, designated avian hepatitis E virus (avian HEV), from chickens with hepatitis-splenomegaly syndrome (HS syndrome) in the United States. Avian HEV is genetically related to, but distinct from human and swine HEVs. To determine the extent of genetic variations and the seroprevalence of avian HEV infection in chicken flocks, we genetically identified and characterized 11 additional avian HEV isolates from chickens with HS syndrome and assessed the prevalence of avian HEV antibodies from a total of 1,276 chickens of different ages and breeds from 76 different flocks in five states (CA, CO, CT, VA, and WI). An ELISA assay using a truncated recombinant avian HEV ORF2 protein as antigen was developed and used to determine avian HEV seroprevalence. About 71% chicken flocks and 30% chickens tested in the study were positive for antibodies to avian HEV. The seroprevalence rate was about 17% in chickens of less than 18 weeks old, but about 36% in adult chickens. With an RT-PCR assay, we tested 21 bile samples from chickens with HS syndrome in CA, CT, NY, and WI for the presence of avian HEV RNA. Twelve of the 21 bile samples were positive for 30-35 nm HEV-like virus particles by electron microscopy (EM). Eleven of the 12 EM-positive bile samples and 6 of the 9 EM-negative bile samples were positive for avian HEV RNA by RT-PCR. The sequences of a 372-bp region within the helicase gene of 11 avian HEV isolates were determined. Sequence analyses revealed that the 11 field isolates of avian HEV shared 78-100% nucleotide sequence identities with each other, 79-88% identities with the prototype avian HEV, 76-80% identities with chicken big liver and spleen disease virus (BLSV) and 56-61% identities with other known strains of human and swine HEV. The data from this study

indicated that, like swine and human HEVs, avian HEV isolates were also heterogeneic, and that avian HEV infection was enzootic in chicken flocks in the United States.

INTRODUCTION

Hepatitis E is an important public health disease in many developing countries and endemic in some industrialized countries including the United States (1, 16, 20, 24, 30-33, 38, 40, 41, 44, 45). The disease is generally transmitted by fecal-oral route through contaminated water (1, 10, 22, 36). Hepatitis E virus (HEV), the causative agent of hepatitis E, is a positive-sense, single-stranded RNA virus (1, 7, 16, 18). The HEV genome contains three open reading frames (ORFs): ORF1 encodes viral non-structural proteins, ORF2 encodes the putative capsid protein and ORF3 encodes a small protein that may be involved in virus replication (1, 42, 44, 45, 49).

Seroepidemiological studies revealed that anti-HEV antibodies are present in numerous animal species including pigs, rodents, chickens, dogs, cows, sheep and goats from both developing and industrialized countries (2, 3, 8, 19, 23, 29-31, 48; N. T. Tien, H. T. Clayson, H. B. Khiem, P. K. Sac, A. L. Corwin, K. S. Myint, and D. W. Vaughn. Abstract, Am. J. Trop. Med. Hyg. **57:**211, 1997; S. A. Tsarev, M. P. Shrestha, J. He, R. M. Scott, D. W. Vaughn, E. T. Clayson, S. Gigliotti, C. F. Longer, and B. L. Innis. Abstract, Am. J. Trop. Med. Hyg. **59:**242, 1998), suggesting that these animal species have been exposed to HEV-like virus. The first animal strain of HEV, swine HEV, was identified and characterized in 1997 from a pig in the United States (26). The prototype swine HEV strain is genetically more closely related to two U.S. human HEV strains than to HEV strains from other geographic regions (7, 26-28, 38, 39). Since 1997, more swine HEV strains have been genetically identified from pigs in the United States, Taiwan, The Netherlands, and Japan (17, 18, 34, 36, 43, 47, 48). Phylogenetic analyses revealed that, like human HEV (25, 38, 39, 44, 45), these swine HEV strains from different geographic regions are also heterogeneic (17, 18, 34, 36, 43, 47, 48). Interspecies HEV transmission has been documented: a U.S. human HEV strain infected pigs and the prototype swine HEV infected non-human primates (11, 28, 46). Recent seroepidemiological studies indicated that individuals working with swine have increased risks of HEV infection (6, 15, 21, 29, 32). Taken together, these data strongly suggest that HEV infection is zoonotic and that there exist animal reservoirs for HEV.

More recently, another animal strain of HEV, designated avian HEV, has been identified and characterized from chickens with hepatitis-splenomegaly (HS) syndrome in the United States (13, 14). The newly discovered avian HEV is genetically related to, but distinct from, other known HEV strains. Unlike swine HEV, which only causes subclinical infection in pigs, avian HEV is associated with HS syndrome in chickens (13, 14). HS syndrome in chickens was first described in 1991 in western Canada and is now recognized in eastern Canada and the United States (37). HS syndrome is characterized by above-normal mortality in laying hens at age of 30-72 weeks. Infected birds usually have red fluid in their abdomens and enlarged livers and spleens (37). Microscopically, liver lesions vary from multifocal patches to areas of extensive hepatic necrosis and hemorrhage (13, 37). We have now successfully reproduced HS syndrome in adult specific-pathogen-free (SPF) chickens by infection with avian HEV (F. F. Fang et al., unpublished data). Since avian HEV is a novel virus and only one strain of avian HEV has been identified in the United States thus far, the molecular and serological epidemiology of this new virus is not known. In this study, we genetically characterized 11 additional avian HEV isolates from chickens with HS syndrome in the United States and assessed the prevalence of anti-avian HEV antibodies in chickens of different ages from five different states.

MATERIALS AND METHODS

Clinical specimens. Bile samples used for genetic identification and characterization of avian HEV isolates in this study were collected from 21 chickens with HS syndrome in California, Connecticut, New York, and Wisconsin from 1993 to 2001 (Table 1). Twelve samples were positive and 9 samples were negative, by electron microscopy (EM) examination for 30-35 nm avian HEV-like virus particles and by EM.

Serum samples used for the seroprevalence study were collected from 1,276 chickens of different ages from 76 chicken flocks located in California, Colorado, Connecticut, Virginia, and Wisconsin (Table 2).

ELISA assay to detect anti-HEV in chickens. A truncated recombinant ORF2 protein of avian HEV was expressed in *E. coli.*, and purified by affinity chromatography (14).

The purified avian HEV ORF2 truncated protein was used as the antigen to standardize an enzyme-linked immunosorbent assay (ELISA) to detect anti-HEV immunoglobulin G (IgG) antibodies in chickens essentially as previously described for the ELISAs used to detect anti-HEV antibodies in humans and swine (26-29,32). The purified ORF2 antigen of avian HEV was coated onto 96-well plates. An HRP-conjugated rabbit anti-chicken IgG (Sigma Chemical Co., St. Louis, MO) was used as the secondary antibody. The cut-off value was determined on the basis of the OD values of the 85 adult chicken sera collected from a commercial SPF flock (Charles River Laboratory Inc., Wilmington, MA) and from two high health university research flocks. The cut-off value was set conservatively at 0.30, which is about 6 standard deviations (SDs) above the mean OD value of the normal chicken sera. All sera from 76 chicken flocks in the five states were tested in duplicate at a dilution of 1:100 in 0.05% Tween-PBS blocking buffer containing 5% non-fat dried milk and 5% goat sera. Sera from SPF chickens were used for negative controls, and convalescent sera from SPF chickens experimentally infected with avian HEV were included as positive controls. In general, the OD values for the positive sera from experimentally infected chickens were above 1.0, whereas those from naturally infected birds were about 0.5.

RT-PCR assay for detection of field isolates of avian HEV. To identify field isolates of avian HEV, it was necessary to develop a RT-PCR assay capable of detecting avian HEV strains with significant genetic variation. Our previous studies showed that the helicase gene is the most conserved between avian HEV and other known strains of HEV (13, 14); therefore primers for RT-PCR were targeted in the helicase gene region. A short stretch of the helicase gene sequence of the Australian chicken big liver and spleen disease virus (BLSV) has been reported, and it shared about 80% nucleotide sequence identity with avian HEV (35). The primers were selected from conserved helicase gene regions based on a sequence alignment of BLSV (35), the prototype strain of avian HEV (12) and other HEV strains. The primer sequences were of BLSV origin: forward primer (B1), 5'- GCTAGGCGACCCGCACCAGAT-3'; reverse primer (B2), 5' GGTTAGCGCAACAATAGCATG-3'. The RT-PCR assay with this set of primers was able to detect the prototype strain of avian HEV, and was therefore used to identify

additional field isolates of avian HEV. Briefly, total RNAs were extracted with TriZol Reagent® (NALGENE) from 100 µl of bile samples from each of the 21 chickens with HS syndrome. Total RNAs were resuspended in DNase- and RNase-free water. Reverse transcription was performed at 42 ºC for 60 min with Superscript II reverse transcriptase® (GIBCO-BRL) using the reverse primer B2. Five µl of the resulting cDNA was amplified using AmpliTaq gold DNA polymerase® (Perkin-Elmer). The PCR parameters included a denaturation at 95 ºC for 9 min, followed by 39 cycles of denaturation for 1 min at 94 ºC, annealing for 1 min at 42 ºC, extension for 1 min at 72 ºC, and a final incubation at 72 ºC for 7 min. Negative and positive controls were included in each set of RT-PCR reactions. The negative control was a water sample treated the same way as the bile samples. The positive control was a fecal stock of avian HEV with a titer of about 10^4 genomic equivalent (GE) per ml.

Nucleotide sequencing. The expected PCR products of field isolates of avian HEV were purified using the glassmilk procedure with a GENECLEAN kit® (Bio 101 Inc. Carlsbad, CA.). PCR products amplified from 11 chicken bile samples originated from chicken flocks in four states were directly sequenced at the Virginia Tech DNA Sequencing Facility. Sequences of the PCR products were determined for both DNA strands.

Development of a more sensitive RT-PCR assay for the detection of avian HEV. The RT-PCR assay described above was primarily based on the nucleotide sequence of BLSV and the prototype strain of avian HEV in helicase gene (35) (12). Therefore, it was possible that more genetically divergent field isolates of avian HEV would not be detected by the RT-PCR assay. With the availability of the sequence information from 11 additional field isolates of avian HEV, we attempted to develop a more sensitive RT-PCR assay that is capable of detecting genetically divergent field strains of avian HEV. Briefly, the resulting sequences of the 11 field isolates of avian HEV and the prototype strain of avian HEV were aligned, and a set of degenerate primers were designed based on the sequence alignment: forward primer (helicase F), 5'-TGGCGCACC(T)GT(A)TTCC(T)CACCG-3', reverse primer (helicase R), 5'-CCTCA(G)TGGACCGTA(T)ATCGACCC-3'. The parameters for the RT-PCR assay

with the degenerate primers helicase F and helicase R included a denaturation at 95 ºC for 9 min, followed by 39 cycles of denaturation for 50 sec at 94 ºC, annealing for 50 sec at 46 ºC, extension for 50 sec at 72 ºC, and a final incubation at 72 ºC for 7 min. Positive and negative controls were the same as the ones described before.

Sequence and phylogenetic analyses. The primer sequences used to amplify the avian HEV isolates were excluded from the sequence and phylogenetic analyses. The resulting 330 bp sequences of the helicase genes of the 11 field isolates of avian HEV were analyzed and compared with the corresponding regions of the prototype avian HEV, BLSV and other known strains of swine and human HEVs available in the GenBank database by the MacVector computer program (Oxford Molecular Inc.). Phylogenetic analysis was conducted with the aid of PAUP program of 4.0 beta version (David Swofford, Smithsonian Institute, Washington, DC, distributed by Sinauer Associate Inc. Sunderland, MA). Heuristic search and mid-point rooting option with 1,000 replicates of random adding was used to produce a phylogenetic tree.

The geographic origins and the GenBank accession numbers of the nucleotide sequences of the HEV strains used in the phylogenetic and sequence analyses are as follows: JRA1 (Japan, AP003430), SWJ570 (Japan, AB073912), Swine HEV (USA, AF082843), US1 (USA, AF060668), US2 (USA, AF060669), T1 (China, AJ272108), Burma (Burma, M73218), Myanmar (Myanmar, D10330), Hyderabad (India, AF076239), Madras (India, X99441), Nepal (Nepal, AF020486), hev037 (India, X98292), Xinjiang (China, D11092), Uigh179 (China, D11093), Hetian (China, L08816), Sar-55 (Pakistan, M80581), Mexico (Mexico, M74506).

Nucleotide sequence accession numbers. The sequences of the helicase gene region of the 11 avian HEV isolates have been deposited in the GenBank database with accession numbers AF531898 through AF531908.

RESULTS AND DISCUSSION

Avian HEV is enzootic in chicken flocks in the United States. The prevalence of anti-HEV in commercial pig herds in the United States has been reported (26, 29).

Seropositivity for swine HEV in pigs is age-dependent: most pigs younger than 2 months of age were seronegative whereas the majority of pigs older than 3 months of age were seropositive (26, 29). In this study, we tested 1,276 serum samples from chickens of various ages in five states for the presence of antibodies to avian HEV (Table 2). The percentage of seropositive chickens varied greatly from flock to flock, ranging from 0% to 100%. Fifty-four of the 76 flocks (71%) tested were positive for anti-HEV IgG. Similar to swine HEV, the prevalence of avian HEV antibodies in adult chickens (older than 18 weeks of age) was higher than that in younger ones: about 17% of chickens less than 18 weeks of age were seropositive, whereas about 36% of adult chickens were seropositive. However, the age-related difference in avian HEV seropositivity in chickens is less definitive than that previously observed in swine (data not shown). Several adult chicken flocks were also found to be negative for antibodies to avian HEV. It is possible that these negative flocks may have better biosecurity measures that could prevent transmission of avian HEV. The antibodies to avian HEV detected in some 1-day-old chicks are likely maternal antibodies, which usually wane at about 3 weeks of age. So far, avian HEV was only isolated from chickens in the United States. The BLSV identified in Australia is genetically and antigenically related to HEV (12, 13, 14), and it is likely that the big liver and spleen disease in Australia (4, 5, 12, 35) and the HS syndrome in North America (13, 14, 37) are caused by variant strains of the same virus.

Detection of Avian HEV by RT-PCR. Twenty-one bile samples from chickens with HS syndrome (increased mortality and decreased egg production) were examined for avian HEV-like virus particles by EM and for avian HEV RNA by RT-PCR. Twelve of them were positive for 30-35 nm virus particles by EM. RT-PCR results with the B1/B2 primers primarily based on BLSV sequence showed that 7 of the 12 EM-positive bile samples and 4 of the 9 EM-negative bile samples were positive for avian HEV RNA (Table 1). The bile samples tested negative with the B1/B2 primers were further tested by a more sensitive RT-PCR assay with degenerate primers helicase F/R based on the sequence alignment of 11 avian HEV isolates and the prototype avian HEV. Additional 4 EM-positive and 2 EM-negative bile samples turned out to be positive for avian HEV RNA (Table 1). The 6 additional positive bile samples detected by the more sensitive RT-

PCR assay with the degenerate primers were confirmed by sequencing (data not shown). Overall, 11 of 12 EM-positive and 6 of 9 EM-negative bile samples from chickens with HS syndrome were positive for avian HEV RNA by RT-PCR, indicating that RT-PCR is a more sensitive method to detect avian HEV than EM. The RT-PCR assay developed in this study should be very useful for future avian HEV studies.

Field isolates of avian HEV are genetically heterogenic. It has been reported that swine and human HEV isolates from different geographic regions are genetically heterogenic (17, 39, 44). The extent of genetic variation of avian HEV is not known since only one strain of avian HEV has been characterized. The PCR products from the 11 avian HEV isolates amplified by B1/B2 primers were sequenced. The 330 bp sequences within the helicase genes were analyzed and compared to each other and to the corresponding region of the prototype avian HEV, BLSV and other known strains of HEV. Sequence analyses revealed that the avian HEV isolates identified in this study shared 78-100% nucleotide sequence identities with each other, 79-88% identities with the prototype avian HEV, 76-80% identities with BLSV and 56-61% identities with other known strains of human and swine HEVs (Table 3.). At the amino acid sequence level, the 11 avian HEV isolates identified in this study displayed 90-100% sequence identities with each other, 89-98% identities with the prototype avian HEV, 78-79% identities with BLSV and 55-62% identities with other known strains of human and swine HEVs.

Phylogenetic analyses revealed that all the U.S. avian HEV isolates identified in this study clustered together with the prototype avian HEV and BLSV but distinct from other known strains of HEV (Fig. 1). Minor branches, indicating heterogeneity, also exist among avian HEV isolates. Whether avian HEV represents a fifth HEV genotype or belongs to a separate genus remains to be determined.

An increasing amount of data suggested that HEV infection is zoonotic. Recently it has been reported that swine veterinarians (29, 32) and other pig handlers (6) may be at higher risk of HEV infection than normal blood donors. Therefore, it will be important to determine if avian HEV infection is also zoonotic, and if chicken handlers have a higher risk of HEV infection as swine handlers do.

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REFERENCES

1. **Aggarwal, R. and K. Krawczynski.** 2000. Hepatitis E: an overview and recent advances in clinical and laboratory research. J. Gastroenterol. Hepatol. **15**:9-20.
2. **Arankalle, V. A., M. K. Goverdhan, and K. Banerjee.** 1994. Antibodies against hepatitis E virus in Old World monkeys. J. Viral. Hepat. **1**:125-129.
3. **Clayson, E. T., B. L. Innis, K. S. Myint, S. Narupiti, D. W. Vaughn, S. Giri, P. Ranabhat, and M. P. Shrestha.** 1995. Detection of hepatitis E virus infections among domestic swine in the Kathmandu Valley of Nepal. Am. J. Trop. Med. Hyg. **53**:228-232.
4. **Crerar, S. K. and G. M. Cross.** 1994. The experimental production of big liver and spleen disease in broiler breeder hens. Aust. Vet. J. **71**: 414-417.
5. **Crerar, S. K. and G. M. Cross.** 1994. Epidemiological and clinical investigations into big liver and spleen disease of broiler breeder hens. Aust. Vet. J. 71: 410-413.
6. **Drobeniuc, J., M. O. Favorov, C. N. Shapiro, B. P. Bell, E. E. Mast, A. Dadu, D. Culver, P. Iarovoi, B. H. Robertson, and H. S. Margolis.** 2001. Hepatitis E virus antibody prevalence among persons who work with swine. J. Infect. Dis. **184**: 1594-1597.
7. **Erker, J. C., S. M. Desai, G. G. Schlauder, G. J. Dawson, and I. K, Mushahwar.** 1999. A hepatitis E virus variant from the United States: molecular characterization and transmission in cynomolgus macaques. J. Gen. Virol. **80**:681-690.
8. **Favorov, M. O., M. Y. Kosoy, S. A. Tsarev, J. E. Childs, and H. S. Margolis**. 2000. Prevalence of antibody to hepatitis E virus among rodents in the United States. J. Infect. Dis. **181**: 449-455.
9. **Garkavenko, O., A. Obriadina, J. Meng, D. A. Anderson, H. J. Benard, B. A. Schroeder, Y. E. Khudyakov, H. A. Fields, and M. C. Croxson.** 2001. Detection and characterization of swine hepatitis E virus in New Zealand. J. Med. Virol. **65**:525-529.
10. **Grimm, A. C. and G. Shay Fout.** 2002. Development of a molecular method to identify hepatitis E virus in water. J. Virol. Methods. **101**: 175-188.

11. **Halbur, P. G., C. Kasorndorkbua, C. Gilbert, D. Guenette, M. B. Potters, R. H. Purcell, S. U. Emerson, T. E. Toth, and X. J. Meng**. 2001. Comparative pathogenesis of infection of pigs with hepatitis E viruses recovered from a pig and a human. J. Clin. Microbiol. **39**: 918-923.
12. **Handlinger, J. H. and W. Williams.** 1988. An egg drop associated with splenomegaly in broiler breeders. Avian Dis. **32** (4): 773-778.
13. **Haqshenas, G., H. L. Shivaprasad, P. R. Woolcock, D. H. Read, and X. J. Meng**. 2001. Genetic identification and characterization of a novel virus related to human hepatitis E virus from chickens with hepatitis-splenomegaly syndrome in the United States. J. Gen. Virol. **82**:2449-2462.
14. **Haqshenas, G., F. F. Huang, M. Fenaux, D. K. Guenette, F. W. Pierson, C. T. Larsen, H. L. Shivaprasad, T. E. Toth, and X. J. Meng.** 2002. The putative capsid protein of the newly identified avian hepatitis E virus shares antigenic epitopes with that of swine and human hepatitis E viruses and the chicken big liver and spleen disease virus. J. Gen. Virol. **83**:2201-2209.
15. **Hsieh, S. Y., X. J. Meng, Y. H. Wu, S. T. Liu, A. W. Tam, D. Y. Lin, and Y. F. Liaw**. 1999. Identity of a novel swine hepatitis E virus in Taiwan forming a monophyletic group with Taiwan isolates of human hepatitis E virus. J. Clin. Microbiol. **37**: 3828-3834.
16. **Huang, C. C., D. Nguyen, J. Fernandez, K. Y. Yun, K. E. Fry, D. W. Bradley, A. W. Tam, and G. R. Reyes**. 1992. Molecular cloning and sequencing of the Mexico isolate of hepatitis E virus (HEV). Virology. **191**: 550-558.
17. **Huang, F. F., G. Haqshenas, D. K. Guenette, P. G. Halbur, S. K. Schommer, F. W. Pierson, T. E. Toth, and X. J. Meng.** 2002**.** Detection by reverse transcription-PCR and genetic characterization of field isolates of swine hepatitis E virus from pigs in different geographic regions of the United States. J. Clin. Microbiol. **40**:1326-1332.
18. **Kabrane-Lazizi, Y., X. J. Meng, R. H. Purcell, and S. U. Emerson**. 1999. Evidence that the genomic RNA of hepatitis E virus is capped. J. Virol. **73**:8848-8850.

19. **Kabrane-Lazizi, Y., J. B. Fine, J. Elm, G. E. Glass, H. Higa, A. Diwan, C. J. Jr. Gibbs, X. J. Meng, S. U. Emerson, and R. H. Purcell**. 1999. Evidence for widespread infection of wild rats with hepatitis E virus in the United States. Am. J. Trop. Med. Hyg. **61**:331-335.
20. **Kabrane-Lazizi, Y., M. Zhang, R. H. Purcell, K. D. Miller, R. T. Davey, and S. U. Emerson**. 2001. Acute hepatitis caused by a novel strain of hepatitis E virus most closely related to United States strains. J. Gen. Virol. **82**:1687-1693.
21. **Karetnyi, Y.V., M. J. Gilchrist, and S. J. Naides.** 1999. Hepatitis E virus infection prevalence among selected populations in Iowa. J. Clin. Virol. **14:** 51-55.
22. **Kasorndorkbua, C., P. G. Halbur, P. J. Thomas, D. K. Guenette, T. E. Toth, and X. J. Meng.** 2002. Use of a swine bioassay and a RT-PCR assay to assess the risk of transmission of swine hepatitis E virus in pigs. J. Virol. Methods. **101**:71-78.
23. **Maneerat, Y., E. T. Clayson, K. S. Myint, G. D. Young, and B. L. Innis.** 1996. Experimental infection of the laboratory rat with the hepatitis E virus. J. Med. Virol. **48**:121-128.
24. **Mast, E. E., I. K. Kuramoto, M. O. Favorov, V. R. Schoening, B. T. Burkholder, C. N. Shapiro, and P. V. Holland.** 1997. Prevalence of and risk factors for antibody to hepatitis E virus seroreactivity among blood donors in Northern California. J. Infect. Dis. **176**:34-40.
25. **Meng, J., J. Pillot, X. Dai, H. A. Fields, and Y. E. Khudyakov.** 1998. Neutralization of different geographic strains of the hepatitis E virus with anti-hepatitis E virus-positive serum samples obtained from different sources. Virology. **249**:316-324**.**
26. **Meng, X.J., R.H. Purcell, P.G. Halbur, J.R. Lehman, D.M. Webb, T.S. Tsareva, J.S. Haynes, B.J. Thacker, and S.U. Emerson**. 1997. A novel virus in swine is closely related to the human hepatitis E virus. Proc. Natl. Acad. Sci. USA **94**: 9860-9865.
27. **Meng, X.J., P.G. Halbur, J.S. Haynes, T.S. Tsareva, J.D. Bruna, R.L. Royer, R.H. Purcell, and S.U. Emerson**. 1998. Experimental infection of pigs with the

newly identified swine hepatitis E virus (swine HEV), but not with human strains of HEV. Arch. Virol. **143**:1405-1415.

28. **Meng, X.J., P.G. Halbur, M.S. Shapiro, S. Govindarajan, J.D. Bruna, I.K. Mushahwar, R.H. Purcell, and S.U. Emerson**. 1998. Genetic and experimental evidence for cross-species infection by the swine hepatitis E virus. J. Virol. **72**:9714-9721.
29. **Meng, X.J., S. Dea, R.E. Engle, R. Friendship, Y.S. Lyoo, T. Sirinarumitr, K. Urairong, D. Wang, D. Wong, D. Yoo, Y. Zhang, R.H. Purcell, and S.U. Emerson**. 1999. Prevalence of antibodies to the hepatitis E virus in pigs from countries where hepatitis E is common or is rare in the human population. J. Med. Virol. **58**:297-302.
30. **Meng, X.J**. 2000. Zoonotic and xenozoonotic risks of hepatitis E virus. Infect. Dis. Rev. **2**:35-41.
31. **Meng, X.J**. 2000. Novel strains of hepatitis E virus identified from humans and other animal species: Is hepatitis E a zoonosis? J. Hepatol. **33**:842-845.
32. **Meng, X.J., B. Wiseman, F. Elvinger, D. Guenette, T.E. Toth, R.E. Engle, S.U. Emerson, and R.H. Purcell.** 2002. Prevalence of antibodies to hepatitis E virus in veterinarians working with swine and in normal blood donors in the United States and other countries. J. Clin. Microbiol. **40**:117-122.
33. **Meng, X.J**. 2002. Swine hepatitis E virus: cross-species infection and risk in xenotransplantation. Curr. Topics Microbiol. Immunol. In press.
34. **Okamoto, H., M. Takahashi, T. Nishizawa, K. Fukai, U. Muramatsu, and A. Yoshikawa.** 2001. Analysis of the complete genome of indigenous swine hepatitis E virus isolated in Japan. Biochem. Biophys. Res. Commun. **289**:929-936.
35. **Payne, C. J., T. M. Ellis, S. L. Plant, A. R. Gregory, and G. E. Wilcox.** 1999. Sequence data suggests big liver and spleen disease virus (BLSV) is genetically related to hepatitis E virus. Vet. Microbiol. **68**:119-125.
36. **Pina S., M. Buti, M. Cotrina, J. Piella, and R. Girones**. 2000. HEV identified in serum from humans with acute hepatitis and in sewage of animal origin in Spain. J. Hepatol. **33**:826-833.

37. **Riddell, C.** 1997. Hepatitis-splenomegaly syndrome. In Diseases of poultry. Edited by B. W. Calnek, H. J. Barnes, C.W. Beard, L. R. McDougald and Y. M. Saif, Iowa State University Press. p1041.

38. **Schlauder, G. G., G. J. Dawson, J. C. Erker, P. Y. Kwo, M. F. Knigge, D. L. Smalley, J. E. Rosenblatt, S. M. Desai, and I. K. Mushahwar**. 1998. The sequence and phylogenetic analysis of a novel hepatitis E virus isolated from a patient with acute hepatitis reported in the United States. J. Gen. Virol. **79**:447-456.

39. **Schlauder, G. G. and I. K. Mushahwar**. 2001. Genetic heterogeneity of hepatitis E virus. J. Med. Virol. **65**:282-292.

40. **Takahashi, K., K. Iwata, N. Watanabe, T. Hatahara, Y. Ohta, K. Baba, and S. Mishiro**. 2001. Full-genome nucleotide sequence of a hepatitis E virus strain that may be indigenous to Japan. Virology. **287**:9-12.

41. **Thomas, D. L., P. O. Yarbough, D. Vlahov, S. A. Tsarev, K. E. Nelson, A. J. Saah, and R. H. Purcell**. 1997. Seroreactivity to hepatitis E virus in areas where the disease is not endemic. J. Clin. Microbiol. **35**:1244-1247.

42. **Tsarev, S. A., S. U. Emerson, G. R. Reyes, T. S. Tsareva, L. J. Legters, I. A. Malik, M. Iqbal, and R. H. Purcell.** 1992. Characterization of a prototype strain of hepatitis E virus. Proc. Natl. Acad. Sci. U S A. **89**:559-563.

43. **Van der Poel, W. H., F. Verschoor, R. van der Heide, M. I. Herrera, A. Vivo, M. Kooreman, and A. M. de Roda Husman.** 2001. Hepatitis E virus sequences in swine related to sequences in humans, The Netherlands. Emerg. Infect. Dis. **7**:970-976**.**

44. **Wang, Y., R. Ling, J. C. Erker, H. Zhang, H. Li, S. Desai, I. K. Mushahwar, and T. J. Harrison**. 1999. A divergent genotype of hepatitis E virus in Chinese patients with acute hepatitis. J. Gen. Virol. **80**:169-177.

45. **Wang, Y., H. Zhang, R. Ling, H. Li, and T. J. Harrison**. 2000. The complete sequence of hepatitis E virus genotype 4 reveals an alternative strategy for translation of open reading frames 2 and 3. J. Gen. Virol. **81**:1675-1686.

46. **Williams, T. P., C. Kasorndorkbua, P. G. Halbur, G. Haqshenas, D. K. Guenette, T. E. Toth, and X. J. Meng.** 2001. Evidence of extrahepatic sites of

replication of the hepatitis E virus in a swine model. J. Clin. Microbiol. **39**:3040-3046.

47. **Wu, J. C., C. M. Chen, T. Y. Chiang, W. H. Tsai, W. J. Jeng, I. J. Sheen, C. C. Lin, and X. J. Meng.** 2002. Spread of hepatitis E virus among different-aged pigs: two-year survey in Taiwan. J. Med. Virol. **66**:488-492.
48. **Yoo D., P. Willson, Y. Pei, M. A. Hayes, A. Deckert, C. E. Dewey, R. M. Friendship, Y. Yoon, M. Gottschalk, C. Yason, and A. Giulivi.** 2001. Prevalence of hepatitis E virus antibodies in Canadian swine herds and identification of a novel variant of swine hepatitis E virus. Clin. Diagn. Lab. Immunol. **8**: 1213-1219.
49. **Zafrullah, M., M. H. Ozdener, S. K. Panda, and S. Jameel.** 1997. The ORF3 protein of hepatitis E virus is a phosphopretein that associated with the cytoskeleton. J. Virol. **71**: 9045-9053.

Fig. 1

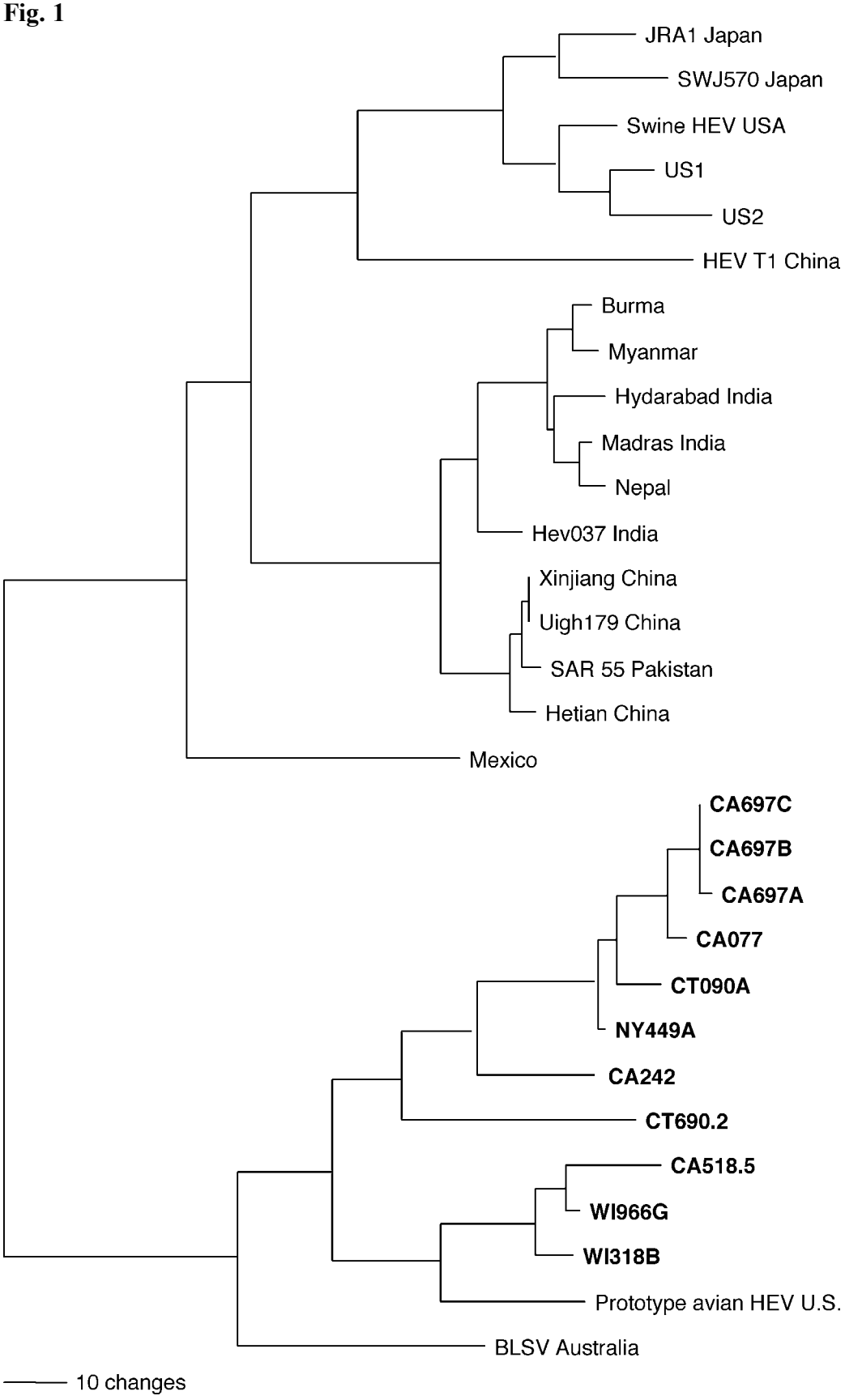
JRA1 Japan
SWJ570 Japan
Swine HEV USA
US1
US2
HEV T1 China
Burma
Myanmar
Hydarabad India
Madras India
Nepal
Hev037 India
Xinjiang China
Uigh179 China
SAR 55 Pakistan
Hetian China
Mexico
CA697C
CA697B
CA697A
CA077
CT090A
NY449A
CA242
CT690.2
CA518.5
WI966G
WI318B
Prototype avian HEV U.S.
BLSV Australia
10 changes

Fig. 1. A phylogenetic tree based on the nucleotide sequences of helicase gene of avian HEV isolates from this study and other selected HEV strains. The tree was constructed with the aid of PAUP program of 4.0 beta version by using a heuristic search with 1,000 replicates of random adding and midpoint rooting option. The scale bar indicating the numbers of character state changes is proportional to the genetic distance. The 11 avian HEV isolates from this study are indicated in boldface type.

Table 1. Detection of avian HEV RNA from bile samples of chickens with HS syndrome in the United States

Strain	Geographic location	Yr of isolation	Breed/Age (wk) of chickens	Clinical Symptom(s)	Test results		
					By EM	RT-PCR with B1/B2 primers	RT-PCR with helicaseF/R primers
WI966B	WI	2000	Layer/60	Decreased egg production and increased mortality	+	-	+
WI966G	WI	2000	Layer/60	Decreased egg production and increased mortality	+	+	+
NY449A	NY	2000	Layer/39	Decreased egg production and increased mortality	+	+	+
CA427	CA	1993	Layer/42	Decreased egg production and increased mortality	+	-	+
CA077	CA	1993	Broiler/56	Decreased egg production and increased mortality	+	+	+
CA242	CA	1994	Broiler/63	Decreased egg production and increased mortality	+	+	+
CA518.3	CA	1997	Broiler/64	Decreased egg production and increased mortality	+	-	+
CA518.5	CA	1997	Broiler/64	Decreased egg production and increased mortality	+	+	+
CA708A	CA	1997	Broiler/56	Decreased egg production and increased mortality	+	-	-
CA889	CA	1996	Layer/51	Decreased egg production and increased mortality	+	-	+
CA697A	CA	2001	Broiler/41	Increased mortality	+	+	+
CA697B	CA	2001	Broiler/41	Increased mortality	+	+	+
CA697C	CA	2001	Broiler/41	Increased mortality	-	+	+
WI318B	WI	2000	Layer/46	Decreased egg production and increased mortality	-	+	+
CT090A	CT	2000	Layer/35	Decreased egg production and increased mortality	-	+	+
CT690.2	CT	2000	Layer/39	Decreased egg production, increased mortality and amyloid arthropathy	-	+	+
CT691.5	CT	2000	Layer/39	Decreased egg production, increased mortality and amyloid arthropathy	-	-	-
CA658.2	CA	2000	Broiler/55	Decreased egg production	-	-	+
CA659.1	CA	2000	Broiler/55	Decreased egg production	-	-	-
CA571.1	CA	2000	Broiler/55	Decreased egg production	-	-	-
CA078A	CA	2000	Layer/80	Has gone through HS syndrome	-	-	+

Table 2. Prevalence of antibodies to avian HEV in chickens of different ages from the United States

Location	Age	No. of birds pos. / No. tested (%)	No. of flocks pos. / No. tested (%)
WI	Adults[a]	20/20 (100)	1/1 (100)
CO	Adults	66/278 (24)	7/7 (100)
CA	<18 wks	58/381 (15)	13/26 (50)
	Adults	138/486 (28)	27/36 (75)
VA	<18 wks	13/24 (54)	1/1 (100)
	Adults	68/70 (97)	3/3 (100)
CT	Adults	17/17 (100)	2/2 (100)

[a] Chickens older than 18 weeks of age are generally considered as adult.

Table 3. Pairwise comparison of the nucleotide sequences of the partial helicase gene of 11 avian HEV isolates identified in this study (in boldface), prototype avian HEV, BLSV and other selected strains of HEV from each of the 4 recognized genotypes

	Sar-55	US2	Swine HEV	Mexico	BLSV	Avian HEV	**CT090A**	**CA518.5**	**CA242**	**CA077**	**CA697C**	**CA697B**	**CA697A**	**NY449A**	**WI318B**	**CT690.2**	**WI966G**
T1	76[a]	75	75	73	56	57	56	57	56	56	56	56	56	56	57	57	56
Sar-55		75	75	78	59	60	59	61	59	59	59	59	60	59	60	59	61
US2			91	72	60	57	59	59	59	60	60	60	60	60	59	59	59
Swine HEV				75	60	58	59	60	60	60	59	59	59	60	59	60	59
Mexico					59	61	60	61	59	60	59	59	60	60	60	60	60
BLSV						77	76	78	79	77	76	76	76	77	78	80	77
Prototype Avian HEV							79	86	80	79	79	79	80	80	88	80	86
CT090A								80	86	94	93	93	93	96	81	83	79
CA518.5									83	80	80	80	80	83	96	81	98
CA242										86	85	85	85	88	83	83	83
CA077											97	97	96	96	81	84	79
CA697C												100	99	95	80	83	78
CA697B													99	95	80	83	78
CA697A														94	80	83	78
NY449A															83	84	81
WI318B																80	96
CT690.2																	81
WI966G																	

Chapter 3

Determination and analyses of the complete genomic sequence of avian hepatitis E virus (avian HEV) and attempts to infect rhesus monkeys with avian HEV

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ABSTRACT

Avian hepatitis E virus (avian HEV), recently identified from a chicken with hepatitis-splenomegaly syndrome in the United States, is genetically and antigenically related to human and swine HEVs. In this study, we completed sequencing the genome and attempted to infect rhesus monkeys with avian HEV. The full-length genome of avian HEV, excluding the poly (A) tail, is 6,654 bp in length, which is about 600 bp shorter than that of human and swine HEVs. Similar to human and swine HEV genomes, the avian HEV genome consists of a short 5' non-coding region (NCR) followed by three partially overlapping open reading frames (ORFs), and a 3' NCR. Avian HEV shared about 50% nucleotide sequence identity in the complete genome, 48-51% identity in ORF1, 46-48% identity in ORF2, and only 29-34% identity in ORF3 with human and swine HEV strains. Significant genetic variations such as deletions and insertions, particularly in ORF1 of avian HEV, were observed. However, motifs in the putative functional domains of ORF1 such as helicase and methyltransferase were relatively conserved between avian HEV and mammalian HEVs, supporting the conclusion that avian HEV is a member of the genus *Hepevirus*. Phylogenetic analyses revealed that avian HEV represented a branch distinct from human and swine HEVs. Swine HEV infected non-human primates and possibly humans, and thus may be zoonotic. We attempted to determine if avian HEV also infected across species by experimentally inoculating two rhesus monkeys with avian HEV. Evidence of virus infection was not observed in the inoculated monkeys as there was no seroconversion, viremia, fecal virus shedding, or serum liver enzyme elevation. The results from this study confirmed that

avian HEV is related to, but distinct from, human and swine HEVs; however, unlike swine HEV, avian HEV is probably not transmissible to non-human primates.

INTRODUCTION

Hepatitis E is an important public health disease in many developing countries of Asia and Africa, and also occurs sporadically in some industrialized countries (Emerson & Purcell, 2003; Kabrane-Lazizi *et al.*, 2001; Schlauder *et al.*, 1999; Takahashi *et al.*, 2002). The disease mainly affects young adults and has a relatively high mortality, up to 25%, in infected pregnant women (Emerson & Purcell, 2003). The causative agent, hepatitis E virus (HEV), is a small, non-enveloped RNA virus that is transmitted via the fecal-oral route, primarily through contaminated water supplies (Emerson & Purcell, 2003). It is the sole member of the genus *Hepevirus*. The genome of HEV is a single-stranded, positive-sense RNA molecule of approximately 7.2 kb, and encodes three open reading frames (ORFs) (Tam *et al.*, 1991). The ORF1, located at the 5' end of the genome, encodes motifs for non-structural proteins, including methyltransferase, protease, helicase and RNA-dependent RNA polymerase (RdRp) (Koonin *et al.*, 1992). The ORF2, located at the 3' end of the genome, encodes the capsid protein (Tam *et al.*, 1991). The small ORF3, which partially overlaps ORF2, encodes a cytoskeleton-associated phosphoprotein (Tyagi *et al.*, 2002; Zafrullah *et al.*, 1997).

The first animal strain of HEV to be sequenced and characterized was isolated from a pig in the United States in 1997, and designated swine HEV (Meng *et al.*, 1997). Since then, numerous strains of HEV have been isolated from pigs in both developing and industrialized countries (Arankalle *et al.*, 2002; Choi *et al.*, 2003; Garkavenko *et al.*, 2001; Hsieh *et al.*, 1999; Huang *et al.*, 2002a; Pei & Yoo, 2002; Takahashi *et al.*, 2003a; van der Poel *et al.*, 2001). Swine HEV is enzootic in swine herds worldwide (Meng, 2003). Seroepidemiological studies showed that pig handlers are at higher risk of HEV infections than normal blood donors (Drobeniuc *et al.*, 2001; Meng *et al.*, 2002). Experimental interspecies transmission of HEV has been demonstrated: swine HEV infected non-human primates and a U.S. strain of human HEV infected pigs (Halbur *et al.*, 2001; Meng *et al.*, 1998a, b). Increasing evidence indicates that pigs are animal reservoirs for HEV and hepatitis E may be zoonotically transmitted to humans (Meng, 2000a, b; Tei *et al.*, 2003).

Big liver and spleen disease (BLS) in chickens was first recognized in Australia in 1980 and considered to be the most economically significant disease affecting commercial broiler breeder flocks in that country (Payne *et al.*, 1999). The causative agent, BLS virus (BLSV), was isolated from diseased chickens in Australia (Payne *et al.*, 1999). Based on the sequence of a very short genomic region, BLSV was found to be genetically related to human HEV (Payne *et al.*, 1999). More recently, Haqshenas *et al.*(2001) identified a strain of HEV, avian HEV, from a chicken with hepatitis-splenomegaly syndrome (HS syndrome) in the United States by using PCR primers primarily based on the sequence of BLSV (Haqshenas et al., 2001). Like BLSV, avian HEV is also distantly related to human HEV (Haqshenas *et al.*, 2001). Avian HEV shared about 80% nucleotide sequence identity with BLSV in a short stretch of sequence available for BLSV but shared only about 57-61% sequence identity with mammalian HEVs in this region (Haqshenas *et al.*, 2001).

The 3' approximate 4 kb sequence of avian HEV genome was determined previously (Haqshenas *et al.*, 2001). It is known that mammalian HEVs encode a methyltransferase at the 5' end of the genome (Koonin *et al*, 1992), and that the genome is capped (Kabrane-Lazizi *et al*, 1999b). Therefore, it was important to identify the remaining 5' end sequence of avian HEV in order to compare it to known HEVs. In this study, we completed the sequencing of avian HEV genome and compared the sequence with those of known human and swine HEVs. We also attempted to infect rhesus monkeys with avian HEV.

MATERIALS AND METHODS

Virus. Avian HEV used in this study was originally recovered from a bile sample of a 56-week-old White Plymouth Rock chicken with HS syndrome (F93-5077) in California (Haqshenas *et al.*, 2001). A standard infectious stock of avian HEV was prepared as a mixture of bile and 10% suspension of feces collected from experimentally infected SPF chickens. This avian HEV stock has an infectivity titer of $5\times10^{5.0}$ 50% chicken infectious doses (CID_{50}) per ml (Sun *et al*., unpublished data), and was used for the rhesus monkey

transmission study. Bile containing avian HEV collected from an experimentally infected SPF chicken was used for construction of cDNA libraries .

Construction of cDNA libraries of avian HEV genomic RNA. The 3' terminal sequence of avian HEV has been previously reported (Haqshenas *et al.*, 2001). To determine the complete genomic sequence, we constructed two cDNA libraries of avian HEV genomic RNA based on the known avian HEV sequences. Synthesis and cloning of cDNA were accomplished by using the SuperScript™ Plasmid System with Gateway™ Technology for cDNA Synthesis and Cloning (Invitrogen). Briefly, total RNAs were extracted with Trizol reagent from a bile sample containing avian HEV. Reverse transcription was performed at 42°C for 1 h with SuperScript II RT reverse transcriptase (Invitrogen) and the *Not* I RT primer-adapter (Table 1) in the first cDNA library, or at 65 °C for 1 h with ThermoScript RT reverse transcriptase (Invitrogen) and the *Not* I RT-1 primer-adapter (Table 1) in the second cDNA library. Primer-adapter *Not* I RT was based on the known terminal sequence of avian HEV with a *Not* I restriction site engineered at the 5' end. Primer-adapter *Not* I RT-1, used in the second cDNA library construction, was based on the 5' sequence of cDNA clones identified in the first cDNA library. The second strand cDNA was synthesized by DNA polymerase I, RNase H and DNA ligase at 16°C for 2 h. After addition of a *Sal* I adapter, the resulting double-stranded cDNA was digested by *Not* I restriction endonuclease. cDNA fractionated by column chromatography was ligated to the vector pSPORT 1 previously digested with *Not* I and *Sal* I. The ligation mixture was transformed into One Shot® TOP 10F' competent *E.coli.* cells (Invitrogen) and 50 µl were spread on LB agar plates containing ampicillin and incubated at 37°C overnight.

Synthesis of digoxygenin-labeled avian HEV-specific cDNA probes for cDNA library screening. Total RNAs extracted from bile containing avian HEV were used to synthesize two avian HEV-specific cDNA probes by RT-PCR with a DIG labeling mix kit (Roche Molecular Biochemicals). Briefly, primers Labeling F1 and Labeling R1 were designed on the basis of the known 3' terminal sequence of avian HEV upstream of the *Not* I RT primer-adapter used for the first cDNA library construction. A DIG-labeled 190

bp cDNA fragment was amplified as the probe with this primer set. The DIG-labeled cDNA probe used for colony screening in the second cDNA library was synthesized as a 100 bp cDNA fragment by the same method with primers Labeling F2 and Labeling R2, which were based on the 5' terminal sequences of cDNA clones identified in the first cDNA library (Table 1).

Identification of authentic cDNA clones containing avian HEV genome by colony hybridization. LB plates containing recombinant bacterial colonies growing at 37°C were chilled at 4°C for 2 h. A marked nylon membrane was then placed on each LB agar plate for 5 min to allow the transfer of the colonies to the membrane. The membranes were removed and dried for 5 min, followed by denaturation with 1.5 M NaCl and 0.5 M NaOH for 10 min, and then neutralization with 1.5 M NaCl and 1 M Tris-HCl (pH 8.0) for 10 min. The plasmid DNA from the recombinant colonies was cross-linked to the membranes by baking at 80°C for 30 min. Prehybridization of each membrane was performed at 55°C for 2 h in a Reichert-Jung water bath shaker. The prehybridization solution was then replaced with 30 ml of hybridization solution containing denatured DIG-labeled avian HEV-specific probes (15 ng/ml) and incubated at 55°C overnight. The hybridized membranes were washed twice, each for 10 min, with 2 × wash solution (2 × SSC, 0.1% SDS) and then twice, each for 15 min, with 0.5 × wash solution.

After colony hybridization, the positive colonies that hybridized to the DIG-labeled avian HEV-specific cDNA probes were detected by a DIG Nucleic Acid Detection Kit (Roche Molecular Biochemicals). Briefly, the hybridized membranes were incubated in a blocking solution, followed by an anti-DIG antibody solution, each for 30 min. After equilibration of the membranes for 5 min in a detection buffer, the membranes were incubated in a freshly prepared substrate solution in the dark. When positive signals appeared, the reaction was stopped by washing the membranes with sterile water. Colonies corresponding to the positive signals on the membranes were matched to the original plates, selected and cultured in LB media containing ampicillin overnight at 37°C in an incubator shaker (New Brunswick Scientific, Model I2400). Plasmid DNA was extracted with a GenElute™ Plasmid Miniprep Kit (Sigma) and verified by restriction enzyme digestion with *Not*I and *Sal*I. Three independent cDNA clones of 508

bp, 540 bp, and 551 bp, respectively, from the first cDNA library and another three independent cDNA clones of 2,440 bp, 2,380 bp, and 2,305 bp, respectively, from the second cDNA library were selected for sequencing.

5' RACE for the identification of the extreme 5' sequence of avian HEV genome. It has been shown that the genomic RNA of HEV is capped at its 5' end (Kabrane-Lazizi *et al.*, 1999b; Zhang *et al.*, 2001). Therefore, to identify the extreme 5' genomic sequence of avian HEV, a RNA Ligase Mediated RACE (RLM-RACE) with FirstChoice™ RLM-RACE Kit (Ambion) was used. Briefly, total RNA extracted from bile containing avian HEV was treated with calf intestinal phosphatase to remove the 5' phosphate from degraded mRNAs but not from viral RNA with a 5' cap structure. The reaction was then treated with tobacco acid pyrophosphatase to remove the cap structure from the viral RNA, followed by ligation of a RNA adapter (5' RACE Adapter) (Table 1) to the viral RNA. The ligated RNA was used as the template for cDNA synthesis with an avian HEV-specific antisense primer (5'AHEV R1) (Table 1) and SuperScript II reverse transcriptase. The resulting cDNA was amplified by a nested PCR with two sense adapter primers supplied in the kit (5' RACE Outer Primer and 5' RACE Inner Primer) and two antisense primers specific to avian HEV (5' AHEV RACE Outer and 5' AHEV RACE Inner) (Table 1).

Nucleotide sequencing and sequence analyses. Three independent cDNA clones from each of the two cDNA libraries were selected for sequencing by using primer walking strategy. The PCR products of 5' RLM-RACE were purified with a GENECLEAN kit (Bio 101 Inc.) and directly sequenced at the Virginia Tech DNA Sequencing Facility. Sequences of the PCR products, and cDNA clones were determined for both DNA strands.

The full-length genomic sequence was assembled and analyzed with the MacVector computer program (Oxford Molecular Inc.). Multiple nucleotide and amino acid sequence alignments of avian HEV and strains of human and swine HEVs with known full-length or near full-length genomic sequences were analyzed with the

MacVector program. GenBank database searches of the avian HEV sequence were carried out with BlastN, BlastP and BlastX with default settings.

Phylogenetic analysis was conducted with the aid of the PAUP program of 4.0 beta version (David Swofford, Smithsonian Institute, Washington, D.C., distributed by Sinauer Associate Inc.). A heuristic search with 1,000 replicates of random adding was used to produce a phylogenetic tree.

Verification of deletions and insertions in avian HEV genome. After the full-length genomic sequence of avian HEV was assembled, numerous deletions and insertions were observed in the ORF1 when aligned with genomic sequences of human and swine HEVs. In order to confirm these deletions and insertions, about 3 kb of ORF1 sequence at the 5' end of the avian HEV genome was amplified directly by RT-PCR as 6 overlapping fragments from a bile sample containing avian HEV. The RT-PCR reactions were performed with the addition of 5% DMSO, using primer pairs ConF1/ConR1, ConF2/ConR2, ConF3/ConR3, ConF4/ConR4, ConF5/ConR5, and ConF6/ConR6, respectively (Table 1).

Experimental inoculation of rhesus monkeys with avian HEV. We have previously infected a chimpanzee and rhesus monkeys with swine HEV (Meng *et al.*, 1998b). To assess whether avian HEV can also infect across species, two rhesus monkeys were each inoculated intravenously with $5\times10^{5.0}$ CID_{50} of avian HEV. Pre-inoculation and weekly serum and fecal samples were collected from both monkeys. Sera were tested for avian HEV RNA by a nested RT-PCR, for IgG antibody to avian HEV by an ELISA and for serum liver enzymes by standard methods. Fecal samples were tested for viral RNA by a nested RT-PCR. The animals were monitored for evidence of viral infection and hepatitis for 22 weeks.

RT-PCR for detection of avian HEV RNA from experimentally inoculated monkeys. In order to determine whether avian HEV RNA was present in fecal and serum samples from the experimentally inoculated monkeys, we performed a nested RT-PCR with avian HEV-specific primers (FAHEVEp/RAHEVEp and FAHEVEpF/RAHEVEpR) (Table 1).

The PCR primers were based on the ORF2 sequence of the avian HEV used in the inoculum. The parameters for each round of the nested PCR included a denaturation at 95°C for 6 min, followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 50 s at 58°C and extension for 1min at 72°C, with a final incubation at 72°C for 7 min. Positive and negative controls were included in each set of RT-PCR amplifications. RNA extraction and other pre-PCR amplification steps were performed in a separate clean room to minimize cross-contamination.

ELISA. A purified truncated recombinant avian HEV ORF2 protein expressed in *E. coli.* was used as the antigen for an ELISA to detect antibody to avian HEV in experimentally inoculated monkeys (Haqshenas *et al.*, 2002; Huang *et al.*, 2002b). All sera from the monkeys before or after inoculation were tested in duplicate at a dilution of 1:100 using peroxidase-labeled goat anti-human IgG (Sigma Chemical Co.) as the secondary antibody.

GenBank accession numbers. The complete genomic sequence of avian HEV has been deposited in NCBI database (GenBank accession number AY535004). The accession numbers of other strains of human and swine HEVs with full-length or near full-length genomic sequences used in the study are: Arkell swine HEV (AY115488), JRA1 (AP003430), JKN-Sap (AB074918), swJ570 (AB073912), prototype swine HEV (AF082843), US1 (AF060668), US2 (AF060669), JMY-Haw (AB074920), HE-JA1 (AB097812), HEVNE8L (D10330), HeBei (M94177), Hyderabad (AF076239), HEVJapan (E17109), Madras (X99441), Haryana (AF459438), Burma (M73218), Xinjiang (D11092), KS2-87 (L25547), hev037 (X98292), Sar-55 (AF444003), T1 (AJ272108), Morocco (AY230202), Mexico (M74506), JSN (AB091395), JJT-Kan (AB091394), HE-JI4 (AB080575), JAK-Sai (AB074915), JKK-Sap (AB074917), and swJ13-1 (AB097811).

RESULTS

Complete sequence and organization of the avian HEV genome. Based on the available 3' terminal sequence, avian HEV shared only about 50% nucleotide sequence

identity with mammalian HEVs (Haqshenas *et al.*, 2001). Therefore, it would be difficult to amplify the avian HEV genome by RT-PCR with degenerate primers based on the sequences of human and swine HEVs. For this reason, we constructed two cDNA libraries of avian HEV genomic RNA. In the first cDNA library, positive colonies containing avian HEV cDNA were detected with a DIG-labeled avian HEV-specific probe and the cDNA inserts were verified by sequencing. However, the positive cDNA inserts in the first cDNA library were all less than 600 bp. Therefore, based on the sequence of the 3 cDNA clones identified in the first cDNA library, a second cDNA library was constructed by using ThermoScript reverse transcriptase, which can melt secondary structures and produce longer cDNA fragments. Clones containing avian HEV cDNA inserts of up to 2.5 kb were detected in the second cDNA library with a DIG-labeled avian HEV-specific probe and the cDNA inserts were confirmed by sequencing. With RLM-RACE, the region at the extreme 5' end of the avian HEV genome was successfully amplified by a nested RT-PCR and sequenced.

The consensus sequence of the avian HEV genome was determined from at least three independent cDNA clones from each of the two cDNA libraries. The resulting consensus sequence in ORF1 was further confirmed by direct sequencing of 6 overlapping RT-PCR products spanning the ORF1 region. An additional 2,723 nucleotides was identified at the 5' end of the avian HEV genome in this study. Combined with the known 3' sequence, the complete genome of avian HEV, excluding the 3' poly (A) tail, is 6,654 nt in length, which is about 600 bp shorter than that of human and swine HEVs. Similar to human and swine HEVs, the genome of avian HEV consists of a short 5' NCR followed by three partially overlapping ORFs, and a 3' NCR. The 5' NCR of avian HEV consists of 24 bp, which is 2-4 bp shorter than that of most human and swine HEV strains (Fig. 1). The ORF1 begins at nucleotide position 25 and ends at position 4,620 and potentially encodes a polyprotein of 1,531 amino acid residues. The ORF2, from position 4,707 to 6,527, comprises 1,821 nucleotides and encodes a putative capsid protein of 606 aa (Haqshenas *et al.*, 2001). The ORF3, from position 4,654 to 4,917, consists of 264 nucleotides and encodes a small protein of 87 aa (Haqshenas *et al.*, 2001) (Fig. 2). As in human and swine HEVs, the ORF2 of avian HEV also overlaps with ORF3. Complete sequence analyses revealed that the predicted

polyprotein encoded by ORF1 of avian HEV contains several putative functional domains including methyltransferase, papain-like cysteine protease, helicase and RdRp, which are also present in human and swine HEVs (Fig. 3). Amino acid sequence comparisons revealed that motifs typical of the helicase superfamily I and of the putative viral methyltransferase found throughout the alpha-like virus supergroup were conserved between avian HEV and mammalian HEVs (Koonin *et al.*, 1992) (Fig. 3). For example, 6 of the 9 aa in motif I, 5 of the 8 aa in motif II, and 7 of 15 aa in motif III of the methyltransferase are identical between avian HEV and mammalian HEVs. Similarly, in the helicase, 9 of the 12 aa in motif I, 11 of the 15 aa in motif V, and 9 of the 11 aa in motif VI are also identical between avian HEV and mammalian HEVs (Fig. 3).

Avian HEV is genetically and phylogenetically related to, but distinct from, human and swine HEVs. The complete genomic sequence of avian HEV was compared to the corresponding sequences of human and swine HEV strains. It was found that avian HEV shared about 50% nucleotide sequence identity in the entire genome, 48-51% identity in ORF1 (Table 2), 46-48% identity in ORF2, and only 29-34% identity in ORF3 with human and swine HEV strains (Haqshenas *et al.*, 2001). At the amino acid sequence level, the putative polyprotein encoded by ORF1 of avian HEV shared 41-42% identity with strains of human and swine HEVs. Sequences of putative functional domains in ORF1 of avian HEV were also compared with those of human and swine HEV strains. The helicase gene was the most conserved between avian HEV and other HEV strains, displaying 56-59% nucleotide sequence identity (Table 2). The ORF1 of avian HEV is 4,596 nt in length, which is 480-521 nt shorter than that of human and swine HEVs. Significant genetic variation, characterized by multiple deletions and insertions, was observed in the ORF1 of avian HEV (Fig. 3). Most of the deletions were located in and between the proposed papain-like cysteine protease domain and the hypervariable region (HVR) of human and swine HEVs. However, the deletions and insertions did not alter the reading frame of avian HEV ORF1.

Phylogenetic analysis based on the complete genomic sequence of HEV confirmed that avian HEV was distantly related to swine and human HEVs. Avian HEV

was segregated into a distinct branch separate from human and swine HEVs of the four known genotypes (Fig. 4).

Failure to infect rhesus monkeys experimentally with avian HEV. Swine HEV was shown to infect rhesus monkeys and a chimpanzee (Meng *et al.*, 1998b), thus we attempted to infect two rhesus monkeys with avian HEV. The two inoculated monkeys were not infected by avian HEV as evidenced by absence of seroconversion, viremia, fecal virus shedding or elevation of serum liver enzymes.

DISCUSSION

HEV was originally misclassified as a calicivirus because of its genomic organization, which superficially resembles that of the *Caliciviridae*. To determine whether the avian HEV shares all of the important characteristics of the *Hepeviridae*, we wished to analyze all of the motifs that now define this new family. The sequence data that we obtained in this study permitted us to make a more thorough comparison of avian HEV and other HEVs: the results suggested that avian HEV shares significant features with other HEVs and is related to them in spite of the overall low sequence homologies. The genome organization of avian HEV is very similar to that of human and swine HEVs. The polyprotein encoded by ORF1 of avian HEV consists of functional domains and motifs similar to those found in human and swine HEVs. The viral methyltransferase found in some single stranded RNA viruses is involved in mRNA capping, and a cap structure at the 5' terminus of mammalian HEVs has been demonstrated (Kabrane-Lazizi *et al.*, 1999b; Okamoto *et al.*, 2001; Zhang *et al.*, 2001). In this study, we used a pyrophosphatase-dependent elongation reaction with RLM-RACE to amplify the extreme 5' genomic sequence of avian HEV, suggesting that avian HEV genomic RNA is also capped. The identification of a methyltransferase gene in the avian HEV genome and the conserved motifs I, II and III in the methyltransferase gene between avian HEV and mammalian HEVs provide important support for classifying avian HEV with other HEVs. The 5' NCRs of HEVs are very conserved among human and swine HEV strains except for 10 additional nucleotides at the 5' terminus of the US2 strain (Zhang *et al.*, 2001). However the 5' NCR of avian HEV is highly divergent from that of human and

swine HEVs. The biological significance of the observed variation in the 5' NCR between avian HEV and other HEV strains is unclear.

The complete avian HEV genome was about 600 bp shorter than that of other HEV strains. Sequence comparison revealed multiple deletions in avian HEV across the genome, but especially in and around the domains of the putative papain-like cysteine protease and the HVR, which in mammalian HEVs, are the most divergent regions. Therefore, deletions and extensive sequence variations in this region of the avian HEV genome are not surprising. Taken together, the evidence suggested that this region may not play a functional role in HEV replication. Importantly, the deletions and the insertions did not cause a frame-shift in ORF1 of avian HEV as might have happened if they were PCR mistakes. It is known that strong secondary structures in GC-rich regions could produce artificial deletions during RT-PCR amplification. To rule out this possibility, six overlapping fragments within the ORF1 were directly amplified from the virus stock by RT-PCR in the presence of DMSO. No additional sequence was identified by direct sequencing of RT-PCR products. For these reasons, we concluded that the observed deletions in ORF1 were not artifacts. The biological significance of these deletions is not known.

Thus far, four major genotypes of HEV have been identified (Emerson & Purcell, 2003): the Burmese-like genotype 1 consisting of Asian and African strains (Arankalle *et al.*, 2002; Tam *et al.*, 1991; Tsarev *et al.*, 1999), the Mexican genotype 2 consisting of a Mexican strain and possibly Nigerian strains (Buisson *et al.*, 2000; Huang *et al.*, 1992), the USA genotype 3 consisting of both human and swine HEV strains identified in countries where hepatitis E is rare (Erker *et al.*, 1999; Meng *et al.*, 1997; Schlauder *et al.*, 1998, 1999; Takahashi *et al.*, 2003a, 3b), and the new genotype 4 consisting of strains of both human and swine HEV from Asia, (Takahashi *et al.*, 2002; Wang *et al.*, 1999). Phylogenetic analysis based on the full-length genomic sequences of 30 HEV strains including human, swine and avian HEV showed that avian HEV belongs to a distinct branch separate from those of genotype 1 to 4 of human and swine HEV strains. It is not known whether avian HEV represents a fifth genotype of HEV or belongs to a separate genus. We have previously shown that avian HEV shared common antigenic epitope(s) in the capsid protein with human and swine HEVs (Haqshenas *et al.*, 2002), and thus it is

possible that avian HEV may belong to a new genotype 5 of HEV. However, additional studies such as cross-neutralization experiments are needed to classify avian HEV definitively.

The seroprevalence of HEV antibody in different animal species has been widely reported (Chandler *et al.*, 1999; Kabrane-Lazizi *et al.*, 1999a; Meng *et al.*, 1999), however the viruses responsible for the seropositivity in these animal species have not been definitively identified except in pigs and chickens (Haqshenas *et al.*, 2001; Meng *et al.*, 1997; Payne *et al.*, 1999). Accumulated evidence suggests that swine HEV infection may be zoonotic (Meng, 2000a, b; Tei *et al.*, 2003). Therefore, in this study we attempted to infect rhesus monkeys with avian HEV. Unlike swine HEV, however, avian HEV was not transmitted to rhesus monkeys. The negative result was not surprising since avian HEV shared only about 50% nucleotide sequence identity with human and swine HEVs. The availability of the complete genomic sequence of avian HEV from this study now affords us an opportunity to construct an infectious cDNA clone of avian HEV and to study molecular mechanism of HEV pathogenesis using chickens as a practical animal model.

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REFERENCES

Arankalle, V. A., Chobe, L. P., Joshi, M. V., Chadha, M. S., Kundu, B. & Walimbe, A. M. (2002). Human and swine hepatitis E viruses from Western India belong to different genotypes. *J Hepatol* **36**, 417-25.

Buisson, Y., Grandadam, M., Nicand, E., Cheval, P., van Cuyck-Gandre, H., Innis, B., Rehel, P., Coursaget, P., Teyssou, R. & Tsarev, S. (2000). Identification of a novel hepatitis E virus in Nigeria. *J Gen Virol* **81**, 903-9.

Chandler, J. D., Riddell, M. A., Li, F., Love, R. J. & Anderson, D. A. (1999). Serological evidence for swine hepatitis E virus infection in Australian pig herds. *Vet Microbiol* **68**, 95-105.

Choi, I. S., Kwon, H. J., Shin, N. R. & Yoo, H. S. (2003). Identification of swine hepatitis E virus (HEV) and prevalence of anti-HEV antibodies in swine and human populations in Korea. *J Clin Microbiol* **41**, 3602-8.

Drobeniuc, J., Favorov, M. O., Shapiro, C. N., Bell, B. P., Mast, E. E., Dadu, A., Culver, D., Iarovoi, P., Robertson, B. H. & Margolis, H. S. (2001). Hepatitis E virus antibody prevalence among persons who work with swine. *J Infect Dis* **184**, 1594-7.

Emerson, S. U. & Purcell, R. H. (2003). Hepatitis E virus. *Rev Med Virol* **13**, 145-54.

Erker, J. C., Desai, S. M., Schlauder, G. G., Dawson, G. J. & Mushahwar, I. K. (1999). A hepatitis E virus variant from the United States: molecular characterization and transmission in cynomolgus macaques. *J Gen Virol* **80 (Pt 3)**, 681-90.

Garkavenko, O., Obriadina, A., Meng, J., Anderson, D. A., Benard, H. J., Schroeder, B. A., Khudyakov, Y. E., Fields, H. A. & Croxson, M. C. (2001). Detection and characterisation of swine hepatitis E virus in New Zealand. *J Med Virol* **65**, 525-9.

Halbur, P. G., Kasorndorkbua, C., Gilbert, C., Guenette, D., Potters, M. B., Purcell, R. H., Emerson, S. U., Toth, T. E. & Meng, X. J. (2001). Comparative pathogenesis of infection of pigs with hepatitis E viruses recovered from a pig and a human. *J Clin Microbiol* **39**, 918-23.

Haqshenas, G., Shivaprasad, H. L., Woolcock, P. R., Read, D. H. & Meng, X. J. (2001). Genetic identification and characterization of a novel virus related to human

hepatitis E virus from chickens with hepatitis-splenomegaly syndrome in the United States. *J Gen Virol* **82**, 2449-62.

Haqshenas, G., Huang, F. F., Fenaux, M., Guenette, D. K., Pierson, F. W., Larsen, C. T., Shivaprasad, H. L., Toth, T. E. & Meng, X. J. (2002). The putative capsid protein of the newly identified avian hepatitis E virus shares antigenic epitopes with that of swine and human hepatitis E viruses and chicken big liver and spleen disease virus. *J Gen Virol* **83**, 2201-9.

Hsieh, S. Y., Meng, X. J., Wu, Y. H., Liu, S. T., Tam, A. W., Lin, D. Y. & Liaw, Y. F. (1999). Identity of a novel swine hepatitis E virus in Taiwan forming a monophyletic group with Taiwan isolates of human hepatitis E virus. *J Clin Microbiol* **37**, 3828-34.

Huang, C. C., Nguyen, D., Fernandez, J., Yun, K. Y., Fry, K. E., Bradley, D. W., Tam, A. W. & Reyes, G. R. (1992). Molecular cloning and sequencing of the Mexico isolate of hepatitis E virus (HEV). *Virology* **191**, 550-8.

Huang, F. F., Haqshenas, G., Guenette, D. K., Halbur, P. G., Schommer, S. K., Pierson, F. W., Toth, T. E. & Meng, X. J. (2002a). Detection by reverse transcription-PCR and genetic characterization of field isolates of swine hepatitis E virus from pigs in different geographic regions of the United States. *J Clin Microbiol* **40**, 1326-32.

Huang, F. F., Haqshenas, G., Shivaprasad, H. L., Guenette, D. K., Woolcock, P. R., Larsen, C. T., Pierson, F. W., Elvinger, F., Toth, T. E. & Meng, X. J. (2002b). Heterogeneity and seroprevalence of a newly identified avian hepatitis E virus from chickens in the United States. *J Clin Microbiol* **40**, 4197-202.

Kabrane-Lazizi, Y., Fine, J. B., Elm, J., Glass, G. E., Higa, H., Diwan, A., Gibbs, C. J., Jr., Meng, X. J., Emerson, S. U. & Purcell, R. H. (1999a). Evidence for widespread infection of wild rats with hepatitis E virus in the United States. *Am J Trop Med Hyg* **61**, 331-5.

Kabrane-Lazizi, Y., Meng, X. J., Purcell, R. H. & Emerson, S. U. (1999b). Evidence that the genomic RNA of hepatitis E virus is capped. *J Virol* **73**, 8848-50.

Kabrane-Lazizi, Y., Zhang, M., Purcell, R. H., Miller, K. D., Davey, R. T. & Emerson, S. U. (2001). Acute hepatitis caused by a novel strain of hepatitis E virus most closely related to United States strains. *J Gen Virol* **82**, 1687-93.

Koonin, E. V., Gorbalenya, A. E., Purdy, M. A., Rozanov, M. N., Reyes, G. R. & Bradley, D. W. (1992). Computer-assisted assignment of functional domains in the nonstructural polyprotein of hepatitis E virus: delineation of an additional group of positive-strand RNA plant and animal viruses. *Proc Natl Acad Sci U S A* **89**, 8259-63.

Meng, X. J., Purcell, R. H., Halbur, P. G., Lehman, J. R., Webb, D. M., Tsareva, T. S., Haynes, J. S., Thacker, B. J. & Emerson, S. U. (1997). A novel virus in swine is closely related to the human hepatitis E virus. *Proc Natl Acad Sci U S A* **94**, 9860-5.

Meng, X. J., Halbur, P. G., Haynes, J. S., Tsareva, T. S., Bruna, J. D., Royer, R. L., Purcell, R. H. & Emerson, S. U. (1998a). Experimental infection of pigs with the newly identified swine hepatitis E virus (swine HEV), but not with human strains of HEV. *Arch Virol* **143**, 1405-15.

Meng, X. J., Halbur, P. G., Shapiro, M. S., Govindarajan, S., Bruna, J. D., Mushahwar, I. K., Purcell, R. H. & Emerson, S. U. (1998b). Genetic and experimental evidence for cross-species infection by swine hepatitis E virus. *J Virol* **72**, 9714-21.

Meng, X. J., Dea, S., Engle, R. E., Friendship, R., Lyoo, Y. S., Sirinarumitr, T., Urairong, K., Wang, D., Wong, D., Yoo, D., Zhang, Y., Purcell, R. H. & Emerson, S. U. (1999). Prevalence of antibodies to the hepatitis E virus in pigs from countries where hepatitis E is common or is rare in the human population. *J Med Virol* **59**, 297-302.

Meng, X. J. (2000a). Novel strains of hepatitis E virus identified from humans and other animal species: is hepatitis E a zoonosis? *J Hepatol* **33**, 842-5.

Meng, X. J. (2000b). Zoonotic and xenozoonotic risks of hepatitis E virus. *Infec Dis Rev* **2**, 35-41.

Meng, X. J., Wiseman, B., Elvinger, F., Guenette, D. K., Toth, T. E., Engle, R. E., Emerson, S. U. & Purcell, R. H. (2002). Prevalence of antibodies to hepatitis E virus in veterinarians working with swine and in normal blood donors in the United States and other countries. *J Clin Microbiol* **40**, 117-22.

Meng, X. J. (2003). Swine hepatitis E virus: cross-species infection and risk in xenotransplantation. *Curr Top Microbiol Immunol* **278**, 185-216.

Okamoto, H., Takahashi, M., Nishizawa, T., Fukai, K., Muramatsu, U. & Yoshikawa, A. (2001). Analysis of the complete genome of indigenous swine hepatitis E virus isolated in Japan. *Biochem Biophys Res Commun* **289**, 929-36.

Payne, C. J., Ellis, T. M., Plant, S. L., Gregory, A. R. & Wilcox, G. E. (1999). Sequence data suggests big liver and spleen disease virus (BLSV) is genetically related to hepatitis E virus. *Vet Microbiol* **68**, 119-25.

Pei, Y. & Yoo, D. (2002). Genetic characterization and sequence heterogeneity of a Canadian isolate of swine hepatitis E virus. *J Clin Microbiol* **40**, 4021-9.

Pina, S., Buti, M., Cotrina, M., Piella, J. & Girones, R. (2000). HEV identified in serum from humans with acute hepatitis and in sewage of animal origin in Spain. *J Hepatol* **33**, 826-33.

Schlauder, G. G., Dawson, G. J., Erker, J. C., Kwo, P. Y., Knigge, M. F., Smalley, D. L., Rosenblatt, J. E., Desai, S. M. & Mushahwar, I. K. (1998). The sequence and phylogenetic analysis of a novel hepatitis E virus isolated from a patient with acute hepatitis reported in the United States. *J Gen Virol* **79 (Pt 3)**, 447-56.

Schlauder, G. G., Desai, S. M., Zanetti, A. R., Tassopoulos, N. C. & Mushahwar, I. K. (1999). Novel hepatitis E virus (HEV) isolates from Europe: evidence for additional genotypes of HEV. *J Med Virol* **57**, 243-51.

Takahashi, M., Nishizawa, T., Yoshikawa, A., Sato, S., Isoda, N., Ido, K., Sugano, K. & Okamoto, H. (2002). Identification of two distinct genotypes of hepatitis E virus in a Japanese patient with acute hepatitis who had not travelled abroad. *J Gen Virol* **83**, 1931-40.

Takahashi, M., Nishizawa, T. & Okamoto, H. (2003a). Identification of a genotype III swine hepatitis E virus that was isolated from a Japanese pig born in 1990 and that is most closely related to Japanese isolates of human hepatitis E virus. *J Clin Microbiol* **41**, 1342-3.

Takahashi, M., Nishizawa, T., Miyajima, H., Gotanda, Y., Iita, T., Tsuda, F. & Okamoto, H. (2003b). Swine hepatitis E virus strains in Japan form four phylogenetic clusters comparable with those of Japanese isolates of human hepatitis E virus. *J Gen Virol* **84**, 851-62.

Tam, A. W., Smith, M. M., Guerra, M. E., Huang, C. C., Bradley, D. W., Fry, K. E. & Reyes, G. R. (1991). Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. *Virology* **185**, 120-31.

Tei, S., Kitajima, N., Takahashi, K. & Mishiro, S. (2003). Zoonotic transmission of hepatitis E virus from deer to human beings. *Lancet* **362**, 371-3.

Tsarev, S. A., Binn, L. N., Gomatos, P. J., Arthur, R. R., Monier, M. K., van Cuyck-Gandre, H., Longer, C. F. & Innis, B. L. (1999). Phylogenetic analysis of hepatitis E virus isolates from Egypt. *J Med Virol* **57**, 68-74.

Tyagi, S., Korkaya, H., Zafrullah, M., Jameel, S. & Lal, S. K. (2002). The phosphorylated form of the ORF3 protein of hepatitis E virus interacts with its non-glycosylated form of the major capsid protein, ORF2. *J Biol Chem* **277**, 22759-67.

van der Poel, W. H., Verschoor, F., van der Heide, R., Herrera, M. I., Vivo, A., Kooreman, M. & de Roda Husman, A. M. (2001). Hepatitis E virus sequences in swine related to sequences in humans, The Netherlands. *Emerg Infect Dis* **7**, 970-6.

Wang, Y., Ling, R., Erker, J. C., Zhang, H., Li, H., Desai, S., Mushahwar, I. K. & Harrison, T. J. (1999). A divergent genotype of hepatitis E virus in Chinese patients with acute hepatitis. *J Gen Virol* **80 (Pt 1)**, 169-77.

Wu, J. C., Chen, C. M., Chiang, T. Y., Tsai, W. H., Jeng, W. J., Sheen, I. J., Lin, C. C. & Meng, X. J. (2002). Spread of hepatitis E virus among different-aged pigs: two-year survey in Taiwan. *J Med Virol* **66**, 488-92.

Zafrullah, M., Ozdener, M. H., Panda, S. K. & Jameel, S. (1997). The ORF3 protein of hepatitis E virus is a phosphoprotein that associates with the cytoskeleton. *J Virol* **71**, 9045-53.

Zhang, M., Purcell, R. H. & Emerson, S. U. (2001). Identification of the 5' terminal sequence of the SAR-55 and MEX-14 strains of hepatitis E virus and confirmation that the genome is capped. *J Med Virol* **65**, 293-5.

Fig. 1

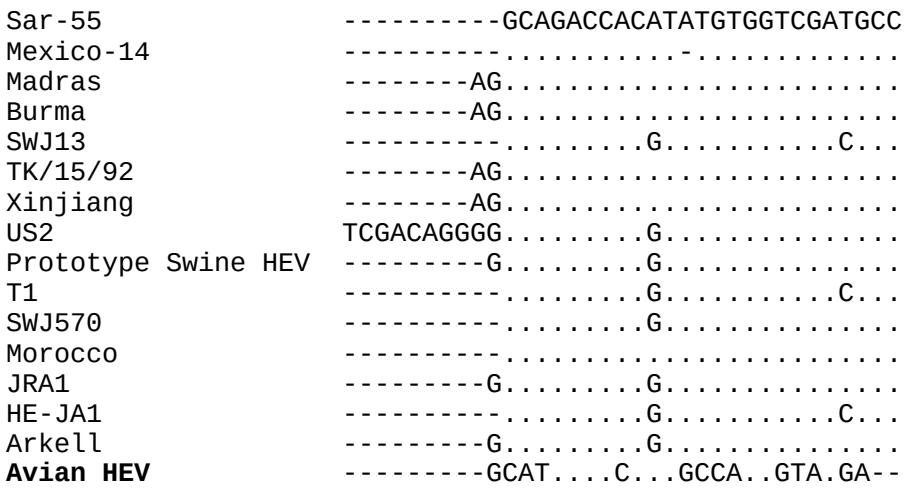

Fig. 1. Nucleotide sequence alignment of the 5' NCR of avian HEV and selected strains of human and swine HEVs. The 5' NCR sequence of Sar-55 human HEV strain is shown on top and only differences are indicated in other strains. The sequence of avian HEV is shown at the bottom. Deletions are indicated by dashes. Identical nucleotide sequences are indicated by dots.

Fig. 2

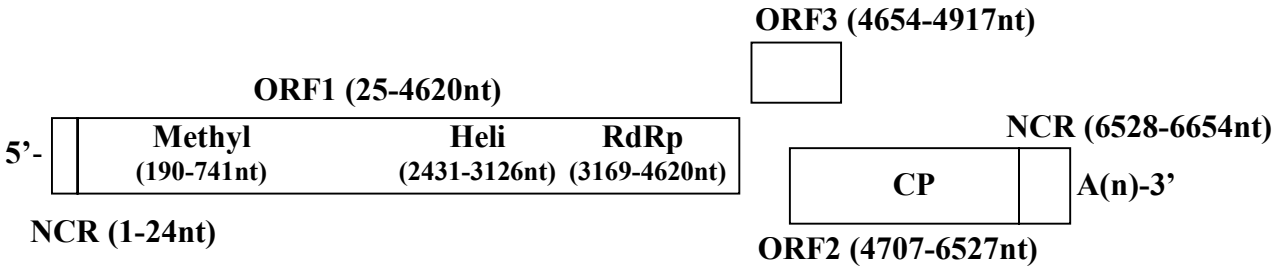

Fig. 2. Schematic diagram of the genomic organization of avian HEV, which contains a short 5' NCR, a 3' NCR and three partially overlapping open reading frames (ORFs): ORF1 encodes non-structural proteins including putative functional domains of methyltransferase (Methyl), helicase (Heli) and RNA dependent RNA polymerase (RdRp); ORF2 encodes putative capsid protein (CP) and ORF3 encodes a small protein with unknown function. The beginning and ending nucleotide (nt) positions of NCRs and ORFs are indicated in parentheses.

Fig. 3

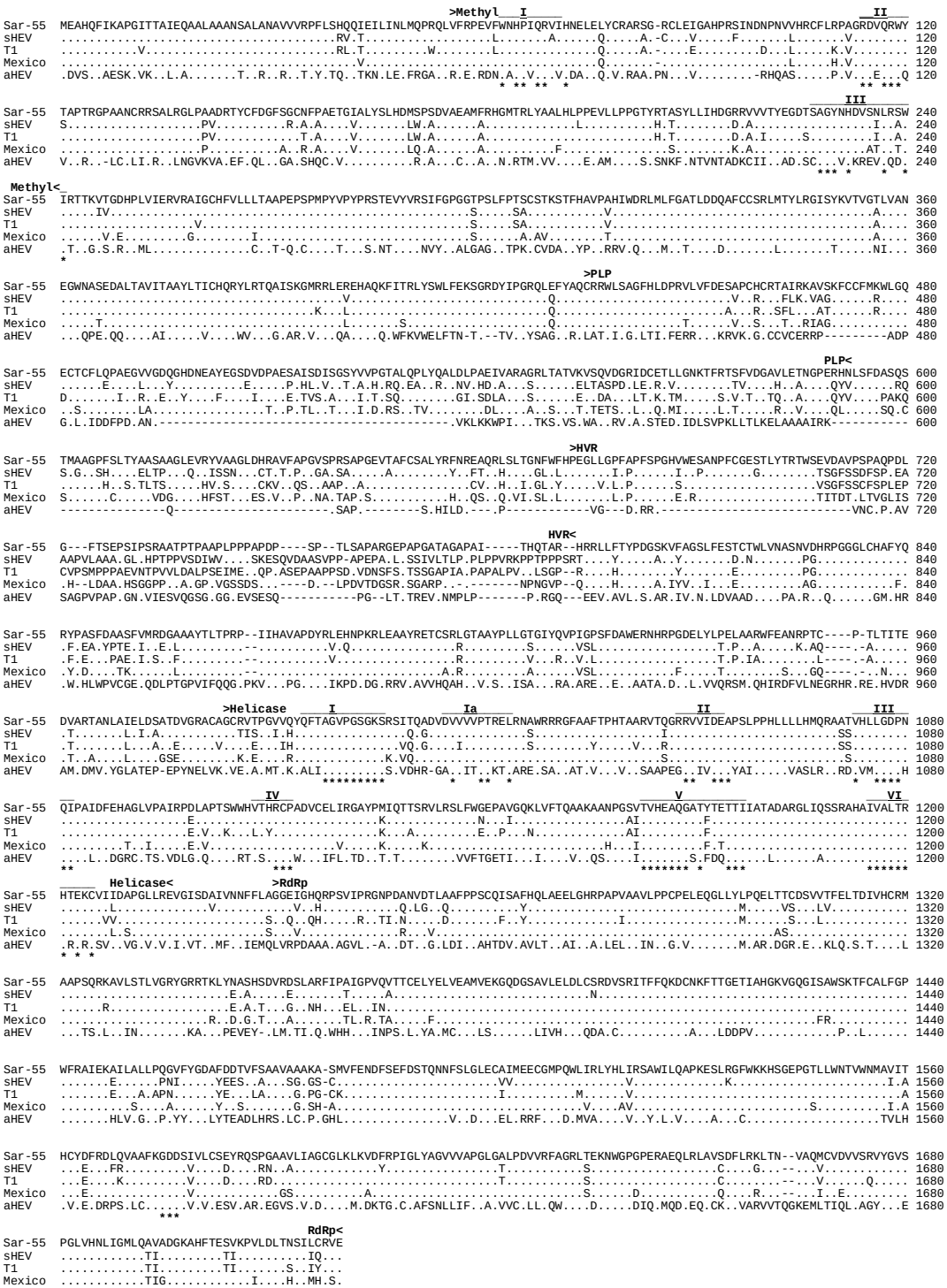
>Methyl___I_____
__II___
Sar-55 MEAHQFIKAPGITTAIEQAALAAANSALANAVVVRPFLSHQQIEILINLMQPRQLVFRPEVFWNHPIQRVIHNELELYCRARSG-RCLEIGAHPRSINDNPNVVHRCFLRPAGRDVQRWY 120
sHEV .....RV.T.....L.....A.....Q.....A.-C...V.....F.....L.....V..... 120
T1 .....RL.T.....W.....L.....Q.....A.-....E.....D...L.....K.V..... 120
Mexico .....V.....Q.....L.....H.V..... 120
aHEV .DVS..AESK.VK..L.A.....T..R..R..T.Y.TQ..TKN.LE.FRGA..R.E.RDN.A..V...V.DA..Q.V.RAA.PN...V.....-RHQAS....P.V...E...Q 120
* ** ** * ** ***
III
Sar-55 TAPTRGPAANCRRSALRGLPAADRTYCFDGFSGCNFPAETGIALYSLHDMSPSDVAEAMFRHGMTRLYAALHLPPEVLLPPGTYRTASYLLIHDGRRVVVTYEGDTSAGYNHDVSNLRSW 240
sHEV S.....PV.....R.A.A....V.....LW.A.....A.....L.....H.T.....D.A.....I..A. 240
T1 .....PV.....T.A....V.....LW.A.....A.....H.T.....D.A.I....S.....I..A. 240
Mexico .....P.....A..R.A....V.....LQ.A.....A.....F.....S.....K.A.....AT..T. 240
aHEV V..R..-LC.LI.R..LNGVKVA.EF.QL..GA.SHQC.V.....R.A...C..A..N.RTM.VV....E.AM....S.SNKF.NTVNTADKCII..AD.SC...V.KREV.QD. 240
*** * * *
Methyl<
Sar-55 IRTTKVTGDHPLVIERVRAIGCHFVLLLTAAPEPSPMPYVPYPRSTEVYVRSIFGPGGTPSLFPTSCSTKSTFHAVPAHIWDRLMLFGATLDDQAFCCSRLMTYLRGISYKVTVGTLVAN 360
sHEV .....IV.....S.....SA.....V.....A.... 360
T1 .....V.....S.....SA.....V.....A.... 360
Mexico .....V.E.....G.....I.....S.....A.AV.....T.....A.... 360
aHEV .T..G.S.R..ML.....C..T-Q.C....T...S.NT....NVY..ALGAG..TPK.CVDA..YP..RRV.Q...M..T....D.....L.....T....NI... 360
*
>PLP
Sar-55 EGWNASEDALTAVITAAYLTICHQRYLRTQAISKGMRRLEREHAQKFITRLYSWLFEKSGRDYIPGRQLEFYAQCRRWLSAGFHLDPRVLVFDESAPCHCRTAIRKAVSKFCCFMKWLGQ 480
sHEV .....Q.....V..R...FLK.VAG.....R.... 480
T1 .....A...R..SFL..AT.....R.... 480
Mexico .....T.....L.....S.....Q.....T.....V..S...T..RIAG..... 480
aHEV ...QPE.QQ....AI.....V....WV...G.AR.V...QA....Q.WFKVWELFTN-T.--TV..YSAG..R.LAT.I.G.LTI.FERR...KRVK.G.CCVCERRP---------ADP 480
PLP<
Sar-55 ECTCFLQPAEGVVGDQGHDNEAYEGSDVDPAESAISDISGSYVVPGTALQPLYQALDLPAEIVARAGRLTATVKVSQVDGRIDCETLLGNKTFRTSFVDGAVLETNGPERHNLSFDASQS 600
sHEV .....E....L...Y.....E.....P.HL.V..T.A.H.RQ.EA..R..NV.HD.A...S.....ELTASPD.LE.R.V.....TV....H..A....QYV.....RQ 600
T1 D.....I..R..E..Y....F....I....E.TVS.A...I.T.SQ.....GI.SDLA...S.....E..DA...LT.K.TM.....S.V.T..TQ..A....QYV....PAKQ 600
Mexico ..S.....LA.....T..P.TL..T...I.D.RS..TV.....DL....A..S...T.TETS..L..Q.MI....L.T.....R..V...QL.....SQ.C 600
aHEV G.L.IDDFPD.AN.----------VKLKKWPI...TKS.VS.WA..RV.A.STED.IDLSVPKLLTLKELAAAAIRK---------- 600
>HVR
Sar-55 TMAAGPFSLTYAASAAGLEVRYVAAGLDHRAVFAPGVSPRSAPGEVTAFCSALYRFNREAQRLSLTGNFWFHPEGLLGPFAPFSPGHVWESANPFCGESTLYTRTWSEVDAVPSPAQPDL 720
sHEV S.G..SH....ELTP...Q..ISSN...CT.T.P..GA.SA.....A.....Y..FT..H....GL.L.....I.P.....I..P.....G.....TSGFSSDFSP.EA 720
T1 .....H..S.TLTS.....HV.S....CKV..QS..AAP..A.....CV..H..I.GL.Y.....V.L.P.....S.....VSGFSSCFSPLEP 720
Mexico S.....C.....VDG....HFST...ES.V..P..NA.TAP.S.....H..QS..Q.VI.SL.L.....L.P.....E.R.....PG.....TITDT.LTVGLIS 720
aHEV ----------Q----------.SAP.--------S.HILD.---.P----------VG---D.RR.----------VNC.P.AV 720
HVR<
Sar-55 G---FTSEPSIPSRAATPTPAAPLPPPAPDP----SP--TLSAPARGEPAPGATAGAPAI-----THQTAR--HRRLLFTYPDGSKVFAGSLFESTCTWLVNASNVDHRPGGGLCHAFYQ 840
sHEV AAPVLAAA.GL.HPTPPVSDIWV....SKESQVDAASVPP-APEPA.L.SSIVLTLP.PLPPVRKPPTPPPSRT....Y.....A..Y.....D.N.....PG..... 840
T1 CVPSMPPPAEVNTPVVLDALPSEIME..QP.ASEPAAPPSD.VDNSFS.TSSGAPIA.PAPALPV..LSGP--R....H.....Y.....E.....PG..... 840
Mexico .H--LDAA.HSGGPP..A.GP.VGSSDS..----D.--LPDVTDGSR.SGARP..-.-------NPNGVP--Q....H.....A.IYV..I...E.....AG.....F. 840
aHEV SAGPVPAP.GN.VIESVQGSG.GG.EVSESQ----------PG--LT.TREV.NMPLP-------P.RGQ---EEV.AVL.S.AR.IV.N.LDVAAD....PA.R..Q.....GM.HR 840
Sar-55 RYPASFDAASFVMRDGAAAYTLTPRP--IIHAVAPDYRLEHNPKRLEAAYRETCSRLGTAAYPLLGTGIYQVPIGPSFDAWERNHRPGDELYLPELAARWFEANRPTC----P-TLTITE 960
sHEV .F.EA.YPTE.I..E.L.....--.....V.Q.....R.....S.....VSL.....T.P..A....K.AQ----.-A..... 960
T1 .F.E...PAE.I.S..F.....--.....V.....R.....V...R..V.L.....T.P.IA.....L----.-A..... 960
Mexico .Y.D...TK.....L.....--.....A.R.....A.....VSL.....F....T.....S...GQ----.--.N... 960
aHEV .W.HLWPVCGE.QDLPTGPVIFQQG.PKV...PG....IKPD.DG.RRV.AVVHQAH..V.S..ISA...RA.ARE..E..AATA.D..L.VVQRSM.QHIRDFVLNEGRHR.RE.HVDR 960
>Helicase ____I_____ ____Ia_____ ____II____ ____III____
Sar-55 DVARTANLAIELDSATDVGRACAGCRVTPGVVQYQFTAGVPGSGKSRSITQADVDVVVVPTRELRNAWRRRGFAAFTPHTAARVTQGRRVVIDEAPSLPPHLLLLHMQRAATVHLLGDPN 1080
sHEV .T.....L.I.A.....TIS..I.H.....Q.G.....S.....I.....SS..... 1080
T1 .T.....L...A..E.....V....E...IH.....VQ.G....I.....S.....Y.....V...R.....SS..... 1080
Mexico .T..A....L....GSE.....K.E...R.....K.VQ.....S.....S..... 1080
aHEV AM.DMV.YGLATEP-EPYNELVK.VE.A.MT.K.ALI.....S.VDHR-GA..IT..KT.ARE.SA..AT.V...V..SAAPEG..IV...YAI.....VASLR..RD.VM....H 1080
********* * ** * ** *** * ****
__ ____IV____ ____V_____ ____VI____
Sar-55 QIPAIDFEHAGLVPAIRPDLAPTSWWHVTHRCPADVCELIRGAYPMIQTTSRVLRSLFWGEPAVGQKLVFTQAAKAANPGSVTVHEAQGATYTETTIIATADARGLIQSSRAHAIVALTR 1200
sHEV .....E.....K.....N..I.....AI.....F..... 1200
T1 .....E.V..K...L.Y.....K...A.....E..P...N.....AI.....F..... 1200
Mexico .....T..I.....E.V.....V.....K.....K.....H...I.....F.T..... 1200
aHEV ....L..DGRC.TS.VDLG.Q....RT.S....W...IFL.TD..T.T.....VVFTGETI...I....V..QS....I.....S.FDQ.....L.....A..... 1200
** *** ******* * *** ******
_____ Helicase< >RdRp
Sar-55 HTEKCVIIDAPGLLREVGISDAIVNNFFLAGGEIGHQRPSVIPRGNPDANVDTLAAFPPSCQISAFHQLAEELGHRPAPVAAVLPPCPELEQGLLYLPQELTTCDSVVTFELTDIVHCRM 1320
sHEV .....L.....V.....V..H.....Q.LG..Q.....Y.....M.....VS...LV..... 1320
T1 .....VV.....S..Q..QH.....R..TI.N.....D.....F..Y.....I.....M.....S...L..... 1320
Mexico .....L.S.....S...V.....R...V.....AS..... 1320
aHEV .R.R.SV..VG.V.V.I.VT..MF..IEMQLVRPDAAA.AGVL.-A..DT..G.LDI..AHTDV.AVLT..AI..A.LEL..IN..G.V.....M.AR.DGR.E..KLQ.S.T....L 1320
* * *
Sar-55 AAPSQRKAVLSTLVGRYGRRTKLYNASHSDVRDSLARFIPAIGPVQVTTCELYELVEAMVEKGQDGSAVLELDLCSRDVSRITFFQKDCNKFTTGETIAHGKVGQGISAWSKTFCALFGP 1440
sHEV .....E.A.....E.....T.....A.....N..... 1440
T1 .....R.....E.A.T...G..NH...EL..IN..... 1440
Mexico .....R..D.G.T...A.....TL.R.TA.....F..... 1440
aHEV ...TS.L..IN.....KA...PEVEY-.LM.TI.Q.WHH...INPS.L.YA.MC...LS.....LIVH...QDA.C.....A...LDDPV.....P..L..... 1440
Sar-55 WFRAIEKAILALLPQGVFYGDAFDDTVFSAAVAAAKA-SMVFENDFSEFDSTQNNFSLGLECAIMEECGMPQWLIRLYHLIRSAWILQAPKESLRGFWKKHSGEPGTLLWNTVWNMAVIT 1560
sHEV .....E.....PNI.....YEES..A...SG.GS-C.....VV.....V.....K.....I.A 1560
T1 .....E...A.APN.....YE...LA....G.PG-CK.....I.....M.....V.....A 1560
Mexico .....S....A.....Y..S.....G.SH-A.....V....AV.....S.....I.A 1560
aHEV .....HLV.G..P.YY...LYTEADLHRS.LC.P.GHL.....V..D...EL.RRF...D.MVA....V..Y.L.V....A...C.....TVLH 1560
Sar-55 HCYDFRDLQVAAFKGDDSIVLCSEYRQSPGAAVLIAGCGLKLKVDFRPIGLYAGVVVAPGLGALPDVVRFAGRLTEKNWGPGPERAEQLRLAVSDFLRKLTN--VAQMCVDVVSRVYGVS 1680
sHEV ...E...FR.....V....D....RN..A.....Y.....T.....S.....C....G.---...V..... 1680
T1 ...E....K.....V....D....RD.....T.....S.....C.....---...V.....Q..... 1680
Mexico ...E.....V.....GS.....A.....S.....D.....Q....R.---...I..E..... 1680
aHEV .V.E.DRPS.LC.....V.V.ESV.AR.EGVS.V.D....M.DKTG.C.AFSNLLIF..A.VVC.LL.QW....D.....DIQ.MQD.EQ.CK..VARVVTQGKEMLTIQL.AGY...E 1680
***
RdRp<
Sar-55 PGLVHNLIGMLQAVADGKAHFTESVKPVLDLTNSILCRVE
sHEV .....TI.....TI.....IQ...
T1 .....TI.....TI.....S..IY...
Mexico .....TIG.....I....H..MH.S.
aHEV V.M.EVVW.A.K.C.AARETLVTNRL...N.SKED-----

Fig. 3. Alignment of the deduced amino acid sequence of avian HEV ORF1 (aHEV) with those of selected HEV strains, one from each of the four genotypes: Sar-55 (genotype 1), Mexico (genotype 2), sHEV (swine HEV, genotype 3), and T1 (genotype 4). The ORF1 sequence of Sar-55 human HEV is shown on the top and only differences in other strains are indicated. Deletions are indicated by dashes. Identical amino acids are indicated by dots. The beginning and ending positions of the putative functional domains in ORF1 of human and swine HEVs are indicated with arrows: methyltransferase (Methyl, positions 56-241), papain-like cysteine protease (PLP, positions 433-593), hypervariable region (HVR, positions 673-802), Helicase (positions 984-1216), and RNA-dependent RNA polymerase (RdRp, positions 1231-1720). The conserved motifs I-VI typical of the helicase superfamily I and the conserved motifs I-III in viral methyltransferase found throughout the alpha-like virus supergroup are indicated. Asterisks indicate identical amino acid residues.

Fig. 4

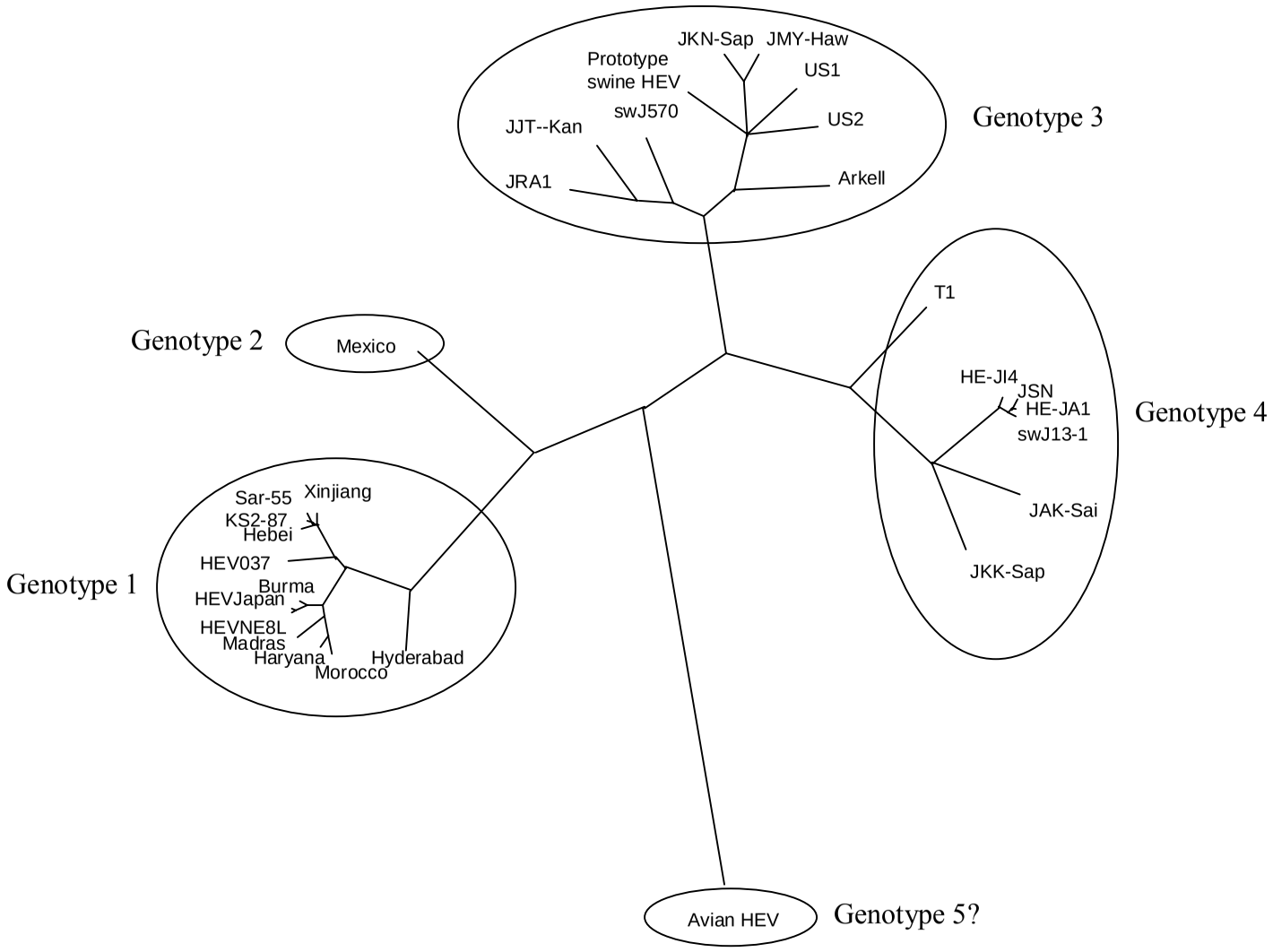
JKN-Sap
JMY-Haw
Prototype swine HEV
US1
swJ570
US2
JJT--Kan
JRA1
Arkell
Genotype 3
T1
Genotype 2
Mexico
HE-JI4
JSN
HE-JA1
swJ13-1
Genotype 4
JAK-Sai
JKK-Sap
Sar-55
Xinjiang
KS2-87
Hebei
HEV037
Burma
HEVJapan
HEVNE8L
Madras
Haryana
Hyderabad
Morocco
Genotype 1
Avian HEV
Genotype 5?
100 changes

Fig. 4. A phylogenetic tree based on the complete genomic sequences of avian HEV and 29 human and swine HEV strains. The tree was constructed with the aid of the PAUP program of 4.0 beta version by using heuristic search with 1,000 replicates of random adding. A scale bar, indicating the number of character state changes, is proportional to the genetic distance.

Table 1. Synthetic oligonucleotide primers used for cDNA amplification, library constructions and screening, and DNA sequencing of the avian HEV genome

Primer	Sequence (5'-3')	Position (nt)
5' AHEVR1	AAA CTC ACG CGC CGC CTG GAC	436-456
5' AHEV RACE Outer	TGG GGG TAA GAA GCA GCG GTG	334-354
5' AHEV RACE Inner	CGC GGC GTA CAT ACT GCT CAA	248-268
5' RACE Adapter	GCU GAU GGC GAU GAA UGA ACA CUG CGU UUG CUG GCU UUG AUG AAA	Non-viral primer
5' RACE Outer Primer	GCT GAT GGC GAT GAA TGA ACA CTG	Non-viral primer
5' RACE Inner Primer	CGC GGA TCC GAA CAC TGC GTT TGC TGG CTT TGA TG	Non-viral primer
Not I RT	TGC GCG GCC GCC TGG GTA AAC ACT ATC T	2918-2945
Labeling R1	ATC TTC TGA CCA ATG GTT TC	2902-2921
Labeling F1	TTG GAT TTC GAC GGA CGC TG	2731-2750
Not I RT-1	ATA GCG GCC GCC CCA CGA TGG TCA ACA G	2504-2531
Labeling R2	ACA CCA GCT ATA AGT GCG TA	2461-2480
Labeling F2	TTA ATT ATG GGC TGG CAA CC	2381-2400
ConF1	GCA TGA CCC CAT GCC AGG GT	1-20
ConR1	CGC ACA CGC CAC GTC CGC AGG	532-552
ConF2	GGT GGG CAT AGC TCT TTA CA	495-514
ConR2	GGT TGA AAT CCC ACG TAA AT	1046-1065
ConF3	TGA TGA TGA CGC CTT CTG TT	1008-1027
ConR3	TCA AAT CCT CCG TGC TAT CA	1542-1561
ConF4	AGT GGG CTC AAG TCA GGG TC	1517-1536
ConR4	ATA ACC TTA GGT GGC CCC TG	2074-2093
ConF5	TTG CGG CGA GGT CCA GGA TT	2028-2047
ConR5	CTC GGG GGC TGC ACT TGC TG	2600-2619
ConF6	CCA CGG CTG TTA CAC CCC AC	2576-2595
ConR6	TGC GCC TCA TGG ACC GTT AT	2962-2981
Seq RL	CAA CAG ACG ACG ACT TGC CG	2489-2509
Seq R1	AAT CCT GGA CCT CGC CGC AA	2028-2047
Seq R2	GCC ACT TTT TCA ACT TCA CTA	1463-1483
Seq R3	GGT TGA AAT CCC ACG TAA AT	1046-1065
Seq R4	CAT GTC GTG TAA GCT GTA AA	509-528
FAHEVEp	CAT CCA CCC CTA CAA GCA TTG AC	5188-5210
RAHEVEp	TAC GCA ACA CAT CCC CTG ACC T	5937-5958
FAHEVEpF	GCC GCT TGG TAT GGT TGA TTT T	5426-5447
RAHEVEpR	GGC ATC CTC AAC CGA CAT ATA C	5777-5798

Table 2. Nucleotide sequence comparison of avian HEV with selected human and swine HEV strains

Genotype	HEV strains	Avian HEV (% nt identity)		
		Full-length	ORF1	Helicase
1	Sar-55	50*	50	59
	Burma	50	50	59
	Xinjiang	50	50	59
	Morocco	51	51	58
2	Mexico	50	50	58
3	US2	49	50	56
	Prototype swine HEV	49	50	56
	Arkell	49	49	56
	JRA1	49	49	56
	SWJ570	50	50	57
4	T1	49	49	57
	SWJ13-1	49	48	57

Chapter 4

Construction and characterization of infectious cDNA clones of a chicken strain of the hepatitis E virus (HEV), avian HEV

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ABSTRACT

Hepatitis E virus (HEV), the causative agent of human hepatitis E, is an important human pathogen, and increasing evidence indicates that hepatitis E is a zoonotic disease. Avian HEV was recently discovered from chickens with hepatitis-splenomegaly syndrome in the United States. Like swine HEV from pigs, avian HEV is also genetically and antigenically related to human HEV. The objective of this study is to construct and characterize an infectious cDNA clone of avian HEV for future study of HEV replication and pathogenesis. Three full-length cDNA clones of avian HEV, pT7-aHEV-5, pT7G-aHEV-10 and pT7G-aHEV-6, were constructed, and their infectivity was tested by *in vitro* transfection of the LMH chicken liver cells and by direct intrahepatic inoculation of specific-pathogen-free (SPF) chickens with capped RNA transcripts from the 3 clones. The results showed that the capped RNA transcripts from each of the clones were replication-competent when transfected into LMH cells as demonstrated by detection of avian HEV antigens with avian HEV-specific antibodies. SPF chickens intrahepatically inoculated with the capped RNA transcripts from each of the 3 clones developed active avian HEV infections as evidenced by seroconversion to avian HEV antibodies, viremia and fecal virus shedding. The results indicated that all 3 cDNA clones of avian HEV are infectious both *in vitro* and *in vivo*. The availability of these infectious clones for a chicken strain of HEV now affords an opportunity to study the mechanisms of HEV cross-species infection and tissue tropism by constructing chimeric viruses among human, swine and avian HEVs.

INTRODUCTION

Hepatitis E virus (HEV), the causative agent of human hepatitis E, is an important public health concern in many developing countries (9). HEV is primarily transmitted via fecal-oral route through contaminated water. The mortality rate associated with HEV infection can reach up to 25% in infected pregnant women (43). HEV is a single-stranded, positive-sense RNA virus of about 7.2 kb in length (43). The viral genome contains three open reading frames (ORFs): ORF1 encodes nonstructural proteins such as a methyltransferase, a papain-like protease, a helicase, and an RNA-dependent RNA polymerase (RdRp), ORF2 encodes a capsid protein, and ORF3 encodes a small protein of unknown functions (21, 43). HEV was classified in the *Caliciviridae* family but was recently declassified and placed in a new genus *Hepevirus (7)*.

Although HEV infections were only sporadic in the United States and other industrialized countries, an unexpected high seroprevalence rate has been reported in healthy individuals (3, 12, 20, 31, 45). Anti-HEV antibodies were also detected in various animal species including pigs, sheep, cows, and rodents, suggesting that HEV or a related agent infects these animals (2, 11, 19, 24, 25, 32, 46). Increasing evidence indicates that HEV is a zoonotic agent and that there exist animal reservoirs for HEV (24). The first animal strain of HEV, swine HEV, was identified from pigs in the United States in 1997 (28). The prototype strain of swine HEV identified in the United States is genetically closely-related to two U.S. strains of human HEV and other genotype 3 strains of human HEV worldwide (22, 37-39). Similarly, numerous swine HEV isolates identified from other countries are also genetically closely-related to strains of human HEV, especially genotype 3 or 4 HEV strains (16, 35, 42, 48). Cross-species infection of genotype 3 human and swine HEVs has been documented: swine HEV infected non-human primates and a US strain of human HEV (US-2 strain) infected pigs (13, 27). Seroepidemiological studies revealed that swine veterinarians and other pig handlers had an increased risk of HEV infection compared to normal blood donors (6, 29, 47). Recently, cluster cases of acute hepatitis E in Japan were epidemiologically linked to consumptions of uncooked or raw pig and deer meats, thus providing more convincing evidence of zoonotic HEV transmission (23, 44, 49).

In 2001, another animal strain of HEV, designated avian HEV, was identified from chickens with hepatitis-splenomegaly (HS) syndrome in the United States (15). Avian HEV shared about 80% nucleotide sequence identity with the Australian big liver and spleen disease virus (BLSV) in a short stretch of sequence available for BLSV (15, 34). The complete genomic sequence of avian HEV has been determined (18). Sequence analysis revealed that avian HEV is genetically related to human HEV with about 50-60% sequence identity (18). Avian HEV also shares common antigenic epitopes on the capsid protein, similar genomic organizations, and conserved functional domains and motifs in ORF1 with human and swine HEVs (15, 18). Avian HEV is enzootic in chicken flocks from the United States and has the ability to cross species barrier and infect turkeys (17, 41). Phylogenetic analysis revealed that avian HEV forms a branch distinct from human and swine HEVs, and thus may represent a fifth genotype of HEV or a new genus (18).

Due to the lack of an efficient cell culture system or a practical animal model for HEV, little is known about the mechanisms of HEV pathogenesis and replication. Two infectious cDNA clones of genotype 1 human HEV have been reported (10, 33). Here we report the construction and characterization of infectious cDNA clones of avian HEV.

MATERIALS AND METHODS

Virus and cells. The virus used in the study was a standard infectious stock of avian HEV with an infectious titer of 5×10^5 50% chicken infectious dose per ml (41). LMH chicken liver cell line (CRL-2117) was purchased from the American Type Culture Collection. The cells were maintained in the Waymouth's MB 752/1 medium (Invitrogen) containing 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37˚C with 5% CO_2.

Construction of initial full-length cDNA clones of avian HEV. Total RNAs were extracted from the avian HEV infectious stock with Trizol reagent, and used for cDNA synthesis with SuperScript II reverse transcriptase (Invitrogen) and specific reverse primers (Table 1). Four fragments were subsequently amplified with platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen) by PCR using 4 sets of PCR primers (Table

1). Fragment I representing the 5' end of viral genome was amplified with the primers XbaIT7F1 and PstI3340R (Table 1). The sense primer XbaIT7F1, 5'-GC**TCTAGA**TAATACGACTCACTATAgcatgaccccatgccagggt-3', contained an engineered *Xba*I restriction enzyme site (bold), a T7 core promoter sequence (underlined), followed by the 5' end of avian HEV sequence (lowercase). Fragment I' was the same as fragment I except for an extra G added between the T7 core promoter and the viral sequence by using sense primer XbaIT7GF1 (Table 1). Fragment III representing the 3' end of the viral genome was amplified with primers EcoRIF5019 and T18MluIXhoIR. The antisense primer, T18MluIXhoIR, introduced 18 adenosine nucleotides, and *Mlu*I and *Xho*I restriction sites, at the 3' end of avian HEV genome. Fragment II covering the internal part of the avian HEV genome was amplified with primers of KpnIF and EcoRIR5258 and partially overlapped with fragments I (or I') and III. By using the convenient restriction sites *BamH*I and *EcoR*I naturally present in the overlapping regions (Fig. 1), these PCR fragments were ligated in order into pGEM-7zf (+) vector between *Xho*I and *Xba*I sites in the polylinker. The full-length cDNA clone assembled with fragments I, II and III was designated as pT7-aHEV, and the one assembled with fragment I', II and III as pT7G-aHEV.

Site-directed mutagenesis to produce three new full-length cDNA clones of avian HEV. The consensus genomic sequence of avian HEV was determined by directly sequencing the RT-PCR products amplified from the viral genome. After determining the complete sequence of clones pT7-aHEV and pT7G-aHEV and comparing them to the consensus sequence, a total of 9 non-silent mutations were found in each of the clones pT7-aHEV and pT7G-aHEV. By utilizing the 3 convenient fragments (I/I', II and III), site-directed mutagenesis with QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) was used to change these non-silent mutations back to the consensus sequence of avian HEV. Silent mutations were kept as genetic markers (Table 2). Three new full-length cDNA clones, pT7-aHEV-5, pT7G-aHEV-6 and pT7G-aHEV-10, were produced. Subsequent sequencing of all 3 new clones revealed that clones pT7G-aHEV-6 and pT7G-aHEV-10 had an identical sequence (Table 2).

***In vitro* transcription.** The 3 full-length cDNA clones were linearized by digestion with *Xho*I, and purified by phenol/chloroform extraction and ethanol precipitation. Capped RNA transcripts were synthesized with mMESSAGE mMACHINE® T7 Kit (Ambion). Briefly, each reaction was performed in a 20 µl reaction mixture containing 2 µg of linerized cDNA template, 2 µl of 10 × reaction buffer, 10 µl of 2 × NTP/Cap, 2 µl of enzyme mix and an additional 1 µl of a 30 mM GTP stock for capping. The mixtures were incubated at 37˚C for 1.5 hr, and 0.5 µl of each reaction product was run in a 0.8% agarose gel to check the quality of the RNA transcripts. RNA transcripts from each cDNA clone were directly used for *in vitro* transfection of LMH chicken liver cells. For intrahepatic inoculation of chickens, the RNA transcripts were diluted 1:4 with cold RNase-, DNase-, and proteinase-free PBS buffer, frozen on dry ice and used for inoculation on the same day.

***In vitro* transfection and immunofluorescent assay (IFA).** Prior to transfection, LMH cells growing on a 12-well plate were washed with Waymouth's MB 752/1 medium. Different amounts of RNA transcripts, 8 µl, 14 µl and 18 µl, from each of avian HEV cDNA clones were mixed with 4 µl of Plus Reagent (Invitrogen) in 25 µl Waymouth's MB 752/1medium. After 15 min reaction, the mixtures were combined with 1 µl of Lipofectamine (Invitrogen) diluted in 25 µl of the Waymouth's MB 752/1 medium and incubated for 15 min. The RNA transcripts, Plus Reagent, and Lipofectamine mixtures were then added onto washed LMH cells covered with 200 µl of Waymouth's MB 752/1 medium. After incubation at 37˚C for 3 hr, 600 µl of fresh culture medium was added, and the transfected cells were incubated at 37˚C.

On day 5 after transfection, the transfected cells were trypsinized and replated on 8-well LabTek chamber slides. Slides were fixed and stained on day 6. Briefly, cells on slides were rinsed with PBS buffer, then fixed with a solution containing 80% acetone and 20% methanol, and air-dried. A 1:100 diluted anti-avian HEV convalescent serum from a SPF chicken experimentally infected with avian HEV was added to the fixed cells, and incubated for 25 min at 37˚C. After washing with cold PBS buffer, a 1:100 diluted *Fluorescein*-labeled goat anti-chicken IgG (KPL) was added and incubated at 37˚C for 25

min. The slides were washed by PBS buffer, covered with fluoromount-G (Southern Biotechnology Associates, Inc), and viewed under a fluorescence microscopy.

Intrahepatic inoculation of SPF chickens with RNA transcripts from 3 full-length cDNA clones of avian HEV. Experiment 1: eight, 6-week-old, SPF chickens that were negative for avian HEV RNA and antibodies, were randomly divided into four groups of 2 each (groups A, B, C, and D). Two chickens in each group were housed in a single isolator. The RNA inocula were quickly thawed and immediately injected, through a percutaneous procedure, into two different sites on each side of the liver with about 100 µl per injection site. Two chickens in group A (#3765 and #3768) were each injected with 400 µl of RNA transcripts from clone pT7-aHEV-5, chickens #2572 and #2568 in group B each with 400 µl of RNA transcripts from clone pT7G-aHEV-6, and chickens #3761 and #3767 in group C each with 400 µl of RNA transcripts from clone pT7G-aHEV-10. The 2 chickens in group D were not inoculated and served as negative controls. Fecal swabs were collected from each chicken every 3 days, and sera were collected every week. All inoculated chickens were necropsied at 9 weeks postinoculation.

Experiment 2: The percutaneous intrahepatic injection of RNA transcripts into chicken livers was a blind procedure. For confirmation purpose, we conducted a second chicken experiment using a surgical approach. In this modified procedure, intrahepatic inoculation was performed using a right parasternal incision, and the RNA transcripts were directly injected into the right lobe of the liver following visualization. Two chickens (#3762 and #4496) of 14 weeks of age in group A' were each injected with 400 µl of RNA transcripts from clone pT7-aHEV-5, and chickens #4493 and #4500 in group C' with 400 µl of RNA transcripts from clone pT7G-aHEV-10. Chickens #2569 and #4494 in group D remained uninoculated to serve as negative controls. Fecal swabs and sera were collected similarly as described in experiment 1. All chickens were necropsied at 6 weeks postinoculation.

Detection of genetic markers in viruses recovered from the experimentally infected chickens. Viruse recovered from fecal material of chickens #3768, #2572, and #3767 at

14 days postinoculation (DPI) was used to amplify selected viral genomic regions by RT-PCR to determine the presence of genetic markers. A nested PCR with external primer pairs F2422 and R2931 and internal primers pairs F2495 and R2919 (Table 1) was used to amplify the helicase gene region containing the genetic markers at nt positions 2586, 2592, 2622, and 2886. Another nested PCR with external primers F4914/R5301, and internal primers F4992/R5254 (Table 1) was used to amplify the ORF2 gene region containing the genetic markers at nt positions 5093, 5102, 5111, and 5210. Primers F5973, R6350 and R6407 (Table 1) were used to detect the genetic marker at nt position 6110 in ORF3. The PCR products were purified with a GENECLEAN kit (Bio 101 Inc) and directly sequenced at the Virginia Tech DNA Sequencing Facility.

ELISA and PCR. Fecal swabs and serum samples were tested by RT-PCR for avian HEV RNA as previously described. Sera were also tested for anti-avian HEV antibodies by ELISA (17, 18).

RESULTS

Construction of three cDNA clones of avian HEV. Initially, two full-length cDNA clones were constructed by ligation of three overlapping fragments covering the entire genome with appropriate restriction enzyme sites (Fig. 1). Clone pT7-aHEV had an *Xba*I restriction site followed by the T7 core promoter sequence immediately proceeding the extreme 5' end of avian HEV genome, and 18 adenines followed by *Mlu*I and *Xho*I restriction sites at the 3' end of avian HEV genome. Compared to clone pT7-aHEV, an extra G was incorporated between the end of core T7 promoter sequence and the 5' end of viral sequence in clone pT7G-aHEV since it has been shown that the extra nucleotide G could affect the transcription efficiency.

Consensus genomic sequence of avian HEV, determined by direct sequencing of RT-PCR products amplified from the viral genome, differed at 24 positions from the sequence obtained from cloned fragments (18). Compared to the consensus sequence, the cDNA clone pT7-aHEV contained 21 mutations including 9 non-silent mutations, and the cDNA clone pT7G-aHEV had 19 mutations including 9 non-silent mutations. The non-silent mutations were changed back to the consensus sequence by using site-directed

mutagenesis to create 3 new clones pT7-aHEV-5, pT7G-aHEV-6, and pT7G-aHEV-10 (Table 2).

Capped RNA transcripts from each of the three clones were replication-competent when transfected into chicken liver cells. RNA transcripts from each of the 3 clones were transcribed *in vitro* in the presence of a cap catalog. The addition of an extra G between the T7 core promoter and the 5' end of viral sequence of clones pT7G-aHEV-6 and pT7G-aHEV-10 had no significant effect on the transcription efficiency as similar amounts of RNA transcripts were produced from each of the 3 clones (data not shown). Capped RNA transcripts from each of the three full-length cDNA clones were subsequently transfected into LMH chicken liver cells to determinate the infectivity. Viral antigens were detected in transfected LMH cells by IFA with anti-avian HEV convalescent serum (Fig. 2). Like Huh7 cells transfected with RNA transcripts from genotype 1 human HEV cDNA clone (pSKHEV-2) (8), the fluorescent signals were mainly located in the cytoplasm of LMH cells. About 10-15% of the cells were positive for avian HEV antigen (Fig. 2), however virus spread from cell to cell was not evident. Mock-transfected cells were negative for viral antigens.

Capped RNA transcripts from three cDNA clones of avian HEV were infectious when intrahepatically injected into the livers of SPF chickens via a percutaneous procedure. All chickens in groups A, B and C inoculated with RNA transcripts from each of the three clones seroconverted to IgG anti-avian HEV at 3-6 weeks postinoculation, indicating that active avian HEV infection had occurred in the inoculated chickens (Fig. 3A). The two negative control chickens (#2569 and #4494) in group D remained seronegative throughout the study (Fig. 3A). Fecal virus shedding was detected variably in all inoculated chickens in groups A, B and C (Table 3). All but one chicken (#2568) began fecal virus shedding at 9 days postinoculation (DPI). Fecal virus shedding for chicken #2568 (group B) did not occur until 24 DPI (Table 3). It is possible that RNA transcripts were not injected into the liver of chicken #2568 (or only small amount was injected) due to the fact that percutaneous procedure was performed blindly. The infection in chicken #2568 could occur as a result of direct contact with the infected bird

(#2572) housed in the same isolator, since seroconversion in chicken #2568 occurred 2 weeks after chicken #2572 had seroconverted (Fig. 3A). Viremia was transient or undetectable in infected chickens (Table 4), which is consistent with previous observations in chickens infected with avian HEV (4), in pigs experimentally infected with swine HEV (26), and in primates experimentally infected with human HEV (13).

Detection of genetic markers in viruses recovered from infected chickens. Viruses recovered from feces of chicken #3768 in group A, chicken #2572 in group B, and chicken #3767 in group C, were tested for the presence of genetic markers for the 3 clones. Sequence analysis revealed that the genetic markers of 10 silent mutations in ORF1, ORF2 and ORF3 were all detected in recovered viruses from chickens #3767, #3768, and #2572. In addition, chicken # 3768 had an extra non-silent mutation at position nt 5089, that resulted a change from alanine to valine.

***In vivo* transfection of chickens by intrahepatic inoculation via laparoscopy with capped RNA transcripts from 2 clones.** Retrospective sequencing and sequence analysis revealed that clones pT7G-aHEV-6 and pT7G-aHEV-10 had an identical full-length genomic sequence. Therefore, only clones pT7-aHEV-5 and pT7G-aHEV-10 were included in the surgical study. Compared to the percutaneous intrahepatic injection procedure in which the liver can not be visualized, the right parasternal approach allowed direct visualization of the liver prior to inoculation. Viral RNA was detected in fecal materials of all 4 injected chickens starting from 9 or 12 DPI (Table 3). Similarly, only 2 of the 4 infected chickens had detectable viremia (Table 4). All 4 chickens seroconverted to avian HEV antibodies, although chicken #4493 had low titer of antibodies, and chicken #4498 had a delayed seroconversion (Fig. 3B).

DISCUSSION

Avian HEV is genetically and antigenically related to human and swine HEVs (14, 15, 18). It shares similar genomic organizations and functional motifs and domains with human and swine HEVs (18). Infectious cDNA clones of genotype 1 human HEV are available (10, 33). We report here the construction and characterization of infectious

cDNA clones of avian HEV. We demonstrated that capped RNA transcripts from 3 full-length cDNA clones of avian HEV were replication-competent when transfected into chicken liver LMH cells, and infectious when intrahepatically inoculated into the livers of SPF chickens. Detection of genetic markers in recovered viruses from infected chickens confirmed that the rescued viruses originated from their respective clones. The availability of these infectious cDNA clones of a chicken strain of HEV now affords us an opportunity to study the mechanisms of cross-species infection and tissue tropism by constructing chimeric viruses among human, swine and avian HEVs.

It has been demonstrated that a cap structure is required for the infectivity of human HEV Sar-55 strain cDNA clones (10). The 5' terminal sequence of the avian HEV genome was amplified by a pyrophosphaphatase-dependent elongation reaction, suggesting that avian HEV genomic RNA is also capped (18). Therefore, capped RNA transcripts were synthesized in this study to test their infectivity *in vivo* and *in vitro*. It is known that an extra nucleotide G at the end of T7 core promoter is important for the *in vitro* transcription efficiency of RNA (30, 36). Therefore, we added an extra G in the clone pT7G-aHEV. In addition, nonviral sequences (*Mlu*I restriction site and a C from digestion by *Xho*I) were also introduced at the 3' end of viral genome. Our results showed that these extra nonviral nucleotides at both 5' and 3' end of avian HEV genome did not affect the infectivity of these clones in chickens or in LMH chicken liver cells. Unexpected mutations, introduced during steps of RT-PCR, cloning or due to infidelity of RNA polymerase during *in vitro* transcription steps, are of concern for the construction of infectious cDNA clones. The silent mutations in these clones had no effect on the infectivity of the clones and were still present in the viruses rescued from infected chickens. A non-silent mutation, not present in the original clone, A5089, was detected in the recovered virus from a chicken intrahepatically inoculated with RNA transcripts from clone pT7-aHEV-5. This non-silent mutation is likely due to the quasispecies nature of the virus, which is evidenced by the heterogeneity of avian HEV isolates from chickens in different regions of the U.S. (17, 40).

Fecal virus shedding, viremia and seroconversion were detected variably in chickens intrahepatically injected with RNA transcripts from each of the cDNA clones, indicating that the clones are infectious. However, viremia was transient or absent.

Chickens without viremia generally had delayed seroconversions or relatively low anti-avian HEV antibody levels. Lack of viremia has also been reported in some hepatitis E patients and in experimentally infected chimpanzees (5). Ultrasound-guided intrahepatic injection was used for *in vivo* testing of infectious clones of human HEV in non-human primates (1, 26). However, this procedure is not applicable for chickens due to their small size. Therefore, we attempted the percutaneous and the surgical methods for intrahepatic injection. Our results showed that both procedures are equally sufficient to induce infection.

Capped RNA transcripts from human HEV Sar-55 strain infectious clone were replication-competent in two human liver cell lines PLC/PRF/5 and Huh-7, and an intestinal cell line Caco-2, although the virus progeny produced from cells transfected with the infectious clone did not spread (8). Cell lines of non-human primates, pigs, and rodent origins transfected with the RNA transcripts from the Sar-55 human HEV clone did not support virus replication, indicating that species-specific factors are involved in virus replication. It has been shown that avian HEV can cross species barrier and infect turkeys, but failed to infect 2 rhesus monkeys (18, 41). Therefore, to test the infectivity of the avian HEV clones *in vitro*, we used a liver cell line of chicken origin, LMH. As expected, RNA transcripts from the avian HEV clones were replication-competent in the LMH cells. Like human HEV Sar-55 infectious clone (8), the fluorescent signals in LMH cells transfected with avian HEV RNA were also detected in the cytoplasma of dividing cells, but the virus progeny did not spread from cell to cell. Nevertheless, the LMH cell culture system is still useful for the study of HEV biology, as we can now test the viability of mutant clones of avian HEV in LMH cells and study the mechanism of HEV replication.

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REFERENCES

1. **Aggarwal, R., S. Kamili, J. Spelbring, and K. Krawczynski.** 2001. Experimental studies on subclinical hepatitis E virus infection in cynomolgus macaques. J Infect Dis **184:**1380-5.
2. **Arankalle, V. A., M. V. Joshi, A. M. Kulkarni, S. S. Gandhe, L. P. Chobe, S. S. Rautmare, A. C. Mishra, and V. S. Padbidri.** 2001. Prevalence of anti-hepatitis E virus antibodies in different Indian animal species. J Viral Hepat **8:**223-7.
3. **Balayan, M. S.** 1997. Epidemiology of hepatitis E virus infection. J Viral Hepat **4:**155-65.
4. **Billam, P., F.F. Huang, Z.F. Sun, D.K. Guenette, F.W. Pierson, T.E. Toth, R. Duncan, F. Elvinger, T.E. Toth and X.J. Meng.** 2004. Systematic pathogenesis and replication of a strain of the hepatitis E virus (HEV) in its natural host: avian HEV infections in specific-pathogen-free adult chickens. J Virol **In press**.
5. **Clayson, E. T., B. L. Innis, K. S. Myint, S. Narupiti, D. W. Vaughn, S. Giri, P. Ranabhat, and M. P. Shrestha.** 1995. Detection of hepatitis E virus infections among domestic swine in the Kathmandu Valley of Nepal. Am J Trop Med Hyg **53:**228-32.
6. **Drobeniuc, J., M. O. Favorov, C. N. Shapiro, B. P. Bell, E. E. Mast, A. Dadu, D. Culver, P. Iarovoi, B. H. Robertson, and H. S. Margolis.** 2001. Hepatitis E virus antibody prevalence among persons who work with swine. J Infect Dis **184:**1594-7.
7. **Emerson, S. U., D. Anderson, A. Arankalle, X. J. Meng, M. Purdy, G. G. Schlauder, and S. A. Tsarev.** 2004. *Hepevirus*. C.M. Fauquet, M.A. Mayo, J. Maniloff, U. Desselberger, and L.A. Ball. Elsevier/Academic Press. p. 851-855.
8. **Emerson, S. U., H. Nguyen, J. Graff, D. A. Stephany, A. Brockington, and R. H. Purcell.** 2004. In vitro replication of hepatitis E virus (HEV) genomes and of an HEV replicon expressing green fluorescent protein. J Virol **78:**4838-46.
9. **Emerson, S. U., and R. H. Purcell.** 2003. Hepatitis E virus. Rev Med Virol **13:**145-54.

10. **Emerson, S. U., M. Zhang, X. J. Meng, H. Nguyen, M. St Claire, S. Govindarajan, Y. K. Huang, and R. H. Purcell.** 2001. Recombinant hepatitis E virus genomes infectious for primates: importance of capping and discovery of a cis-reactive element. Proc Natl Acad Sci U S A **98:**15270-5.

11. **Favorov, M. O., M. Y. Kosoy, S. A. Tsarev, J. E. Childs, and H. S. Margolis.** 2000. Prevalence of antibody to hepatitis E virus among rodents in the United States. J Infect Dis **181:**449-55.

12. **Fukuda, S., J. Sunaga, N. Saito, K. Fujimura, Y. Itoh, M. Sasaki, F. Tsuda, M. Takahashi, T. Nishizawa, and H. Okamoto.** 2004. Prevalence of antibodies to hepatitis E virus among Japanese blood donors: identification of three blood donors infected with a genotype 3 hepatitis E virus. J Med Virol **73:**554-61.

13. **Halbur, P. G., C. Kasorndorkbua, C. Gilbert, D. Guenette, M. B. Potters, R. H. Purcell, S. U. Emerson, T. E. Toth, and X. J. Meng.** 2001. Comparative pathogenesis of infection of pigs with hepatitis E viruses recovered from a pig and a human. J Clin Microbiol **39:**918-23.

14. **Haqshenas, G., F. F. Huang, M. Fenaux, D. K. Guenette, F. W. Pierson, C. T. Larsen, H. L. Shivaprasad, T. E. Toth, and X. J. Meng.** 2002. The putative capsid protein of the newly identified avian hepatitis E virus shares antigenic epitopes with that of swine and human hepatitis E viruses and chicken big liver and spleen disease virus. J Gen Virol **83:**2201-9.

15. **Haqshenas, G., H. L. Shivaprasad, P. R. Woolcock, D. H. Read, and X. J. Meng.** 2001. Genetic identification and characterization of a novel virus related to human hepatitis E virus from chickens with hepatitis-splenomegaly syndrome in the United States. J Gen Virol **82:**2449-62.

16. **Hsieh, S. Y., X. J. Meng, Y. H. Wu, S. T. Liu, A. W. Tam, D. Y. Lin, and Y. F. Liaw.** 1999. Identity of a novel swine hepatitis E virus in Taiwan forming a monophyletic group with Taiwan isolates of human hepatitis E virus. J Clin Microbiol **37:**3828-34.

17. **Huang, F. F., G. Haqshenas, H. L. Shivaprasad, D. K. Guenette, P. R. Woolcock, C. T. Larsen, F. W. Pierson, F. Elvinger, T. E. Toth, and X. J. Meng.** 2002. Heterogeneity and seroprevalence of a newly identified avian

hepatitis e virus from chickens in the United States. J Clin Microbiol **40:**4197-202.

18. **Huang, F. F., Z. F. Sun, S. U. Emerson, R. H. Purcell, H. L. Shivaprasad, F. W. Pierson, T. E. Toth, and X. J. Meng.** 2004. Determination and analysis of the complete genomic sequence of avian hepatitis E virus (avian HEV) and attempts to infect rhesus monkeys with avian HEV. J Gen Virol **85:**1609-18.
19. **Kabrane-Lazizi, Y., J. B. Fine, J. Elm, G. E. Glass, H. Higa, A. Diwan, C. J. Gibbs, Jr., X. J. Meng, S. U. Emerson, and R. H. Purcell.** 1999. Evidence for widespread infection of wild rats with hepatitis E virus in the United States. Am J Trop Med Hyg **61:**331-5.
20. **Kamel, M. A., H. Troonen, H. P. Kapprell, A. el-Ayady, and F. D. Miller.** 1995. Seroepidemiology of hepatitis E virus in the Egyptian Nile Delta. J Med Virol **47:**399-403.
21. **Koonin, E. V., A. E. Gorbalenya, M. A. Purdy, M. N. Rozanov, G. R. Reyes, and D. W. Bradley.** 1992. Computer-assisted assignment of functional domains in the nonstructural polyprotein of hepatitis E virus: delineation of an additional group of positive-strand RNA plant and animal viruses. Proc Natl Acad Sci U S A **89:**8259-63.
22. **Kwo, P. Y., G. G. Schlauder, H. A. Carpenter, P. J. Murphy, J. E. Rosenblatt, G. J. Dawson, E. E. Mast, K. Krawczynski, and V. Balan.** 1997. Acute hepatitis E by a new isolate acquired in the United States. Mayo Clin Proc **72:**1133-6.
23. **Matsuda, H., K. Okada, K. Takahashi, and S. Mishiro.** 2003. Severe hepatitis E virus infection after ingestion of uncooked liver from a wild boar. J Infect Dis **188:**944.
24. **Meng, X. J.** 2000. Novel strains of hepatitis E virus identified from humans and other animal species: is hepatitis E a zoonosis? J Hepatol **33:**842-5.
25. **Meng, X. J., S. Dea, R. E. Engle, R. Friendship, Y. S. Lyoo, T. Sirinarumitr, K. Urairong, D. Wang, D. Wong, D. Yoo, Y. Zhang, R. H. Purcell, and S. U. Emerson.** 1999. Prevalence of antibodies to the hepatitis E virus in pigs from

countries where hepatitis E is common or is rare in the human population. J Med Virol **59:**297-302.

26. **Meng, X. J., P. G. Halbur, J. S. Haynes, T. S. Tsareva, J. D. Bruna, R. L. Royer, R. H. Purcell, and S. U. Emerson.** 1998. Experimental infection of pigs with the newly identified swine hepatitis E virus (swine HEV), but not with human strains of HEV. Arch Virol **143:**1405-15.
27. **Meng, X. J., P. G. Halbur, M. S. Shapiro, S. Govindarajan, J. D. Bruna, I. K. Mushahwar, R. H. Purcell, and S. U. Emerson.** 1998. Genetic and experimental evidence for cross-species infection by swine hepatitis E virus. J Virol **72:**9714-21.
28. **Meng, X. J., R. H. Purcell, P. G. Halbur, J. R. Lehman, D. M. Webb, T. S. Tsareva, J. S. Haynes, B. J. Thacker, and S. U. Emerson.** 1997. A novel virus in swine is closely related to the human hepatitis E virus. Proc Natl Acad Sci U S A **94:**9860-5.
29. **Meng, X. J., B. Wiseman, F. Elvinger, D. K. Guenette, T. E. Toth, R. E. Engle, S. U. Emerson, and R. H. Purcell.** 2002. Prevalence of antibodies to hepatitis E virus in veterinarians working with swine and in normal blood donors in the United States and other countries. J Clin Microbiol **40:**117-22.
30. **Milligan, J. F., D. R. Groebe, G. W. Witherell, and O. C. Uhlenbeck.** 1987. Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. Nucleic Acids Res **15:**8783-98.
31. **Moaven, L., M. Van Asten, N. Crofts, and S. A. Locarnini.** 1995. Seroepidemiology of hepatitis E in selected Australian populations. J Med Virol **45:**326-30.
32. **Okamoto, H., M. Takahashi, T. Nishizawa, R. Usui, and E. Kobayashi.** 2004. Presence of antibodies to hepatitis E virus in Japanese pet cats. Infection **32:**57-8.
33. **Panda, S. K., I. H. Ansari, H. Durgapal, S. Agrawal, and S. Jameel.** 2000. The in vitro-synthesized RNA from a cDNA clone of hepatitis E virus is infectious. J Virol **74:**2430-7.

34. **Payne, C. J., T. M. Ellis, S. L. Plant, A. R. Gregory, and G. E. Wilcox.** 1999. Sequence data suggests big liver and spleen disease virus (BLSV) is genetically related to hepatitis E virus. Vet Microbiol **68:**119-25.

35. **Pina, S., M. Buti, M. Cotrina, J. Piella, and R. Girones.** 2000. HEV identified in serum from humans with acute hepatitis and in sewage of animal origin in Spain. J Hepatol **33:**826-33.

36. **Sasaki, J., Y. Kusuhara, Y. Maeno, N. Kobayashi, T. Yamashita, K. Sakae, N. Takeda, and K. Taniguchi.** 2001. Construction of an infectious cDNA clone of Aichi virus (a new member of the family Picornaviridae) and mutational analysis of a stem-loop structure at the 5' end of the genome. J Virol **75:**8021-30.

37. **Schlauder, G. G., G. J. Dawson, J. C. Erker, P. Y. Kwo, M. F. Knigge, D. L. Smalley, J. E. Rosenblatt, S. M. Desai, and I. K. Mushahwar.** 1998. The sequence and phylogenetic analysis of a novel hepatitis E virus isolated from a patient with acute hepatitis reported in the United States. J Gen Virol **79 (Pt 3):**447-56.

38. **Schlauder, G. G., S. M. Desai, A. R. Zanetti, N. C. Tassopoulos, and I. K. Mushahwar.** 1999. Novel hepatitis E virus (HEV) isolates from Europe: evidence for additional genotypes of HEV. J Med Virol **57:**243-51.

39. **Schlauder, G. G., and I. K. Mushahwar.** 2001. Genetic heterogeneity of hepatitis E virus. J Med Virol **65:**282-92.

40. **Sun, Z. F., C. T. Larsen, A. Dunlop, F. F. Huang, F. W. Pierson, T. E. Toth, and X. J. Meng.** 2004. Genetic identification of avian hepatitis E virus (HEV) from healthy chicken flocks and characterization of the capsid gene of 14 avian HEV isolates from chickens with hepatitis-splenomegaly syndrome in different geographical regions of the United States. J Gen Virol **85:**693-700.

41. **Sun, Z. F., C. T. Larsen, F. F. Huang, P. Billam, F. W. Pierson, T. E. Toth, and X. J. Meng.** 2004. Generation and infectivity titration of an infectious stock of avian hepatitis E virus (HEV) in chickens and cross-species infection of turkeys with avian HEV. J Clin Microbiol **42:**2658-62.

42. **Takahashi, M., T. Nishizawa, H. Miyajima, Y. Gotanda, T. Iita, F. Tsuda, and H. Okamoto.** 2003. Swine hepatitis E virus strains in Japan form four

phylogenetic clusters comparable with those of Japanese isolates of human hepatitis E virus. J Gen Virol **84:**851-62.

43. **Tam, A. W., M. M. Smith, M. E. Guerra, C. C. Huang, D. W. Bradley, K. E. Fry, and G. R. Reyes.** 1991. Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. Virology **185:**120-31.
44. **Tei, S., N. Kitajima, K. Takahashi, and S. Mishiro.** 2003. Zoonotic transmission of hepatitis E virus from deer to human beings. Lancet **362:**371-3.
45. **Thomas, D. L., P. O. Yarbough, D. Vlahov, S. A. Tsarev, K. E. Nelson, A. J. Saah, and R. H. Purcell.** 1997. Seroreactivity to hepatitis E virus in areas where the disease is not endemic. J Clin Microbiol **35:**1244-7.
46. **Wang, Y. C., H. Y. Zhang, N. S. Xia, G. Peng, H. Y. Lan, H. Zhuang, Y. H. Zhu, S. W. Li, K. G. Tian, W. J. Gu, J. X. Lin, X. Wu, H. M. Li, and T. J. Harrison.** 2002. Prevalence, isolation, and partial sequence analysis of hepatitis E virus from domestic animals in China. J Med Virol **67:**516-21.
47. **Withers, M. R., M. T. Correa, M. Morrow, M. E. Stebbins, J. Seriwatana, W. D. Webster, M. B. Boak, and D. W. Vaughn.** 2002. Antibody levels to hepatitis E virus in North Carolina swine workers, non-swine workers, swine, and murids. Am J Trop Med Hyg **66:**384-8.
48. **Wu, J. C., C. M. Chen, T. Y. Chiang, W. H. Tsai, W. J. Jeng, I. J. Sheen, C. C. Lin, and X. J. Meng.** 2002. Spread of hepatitis E virus among different-aged pigs: two-year survey in Taiwan. J Med Virol **66:**488-92.
49. **Yazaki, Y., H. Mizuo, M. Takahashi, T. Nishizawa, N. Sasaki, Y. Gotanda, and H. Okamoto.** 2003. Sporadic acute or fulminant hepatitis E in Hokkaido, Japan, may be food-borne, as suggested by the presence of hepatitis E virus in pig liver as food. J Gen Virol **84:**2351-7.

Fig. 1

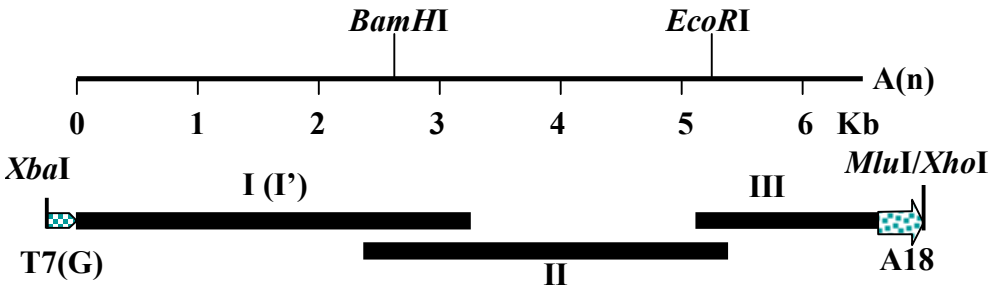


Fig. 1. Construction of full-length cDNA clones of avian HEV. *BamH*I and *EcoR*I are the unique restriction sites naturally present in avian HEV genome, and were utilized to construct the full-length cDNA clones. A stretch of 18 poly(A) tail, *Mlu*I and *Xho*I sites were introduced at the 3' end of the genome. *Xba*I site and a T7 core promoter sequence were engineered at the 5' end in fragment I. *Xba*I site, a T7 core promote sequence and an additional G were added at the 5' end of fragment I'. Fragments I, II and III were ligated in order into pGEM-7zf (+) vector to produce clone pT7-aHEV, and fragments I', II and III were ligated to create clone pT7G-aHEV.

Fig. 2

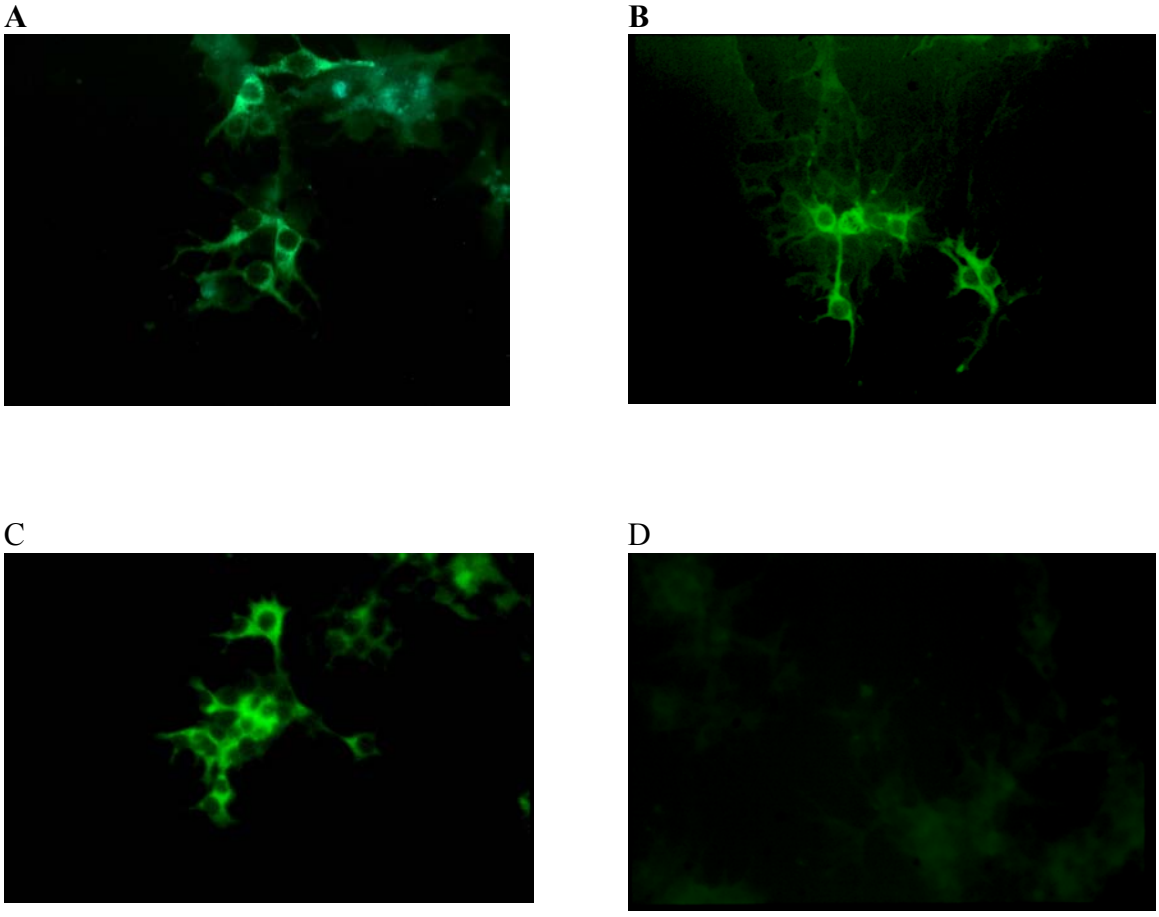
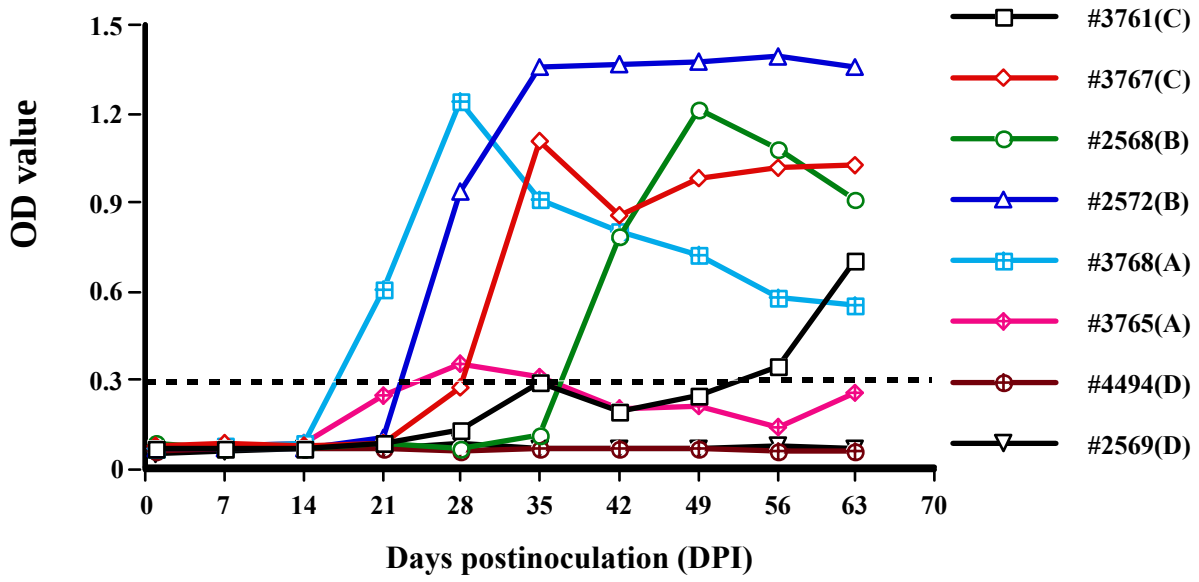


Fig. 2. Immunofluorescent staining of LMH chicken liver cells transfected with RNA transcripts from the clones of pT7-aHEV-5 (panel A), pT7G-aHEV-6 (panel B) and pT7G-aHEV-10 (panel C) or nontransfected cells as negative control (panel D).

Fig. 3

A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1.2
0.9
0.6
0.3
0
0
7
14
21
28
35
42
49
56
63
70
Days postinoculation (DPI)
#3761(C)
#3767(C)
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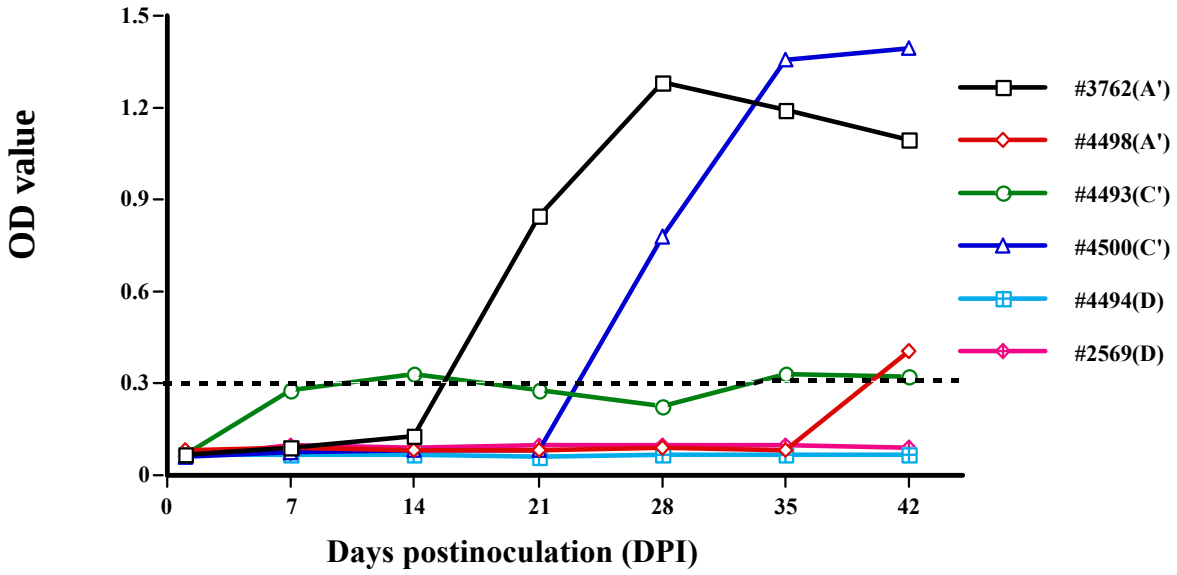
#2572(B)
#3768(A)
#3765(A)
#4494(D)
#2569(D)

B


OD value
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1.2
0.9
0.6
0.3
0
0
7
14
21
28
35
42
Days postinoculation (DPI)
#3762(A')
#4498(A')
#4493(C')
#4500(C')
#4494(D)
#2569(D)

Fig. 3. Seroconversion to IgG anti-avian HEV in chickens intrahepatically injected with capped RNA transcripts from avian HEV clones. IgG anti-avian HEV is plotted as ELISA OD value (A405) (Y axle) at different days postinoculation (DPI) (X axle), and the cut-off value is 0.3. (A) Chickens were intrahepatically injected with RNA transcripts from each of the 3 clones by percutaneous approach: chickens #3761, #3767 with clone pT7G-aHEV-10, chickens #3765, #3768 with clone pT7-aHEV-5, and chickens #2572, #2568 with clone pT7G-aHEV-6. Chickens #4494 and #2569 were uninoculated as negative controls. (B) Chickens were intrahepatically injected with RNA transcripts of 2 clones by surgical approach: chickens #4493 and #4500 with clone pT7G-aHEV-10, chickens #3762, #4498 with clone pT7-aHEV-5, and chickens #2569 and #4494 served as uninoculated, negative controls.

Table 1. Oligonucleotide primers used for the construction of avian HEV infectious cDNA clones

Primer ID	Sequence (5'-3')[a]
XbaIT7F1	gctctagataatacgactcactataGCATGACCCCATGCCAGGGT
XbaIT7GF1	gctctagataatacgactcactatagGCATGACCCCATGCCAGGGT
PstI3340R	ATAATAGGCCCTGCTCCAATAC
KpnIF	GTTGATGCCACGTTCTACCCTG
EcoRIR5258	TATAGTCAGCAAACCAGCAGAG
EcoRIF5019	GTGTCACCGTTAATGCCGCTTC
T18MluIXhoIR	ctcgagacgcgttttttttttttttttttttACTATGCCCGAGATG
F2422	CGGGCTATGGCTGACATGGTT
F2495	AGTCGTCGTCTGTTGACCAT
R2919	CCACCTGGGTAAACACTATC
R2931	TGCGCCTCATGGACCGTTATG
F4914	GTAGATCAAGCCGGTGCCGTGC
F4992	ACCAATGCCTTGTTTATGCCG
R5254	GTCAGCAAGCCAGCAGAGCCCG
R5301	CGGACCGCCAACCATTGTTCTT
F5973	CAGTATGTGACTAACGCCGA
R6350	GATCACTAGCTTGGATTGGC
R6407	GAGGCAGACAGGTGGTACAG

a, Lowercase letters indicate non-viral sequence.

Table 2. Silent mutations used as genetic markers in the 3 cDNA clones compared to the consensus sequence of avian HEV genome

Fragment	Genome region	consensus	pT7-aHEV-5	pT7G-aHEV-6	pT7G-aHEV-10
I(I')	Methyltransferase	450 T	G	T	T
	Hypervariable	1908 C	T	C	C
	Helicase	2586 T	C	C	C
		2592 C	T	T	T
		2622 C	T	T	T
		2886 T	C	C	C
II	ORF2	5093 G	A	A	A
		5102 G	A	A	A
		5111 G	A	A	A
		5174 C	T	T	T
		5210 T	C	C	C
III	ORF3	6110 T	A	A	A

Table 3. Fecal virus shedding in SPF chickens intrahepatically injected with RNA transcripts of the avian HEV cDNA clones

Clone ID	Group	Chicken#	Days postinoculation (DPI)														
			0	3	6	9	12	15	18	21	24	27	30	33	36	39	42
pT7-aHEV-5	A	3768[a]	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-
		3765[a]	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
	A'	3762[b]	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-
		4498[b]	-	-	-	+	-	-	+	+	-	-	-	-	-	-	-
pT7G-aHEV-6	B	2568[a]	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-
		2572[a]	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
pT7G-aHEV-10	C	3761[a]	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-
		3767[a]	-	-	-	+	+	-	+	+	+	+	+	-	-	-	-
	C'	4493[b]	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
		4500[b]	-	-	-	-	+	-	+	+	+	+	-	+	+	-	-
Uninoculated	D	4494,2569	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

a, intrahepatic injection of RNA inocula via percutaneous approach;
b, intrahepatic injection of RNA inocula via surgical approach.

Table 4. Detection of viremia in SPF chickens intrahepatically injected with RNA transcripts of avian HEV cDNA clones

Clone ID	Group	Chicken#	Days postinoculation (DPI)						
			0	7	14	21	28	35	42
pT7-aHEV-5	A	3768[a]	-	-	-	-	-	-	-
		3765[a]	-	-	+	-	-	-	-
	A'	3762[b]	-	-	+	+	-	-	-
		4498[b]	-	-	-	-	-	-	-
pT7G-aHEV-6	B	2568[a]	-	-	-	-	-	-	-
		2572[a]	-	-	+	+	+	+	+
pT7G-aHEV-10	C	3761[a]	-	-	-	-	-	-	-
		3767[a]	-	-	-	+	+	-	-
	C'	4493[b]	-	-	-	-	-	-	-
		4500[b]	-	-	-	+	+	+	+
Uninoculated	D	4494, 2569	-	-	-	-	-	-	-

a, intrahepatic injection of RNA inocula via percutaneous approach;
b, intrahepatic injection of RNA inocula via surgical approach.

Chapter 5

Detection by Reverse Transcription-PCR and Genetic Characterization of Field Isolates of Swine Hepatitis E Virus from Pigs in Different Geographic Regions of the United States

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ABSTRACT

Hepatitis E virus (HEV) is an important public health concern in many developing countries. HEV is also endemic in some industrialized counties including the United States. With our recent discovery of swine HEV in pigs that is genetically closely related to human HEV, hepatitis E is now considered as a zoonotic disease. Human strains of HEV are genetically heterogenetic. So far in the United States, only one strain of swine HEV has been identified and characterized from a pig. To determine the extent of genetic variations and the nature of swine HEV infections in U.S. pigs, we developed a universal RT-PCR assay that is capable of detecting genetically divergent strains of HEV. By using this universal RT-PCR assay, we tested fecal and serum samples of pigs of 2 to 4 months of age from 37 different U.S. swine farms for the presence of swine HEV RNA. Thirty-four of the 96 pigs (35%) and 20 of the 37 swine herds (54%) tested were positive for swine HEV RNA. The sequences of a 348-bp region within the ORF2 gene of 27 swine HEV isolates from different geographic regions were determined. Sequence analyses revealed that the 27 U.S. swine HEV isolates shared 88 to 100% nucleotide sequence identities to each other and 89% to 98% identities with the prototype U.S. strain of swine HEV. These U.S. swine HEV isolates are only distantly related to the Taiwanese strains of swine HEV with about 74 to 78% nucleotide sequence identities, to most known human strains of HEV worldwide, with <79% sequence identities, and to avian HEV, with 54-56% sequence identities. Phylogenetic analysis showed that all the U.S. swine HEV isolates identified in this study clustered in the same genotype with the

prototype U.S. swine HEV and the two U.S. strains of human HEV. The data from this study indicated that swine HEV is widespread and enzootic in U.S. swine herds, and that, as with human HEV, swine HEV isolates from different geographic regions of the world are also genetically heterogenic. These data further raise potential concerns for zoonosis, xenozoonosis and food safety.

INTRODUCTION

Hepatitis E is a very important public health disease in many developing countries and is also endemic in some industrialized countries including the United States (1, 26, 31, 33). The disease primarily affects young adults, and reportedly has a mortality rate of up to 25% in pregnant women (8, 12, 31). Hepatitis E virus (HEV), the causative agent of hepatitis E, is primarily transmitted by the fecal-oral route through contaminated water or water supplies (1, 31). HEV is a positive-sense, single-strand RNA virus without an envelope. The virus remains unclassified although it was once classified in the family *Caliciviridae* (13, 30). The genome of HEV is approximately 7.5 kb and contains three open reading frames (ORFs) and a short 5' and 3' non-translated region. ORF1 is the largest of the three ORFs and encodes nonstructural proteins, ORF2 encodes the putative capsid protein and ORF3 encodes a small protein of unknown function (1, 11, 17, 20, 31, 38, 44). In the United States, sporadic cases of acute hepatitis E in patients with no known epidemiological exposure have been reported (4, 32). Anti-HEV antibodies have been detected in a significant proportion of healthy individuals in the United States (19, 25, 28, 36). The existence of an animal reservoir for HEV has been proposed (25-27).

Swine hepatitis E virus (swine HEV), the first animal strain of HEV, was identified and characterized in 1997 from a pig in the United States (21). It has been shown that swine HEV is very closely related to the two U.S. strains of human HEV (US1, US2) but is genetically distinct from other known strains of HEV worldwide (4, 21, 24, 32). Interspecies transmission of HEV has been documented: swine HEV infects non-human primates and the US2 strain of human HEV infects pigs (7, 23). More recently, a strain of avian HEV was identified from chickens with Hepatitis-Splenomegaly Syndrome in the United States (9). Avian HEV is genetically related to but distinct from other known HEV strains (9). The single U.S. swine HEV strain and the several Taiwanese swine HEV strains identified thus far are more closely related to strains of human HEV from the same geographic regions than to those from distant regions (10, 23, 43). Most recently, we showed that swine veterinarians in the United States (28) and other pig handlers in China, Taiwan and Thailand (10, 24) are at increased risk of zoonotic HEV infections, suggesting that swine are animal reservoirs for HEV.

The relatively high prevalence of anti-HEV in human populations not at apparent risk of exposure to swine HEV (28), suggests that multiple sources of exposure to HEV may exist in the general U.S. population and that swine are not the only animal reservoir for HEV. Anti-HEV has been detected in wild-caught rats in the United States and other countries (6, 14, 18, 39). In Vietnam, anti-HEV has been detected in 44% of chickens, 36% of pigs, 27% of dogs and 9% of rats (37). Favorov et al (5) reported that anti-HEV is detected in about 29 to 62% of cows from three HEV endemic countries (Somalia, Tajikistan and Turkmenistan) and in 12% of cows in a non-endemic country (Ukraine). In Turkmenistan, about 42 to 67% of the sheep and goats were also found to be positive for IgG anti-HEV. Naturally acquired anti-HEV was also detected in rhesus macaques (2). These serological data strongly suggest that these animal species have been exposed to HEV (or a related agent), and that they could also serve as animal reservoirs for HEV.

Human strains of HEV are very heterogeneic with at least 4 distinct genotypes worldwide (9, 32-33). Most human strains of HEV identified from the same geographic regions tended to cluster together, although a few strains from the same geographic region differed significantly in their genomic sequences (33, 40-41). Since only one strain of swine HEV has been identified and characterized from a pig in the United States (21), the extent of genetic variations among swine HEV isolates and the nature of swine HEV infections in the U.S. swine herds are not known. The objectives of this study were to develop a universal RT-PCR assay capable of detecting genetically divergent strains of HEV, and to genetically identify and characterize field isolates of swine HEV from pigs in different geographic regions of the United States.

MATERIALS AND METHODS

Clinical specimens. Fecal and serum samples used in this study were collected from 95 pigs of 2-4 months of age, and one 7-month-old pig from 37 different herds in 6 U.S. States (AR, IA, MI, MO, NC, and OK). Seventy fecal samples were obtained from pigs submitted to the Iowa State University Veterinary Diagnostic Laboratory. Sixteen serum and 10 fecal samples were obtained from pigs submitted to the University of Missouri Veterinary Medical Diagnostic Laboratory. The pigs were submitted to the Diagnostic

Laboratories for a wide variety of health problems that are not related to swine HEV infection. Fecal samples from rectal swabs were re-suspended in 10% calcium- and magnesium-free phosphate-buffered saline (PBS). Serum and fecal samples were stored at -70°C until analyzed.

Primer design for RT-PCR. To develop a "universal" RT-PCR assay that is capable of detecting HEV strains with significant sequence variations, 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 was performed (21). Based upon the multiple sequence alignment, two sets of degenerate HEV primers were designed for the 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 RT-PCR was 348-bp.

In addition, a published nested RT-PCR assay (16, 22, 42) specific for the prototype U.S. strain of swine HEV was also used in this study. The primers for the specific RT-PCR assay were based upon the published sequence of the prototype U.S. strain of swine HEV (21): external primer set 3329 (forward, 5'-AGCTCCTGTACCTGA TGTTGACTC-3'), and 3330 (reverse, 5'-CTACAGAGCGCCAGCCTTGATTGC-3'); internal primer set 3331 (forward, 5'-GCTCACGTCATCTGTCGCTGCTGG-3'), and 3332 (reverse, 5'-GGGCTGAACCAAAATCCTGACATC-3').

Development and standardization of the universal HEV RT-PCR assay for detection of field isolates of swine HEV. Since HEV strains (including swine HEV) identified from different geographic regions are genetically heterogenic, a universal HEV RT-PCR assay with degenerate HEV primers was developed in this study to detect genetically divergent strains of HEV. To evaluate if the universal RT-PCR assay with degenerate HEV primers could detect known strains of HEV with significant sequence variations, total RNAs were extracted with TriZol Reagent® (GIBCO-BRL) from 100 µl of the US2

strain of human HEV (4, 32), the Pakistani strain (Sar-55) of human HEV (38), and the prototype U.S. strain of swine HEV (21). Total RNAs were resuspended in DNase- and RNase-free water. Reverse transcription was performed at 42°C for 60 min with Superscript II reverse transcriptase® (GIBCO-BRL) using reverse primer 3157N. Five µl of the resulting cDNA was amplified by the universal RT-PCR assay using Ampli*Taq* gold DNA polymerase® (Perkin–Elmer). The PCR parameters for the first round PCR with primers 3156N and 3157N included a denaturation step at 95°C for 9 min, followed by 39 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 42°C, extension for 2 min at 72°C, and a final incubation at 72°C for 7 min. The parameters for the second round PCR were similar except primers 3158N and 3159N were used.

To determine the sensitivity of the universal RT-PCR assay, the prototype U.S. strain of swine HEV (21-23) with a known infectious titer of $10^{4.5}$ 50% pig infectious dose (PID_{50}) per ml was serially diluted 10-fold in PBS. Total RNAs extracted from 100 µl of each dilution were tested using the universal RT-PCR assay, as well as with a published RT-PCR assay (22, 42) specific for the prototype U.S. strain of swine HEV. The PCR parameters for the universal PCR assay are the same as described above. The first- and second-round PCR parameters for the RT-PCR assay specific for the prototype swine HEV were similar, with an initial denaturation step at 95°C for 9 min, followed by 39 cycles of denaturation for 1 min at 94°C, annealing for 1.5 min at 54°C, extension for 1 min at 72°C, and a final incubation at 72°C for 7 min.

After the universal RT-PCR assay with degenerate HEV primers was standardized, 80 fecal and 16 serum samples from pigs in 37 different herds of different geographic regions (Table 1) were tested by the universal RT-PCR assay. Negative and positive controls were included in each set of PCR reactions. The negative control was water treated the same way as the fecal suspensions and sera. The positive control was the prototype U.S. strain of swine HEV. The amplified PCR products were examined by agarose gel electrophoresis.

Nucleotide sequencing. The expected PCR products amplified from fecal or serum samples of pigs were purified using the glassmilk procedure with a GENECLEAN kit ® (Bio 101 Inc.). PCR products amplified from 27 selected pigs were directly sequenced at

the Virginia Tech DNA Sequencing Facility. Sequences of the PCR products were determined for both DNA strands.

Sequence and phylogenetic analyses. The primer sequences used to amplify the field isolates of swine HEV were excluded for the final sequence and phylogenetic analyses. The resulting 304 bp sequences in the ORF2 genes of the 27 U.S. isolates of swine HEV were analyzed and compared with the corresponding regions of other known human, swine and avian HEV strains available in the GenBank by the MacVector computer program (Oxford Molecular Inc) (Table 2). The percentages of nucleotide and amino acid sequence identities among different HEV strains were determined with the MacVector program. Phylogenetic analysis was conducted with the aid of the PAUP program of 4.0 beta version (David L. Swofford, Smithsonian Institute, Washington, DC, distributed by Sinauer Associates Inc., Sunderland, MA). Heuristic search with 1,000 replicates was used to produce a phylogenetic tree.

The geographic origins and the GenBank accession numbers of the nucleotide sequences of the HEV strains used in the phylogenetic and sequence analyses are as follows: TK78/87 (AF020608, Nepal), TK104/91 (AF020603, Nepal), TK15/92 (AF020604, Nepal), Nep4/94 (AF020607, Nepal), TK4/95 (AF020606, Nepal), Hyderabad (AF076239, India), M75 (AF093894, India), AKL-90 (AF124407, India), Y67 (AF093892, India), Madras (X99441, India), Hev037 (X98292, India), Sar-55 (M80581, Pakistan), abb-2B (U40044, Pakistan), Vietnam (AF170450), Burma (M73218), JRA1 (AP003430, Japan), JPHEV (E17109, Japan), Uigh179 (D11093, China), Lanzhou (AF141652, China), K52-87 (L25595, China), Hetian (L08816, China), Ch-T11 (AF151962, China), Ch-T21 (AF151963, China), Ch-T1 (AJ272108, China), Tw6310e (AF117279, Taiwan), Tw8e-2 (AF117275, Taiwan), Tw6196e (AF117278, Taiwan), Tw2494e (AF117276, Taiwan), Tw5483e (AF117277, Taiwan), Tw32sw (AF117280, Taiwan swine HEV), Tw74sw (AF117281, Taiwan swine HEV), Morocco (AF065061, Morocco), Mexico (M74506), VH1 (AF195061, Spain), VH2 (AF195062, Spain), E11 (AF195063, Spain), prototype swine HEV (AF082843, USA), HEV-US1 (AF060668, USA), HEV-US2 (AF060669, USA), and Avian HEV (AY043166, USA).

Nuclotide sequence accession number. The resulting sequences of the 27 swine HEV isolates described in "Nucleotide sequencing" above have been deposited with the GenBank database under accession numbers AF466659 to AF466685.

RESULTS

Sensitivity and specificity of the universal RT-PCR assay. To evaluate if the universal RT-PCR assay developed in this study is capable of detecting different strains of HEV, we tested three genetically divergent HEV strains available to us: the human Sar-55 strain (38), the US2 human HEV strain (4, 32) and the prototype swine HEV strain (21). These three divergent HEV strains differed in their ORF2 gene sequences by at least 20% (23). Total RNAs extracted from the three HEV strains were all tested positive by the universal RT-PCR assay with degenerate HEV primers. We designed this RT-PCR assay with degenerate HEV primers "universal RT-PCR assay" to distinguish it from the published RT-PCR assay (22, 42) which is specific for the single prototype swine HEV isolate.

To evaluate the sensitivity of the universal RT-PCR assay, we compared it with our published RT-PCR assay specific for the prototype U.S. strain of swine HEV (22, 42) to determine its ability to detect a serially diluted virus stock of the prototype U.S. swine HEV strain. The sensitivity of our published RT-PCR assay specific for the prototype swine HEV has been previously determined using an *in vitro* synthesized HEV RNA (42). In this study, the sensitivities of the two assays were compared by using an infectious virus stock of the prototype swine HEV with a known infectious titer. The published RT-PCR assay specific for the prototype swine HEV isolate detected about 3.2 PID_{50} of swine HEV while the universal RT-PCR assay detected about 31.6 PID_{50} of swine HEV. Therefore, the sensitivities of the two assays are comparable, as there is only a 1-log difference in the detection of the prototype swine HEV.

Genetic identification of field isolates of swine HEV from young pigs in different geographic regions of the United States. Our previous seroepidemiological studies indicated that pigs in the United States become infected by swine HEV between the ages

of 2 and 4 months (21, 24). Therefore, young pigs of 2-4 month-of-age were chosen for this study. Since the universal HEV RT-PCR assay developed in this study is capable of detecting genetically divergent strains of HEV, it was therefore used to detect field isolates of swine HEV from fecal and serum samples randomly collected from pigs in different geographic regions of the United States. The results showed that 34 of the 96 pigs (35%) and 20 of the 37 swine herds (54%) tested in this study were positive for swine HEV RNA. The one 7-month-old pig (no. 01-9913) from a farm in Oklahoma was also tested positive for swine HEV RNA (Table 1).

Sequence analyses. The PCR products from 27 of the 34 positive pigs were sequenced. The PCR products from the other 7 positive pigs were not sequenced as they were from the same herds as those sequenced. The 304-bp sequence within the ORF2 genes of the 27 swine HEV isolates were analyzed and compared to each other as well as to other known human, swine and avian strains of HEV (Table 2). Sequence analyses revealed that these U.S. swine HEV isolates shared 88-100% nucleotide sequence identities with each other, 89-98% identities with the prototype U.S. swine HEV strain, 89-96% identities with two U.S. human HEV strains (US1 and US2), 85-88% identities with a Japanese strain of human HEV (JRA1) thought to be of swine origin (36), and 81-86% identities with a Spanish strain of HEV (E11) also thought to be of swine origin (29) (Table 2). However, the U.S. swine HEV isolates were genetically divergent from the Taiwanese strains of swine HEV (Tw32sw, Tw74sw) with about 74-78% sequence identities (10, 43) and from other known human strains of HEV (< 79% sequence identities) (Table 2). The swine HEV isolates shared 54-56% nucleotide sequence identities with the newly identified avian HEV. In general, swine HEV isolates identified from the same geographic region displayed higher percentages of sequence identities than to those from different geographic regions (Table 2). Most of these nucleotide changes were found to represent silent mutations and did not result in significant differences at the amino acid level. These U.S. swine HEV isolates displayed 91-100% amino acid sequence identities with other swine and human HEV strains but only 52-56% amino acid sequence identities with the avian HEV.

Phylogenetic analysis. Phylogenetic analysis showed that all the U.S. swine HEV isolates identified in this study clustered in the same genotype with the prototype U.S. swine HEV strain and with the US1 and US2 strains of human HEV (Fig. 1). Swine HEV isolates identified from the same geographic region tended to cluster together, and thus minor branches among these U.S. swine and human HEV isolates were observed (Fig. 1). Besides the two U.S. strains of human HEV (US1, US2), the U.S. swine HEV isolates appeared to be more closely related to the Japanese strain of human HEV (JRA1) and a Spanish strain of HEV (E11) (29, 36) than to other strains of HEV worldwide. The Taiwanese strains of swine HEV were genetically distinct from the U.S. swine HEV strains but closely related to the Taiwanese strains of human HEV. The U.S. swine HEV isolates were also distinct from the recently discovered avian HEV.

DISCUSSION

Recently, numerous novel strains of human HEV have been identified from patients with acute hepatitis in both developing and industrialized countries (4, 10, 15, 29). The intriguing fact is that these novel strains of human HEV are genetically distinct from each other and from other known strains of HEV. The sources of these novel HEV strains are not known, however, it is hypothesized that they may be of animal origin since there exist several potential animal reservoirs for HEV (2-3, 5-6, 14, 25-27, 34, 39). Genetic identifications of swine HEV from pigs (21) and avian HEV from chickens (9) have given credence to this hypothesis. Recently, in a well controlled large-scale seroepidemiological study, we showed that swine veterinarians in the United States were 1.51 times more likely to be anti-HEV positive than normal U.S. blood donors (28). We found that there was a difference in anti-HEV prevalence in both swine veterinarians and normal blood donors among 8 selected states, with subjects from Minnesota (a major swine state) six times more likely to be anti-HEV positive than those from Alabama (a traditionally non-swine state). Age was not a factor for the observed differences from state to state (28). These data provide compelling evidence that swine HEV infects humans.

Since the identification and characterization of the first strain of swine HEV from a U.S. pig, several additional strains of swine HEV have been identified from pigs in Taiwan (10, 43). The Taiwanese swine HEV strains are genetically distinct from the prototype U.S. swine HEV strain. Since only one strain of swine HEV has been identified from a pig in the United States, the extent of genetic variation among swine HEV isolates and the nature of swine HEV infection in pigs in the United States are not known. In order to identify field isolates of swine HEV from pigs in the United States, a sensitive and broadly reactive diagnostic assay is needed since swine HEV infection in pigs is subclinical. In this study, we developed a universal RT-PCR assay with degenerate HEV primers that is capable of detecting genetically divergent strains of HEV. We showed that the sensitivity of the universal RT-PCR assay was comparable to that of the published RT-PCR assay (22, 42) specific for the prototype U.S. swine HEV strain. Therefore, this universal HEV RT-PCR assay was used for the detection of genetically divergent strains of HEV from pigs in different geographic regions.

By using this universal RT-PCR assay, we tested fecal and serum samples for swine HEV RNA from pigs of 2 to 4 months of age from 37 herds in different geographic regions of the United States. About 35% of the pigs and 54% of herds tested in this study are positive for swine HEV RNA, indicating that swine HEV infection is enzootic and widespread in the United States. It is possible that the negative herds tested by RT-PCR may acquire swine HEV infection at different time points (other than 2-4 months of age) or these negative herds may have better biosecurity measures that could prevent transmission of swine HEV. The swine samples used in this study were submitted to Veterinary Diagnostic Laboratories for diagnoses of diseases unrelated to swine HEV infection. There was no apparent correlation between clinical signs and the presence or absence of swine HEV RNA in the feces. This is consistent with our earlier findings that swine HEV causes only subclinical infection in naturally (21) and experimentally (7, 22) infected pigs.

The U.S. swine HEV isolates identified from pigs in different geographic regions shared significant nucleotide sequence identities with each other (88-100%), and with the prototype U.S. strain of swine HEV (89-98%). Swine HEV isolates identified from the same pig farm or the same geographic region tended to be more closely related to each

other than to those from different farms and geographic regions. Phylogenetic analysis revealed that all the U.S. swine HEV isolates from this study clustered with the US1 and US2 strains of human HEV and the prototype U.S. strain of swine HEV, but were distinct from the Taiwanese strains of swine HEV and most strains of human HEV from other countries. These data indicated that, as is with human HEV strains (33), swine HEV strains from different geographic regions of the world are also heterogeneic.

The enzootic nature of swine HEV infection in pigs in the United States and its ability to infect across species raise potential concerns for zoonosis as well as for food and environmental safety (25-26, 34). Since the infected pigs shed virus in feces, swine fouling of irrigation or coastal waters with manure could cause contamination of produce or shellfish (34). Fecal contamination of pork products in meat packing plants might also serve as a source of HEV infection (34). In addition, since pigs have been considered as preferable organ donors for xenotransplantation, the ability of swine HEV to infect across species also poses a concern for xenozoonosis (27). The universal HEV RT-PCR assay developed in this study will be very useful to screen swine HEV infection in xenograft donor pigs.

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REFERENCES

1. **Aggarwal, R. and K. Krawczynski.** 2000. Hepatitis E: an overview and recent advances in clinical and laboratory research. J. Gastroenterol. Hepatol. **15**:9-20.
2. **Arankalle, V.A., M.K. Goverdhan, and K. Banerjee**. 1994. Antibodies against hepatitis E virus in Old World monkeys. J. Viral. Hepat. **1**:125-129.
3. **Clayson, E.T., B.L. Innis, K.S. Myint, S. Narupiti, D.W. Vaughn, S. Giri, P. Ranabhat, and M.P. Shrestha**. 1995. Detection of hepatitis E virus infections among domestic swine in the Kathmandu Valley of Nepal. Am. J. Trop. Med. Hyg. **53**:228-232.
4. **Erker, J.C., S.M. Desai, G.G. Schlauder, G.J. Dawson, and I.K, Mushahwar**. 1999. A hepatitis E virus variant from the United States: molecular characterization and transmission in cynomolgus macaques. J. Gen. Virol. **80**:681-90.
5. **Favorov, M.O., O. Nazarova, and H.S. Margolis**. 1998. Is hepatitis E an emerging zoonotic disease ? Am. J. Trop. Med. Hyg. **59**:242 (abstract).
6. **Favorov, M.O., M.Y. Kosoy, S.A. Tsarev, J.E. Childs, and H.S. Margolis**. 2000. Prevalence of antibody to hepatitis E virus among rodents in the United States. J. Infect. Dis. **181**:449-455.
7. **Halbur, P.G., C. Kasorndorkbua, C. Gilbert, D. Guenette, M. B. Potters, R. H. Purcell, S. U. Emerson, T.E. Toth, and X. J. Meng**. 2001. Comparative pathogenesis of infection of pigs with hepatitis E viruses recovered from a pig and a human. J. Clin. Microbiol. **39**:918-923.
8. **Hamid, S.S., S.M. Jafri, H. Khan, H. Shah, Z. Abbas, and H.A. Fields**. 1996. Fulminant hepatic failure in pregnant women: acute fatty liver or acute viral hepatitis? Hepatology **25**:20-27.
9. **Haqshenas, G., H.L. Shivaprasad, P.R. Woolcock, D.H. Read, and X.J. Meng**. 2001. Genetic identification and characterization of a novel virus related to human hepatitis E virus from chickens with hepatitis-splenomegaly syndrome in the United States. J. Gen. Virol. **82**:2449-2462.

10. **Hsieh, S.Y., X.J. Meng, Y.H. Wu, S.T. Liu, A.W. Tam, D.Y. Lin, and Y.F. Liaw**. 1999. Identity of a novel swine hepatitis E virus in Taiwan forming a monophyletic group with Taiwan isolates of human hepatitis E virus. J. Clin. Microbiol. **37**:3828-3834.
11. **Huang, C.C., D. Nguyen, J. Fernandez, K.Y. Yun, K.E. Fry, D.W. Bradley, A.W. Tam, and G.R. Reyes**. 1992. Molecular cloning and sequencing of the Mexico isolate of hepatitis E virus (HEV). Virology **191**:550-558
12. **Hussaini, S.H., S.J. Skidmore, P. Richardson, L.M. Sherratt, B.T. Cooper, and J.G. O'Grady**. 1997. Severe hepatitis E infection during pregnancy. J. Viral. Hepat. **4**:51-54.
13. **Kabrane-Lazizi, Y., X.J. Meng, R.H. Purcell, and S.U. Emerson**. 1999. Evidence that the genomic RNA of hepatitis E virus is capped. J. Virol. **73**:8848-8850.
14. **Kabrane-Lazizi, Y., J.B. Fine, J. Elm, G.E. Glass, H. Higa, A. Diwan, C.J. Gibbs Jr., X.J. Meng, S.U. Emerson, and R.H. Purcell**. 1999. Evidence for wide-spread infection of wild rats with hepatitis E virus in the United States. Am. J. Trop. Med. Hyg. **61**:331-335.
15. **Kabrane-Lazizi, Y., M. Zhang, R.H. Purcell, K.D. Miller, R.T. Davey, and S.U. Emerson**. 2001. Acute hepatitis caused by a novel strain of hepatitis E virus most closely related to United States strains. J. Gen. Virol. **82**:1687-93.
16. **Kasorndorkbua, C., P.G. Halbur, D.K. Guenette, T.E. Toth, and X. J. Meng**. 2001. Use of a swine bioassay and a RT-PCR assay to assess the risk of transmission of swine hepatitis E virus in pigs. J. Virol. Methods. In Press.
17. **Khudyakov, Y.E., E.N. Lopareva, D.L. Jue, T.K. Crews, S.P. Thyagarajan, and H. A. Fields**. 1999. Antigenic domains of the open reading frame 2-encoded protein of hepatitis E virus. J. Clin. Microbiol. **37**:2863-2871.
18. **Maneerat, Y., E.T. Clayson, K.S.A. Myint, G.D. Young, and B.L. Innis.** 1996. Experimental infection of the laboratory rat with the hepatitis E virus. J. Med. Virol. **48**:121-128.
19. **Mast, E.E., I.K. Kuramoto, M.O. Favorov, V.R. Schoening, B.T. Burkholder, C.N. Shapiro, and P.V. Holland.** 1997. Prevalence of and risk factors for

antibody to hepatitis E virus seroreactivity among blood donors in Northern California. J. Infect. Dis. **176**:34-40.

20. **Meng, J., J. Pillot, X. Dai, H.A. Field, and Y.E. Khudyakov**. 1998. Neutralization of different geographic strains of the hepatitis E virus with anti-hepatitis E virus-positive serum samples obtained from different sources. Virology. **249**:316-324**.**
21. **Meng, X.J., R.H. Purcell, P.G. Halbur, J.R. Lehman, D.M. Webb, T.S. Tsareva, J.S. Haynes, B.J. Thacker, and S.U. Emerson**. 1997. A novel virus in swine is closely related to the human hepatitis E virus. Proc. Natl. Acad. Sci. USA **94**:9860-9865.
22. **Meng, X.J., P.G. Halbur, J.S. Haynes, T.S. Tsareva, J.D. Bruna, R.L. Royer, R.H. Purcell, and S.U. Emerson**. 1998. Experimental infection of pigs with the newly identified swine hepatitis E virus (swine HEV), but not with human strains of HEV. Arch. Virol. **143**:1405-1415.
23. **Meng, X.J., P.G. Halbur, M.S. Shapiro, S. Govindarajan, J.D. Bruna, I.K. Mushahwar, R.H. Purcell, and S.U. Emerson**. 1998. Genetic and experimental evidence for cross-species infection by the swine hepatitis E virus. J. Virol. **72**:9714-9721.
24. **Meng, X.J., S. Dea, R.E. Engle, R. Friendship, Y.S. Lyoo, T. Sirinarumitr, K. Urairong, D. Wang, D. Wong, D. Yoo, Y. Zhang, R.H. Purcell, and S.U. Emerson**. 1999. Prevalence of antibodies to the hepatitis E virus in pigs from countries where hepatitis E is common or is rare in the human population. J. Med. Virol. **58**:297-302.
25. **Meng, X.J**. 2000. Zoonotic and xenozoonotic risks of hepatitis E virus. Infect. Dis. Rev. **2**:35-41.
26. **Meng, X.J**. 2000. Novel strains of hepatitis E virus identified from humans and other animal species: Is hepatitis E a zoonosis? J. Hepatol. **33**:842-845.
27. **Meng, X.J**. 2002. Swine hepatitis E virus: cross-species infection and risk in xenotransplantation. Curr. Topics Microbiol. Immunol. In press.
28. **Meng, X.J., B. Wiseman, F. Elvinger, D.K. Guenette, T. E. Toth, R.E. Engle, S. U. Emerson, and R.H. Purcell**. 2002. Prevalence of antibodies to the hepatitis E

virus in veterinarians working with swine and in normal blood donors in the United States and other countries. J. Clin. Microbiol. **40**:117-122.

29. **Pina S., M. Buti, M. Cotrina, J. Piella, and R. Girones**. 2000. HEV identified in serum from humans with acute hepatitis and in sewage of animal origin in Spain. J. Hepatol. **33**:826-833.
30. **Pringle, C**. 1998. Minutes of the 27th International Committee on Taxonomy of Viruses Meeting. Arch. Virol. **143**:1449-1459.
31. **Purcell, R.H**. 1996. Hepatitis E virus. In: Fields BN, Knipe DM, Howley PM et al. (eds), Fields Virology. 3rd ed. Vol 2. Lippincott-Raven Publishers, Philadelphia, pp2831-2843.
32. **Schlauder, G.G., G.J. Dawson, J.C. Erker, P.Y. Kwo, M.F. Knigge, D.L. Smalley, J.E. Rosenblatt, S.M. Desai, and I.K. Mushahwar**. 1998. The sequence and phylogenetic analysis of a novel hepatitis E virus isolated from a patient with acute hepatitis reported in the United States. J. Gen. Virol. **79**:447-456.
33. **Schlauder, G.G., and I.K. Mushahwar**. 2001. Genetic heterogeneity of hepatitis E virus. J. Med. Virol. **65**:282-92.
34. **Smith, J.L**. 2001. A review of hepatitis E virus. J. Food Prot. **64**:572-586.
35. **Takahashi, K., K. Iwata, N. Watanabe, T. Hatahara, Y. Ohta, K. Baba, and S. Mishiro**. 2001. Full-genome nucleotide sequence of a hepatitis E virus strain that may be indigenous to Japan. Virology **287**:9-12.
36. **Thomas, D.L., P.O. Yarbough, D. Vlahov, S.A. Tsarev, K.E. Nelson, A.J. Saah, and R.H. Purcell**. 1997. Seroreactivity to hepatitis E virus in areas where the disease is not endemic. J. Clin. Microbiol. **35**:1244-1247.
37. **Tien, N.T., H.T. Clayson, H.B. Khiem, P.K. Sac, A.L. Corwin, K.S. Myint, and D.W. Vaughn**. 1997. Detection of immunoglobulin G to the hepatitis E virus among several animal species in Vietnam. Am. J. Trop. Med. Hyg. **57**:211 (abstract).
38. **Tsarev, S. A., S. U. Emerson, G. R. Reyes, T. S. Tsareva, L. J. Legters, I. A. Malik, M. Iqbal, and R. H. Purcell**. 1992. Characterization of a prototype strain of hepatitis E virus. Proc. Natl. Acad. Sci. U S A. **89**:559-563.

39. Tsarev, S.A., M.P. Shrestha, J. He, R.M. Scott, D.W. Vaughn, E.T. Clayson, S. Gigliotti, C.F. Longer, and B.L. Innis. 1998. Naturally acquired hepatitis E virus (HEV) infection in Nepalese rodents. Am. J. Trop. Med. Hyg. **59**:242 (abstract).

40. Wang, Y., R. Ling, J.C. Erker, H. Zhang, H. Li, S. Desai, I.K. Mushahwar, and T.J. Harrison. 1999. A divergent genotype of hepatitis E virus in Chinese patients with acute hepatitis. J. Gen. Virol. **80**:169-77.

41. Wang, Y., H. Zhang, R. Ling, H. Li, and T.J. Harrison. 2000. The complete sequence of hepatitis E virus genotype 4 reveals an alternative strategy for translation of open reading frames 2 and 3. J. Gen. Virol. **81**:1675-1686.

42. Williams, T.P.E., C. Kasorndorkbua, P.G. Halbur, G. Haqshenas, D.K. Guenette, T.E. Toth, and X.J. Meng. 2001. Evidence of extrahepatic sites of replication of the hepatitis E virus in a swine model. J. Clin. Microbiol. **39**:3040-3046.

43. Wu, J.C., C.M. Chen, T.Y. Chiang, I.J. Sheen, J.Y. Chen, W.H. Tsai, Y.H. Huang, and S.D. Lee. 2000. Clinical and epidemiological implications of swine hepatitis E virus infection. J. Med. Virol. **60**:166-171.

44. Zafrullah, M., M.H. Ozdener, S.K. Panda, and S. Jameel. 1997. The ORF3 protein of hepatitis E virus is a phosphoprotein that associated with the cytoskeleton. J. Virol. **71**:9045-9053.

Fig. 1

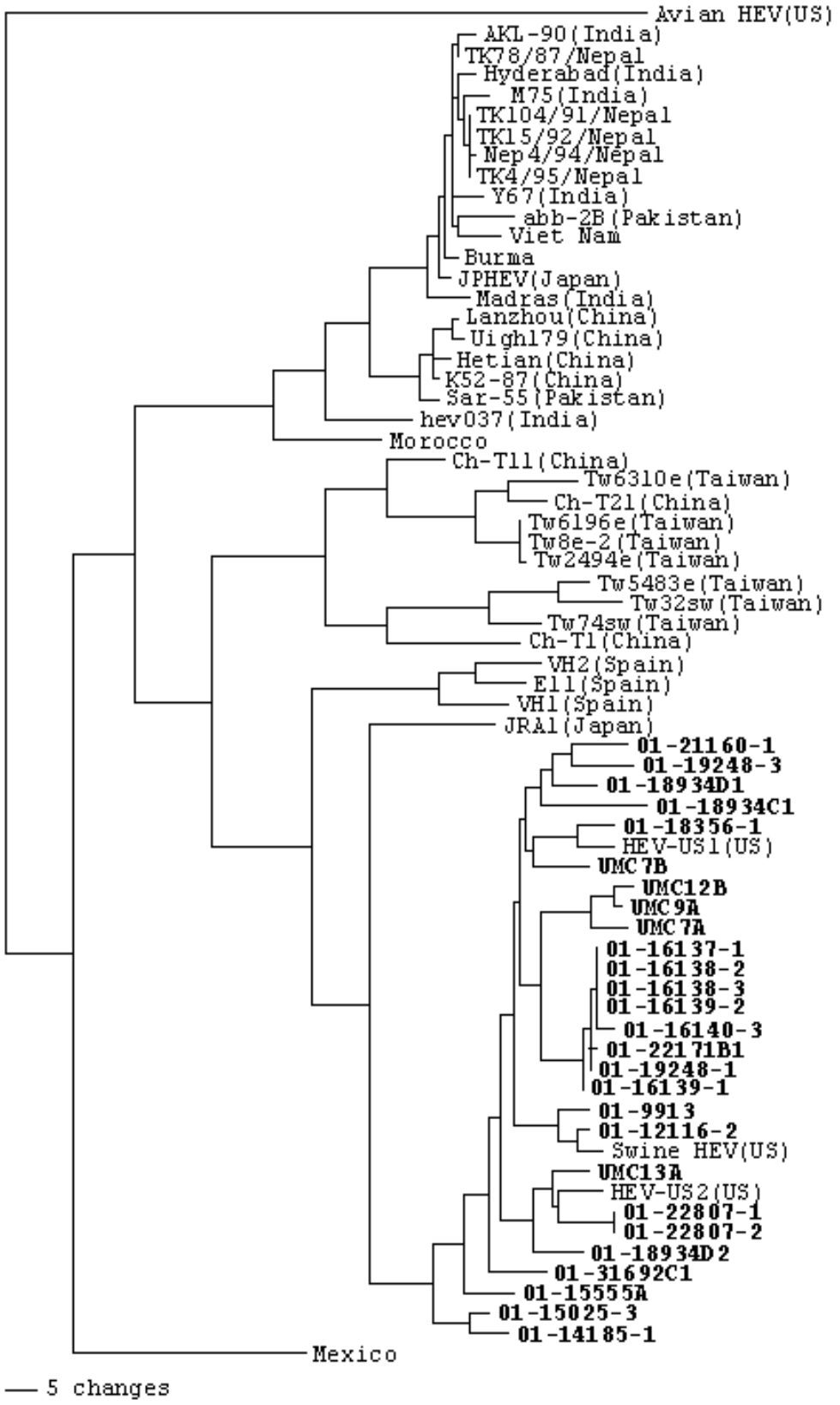
Avian HEV(US)
AKL-90(India)
TK78/87/Nepal
Hyderabad(India)
M75(India)
TK104/91/Nepal
TK15/92/Nepal
Nep4/94/Nepal
TK4/95/Nepal
Y67(India)
abb-2B(Pakistan)
Viet Nam
Burma
JPHEV(Japan)
Madras(India)
Lanzhou(China)
Uighl79(China)
Hetian(China)
K52-87(China)
Sar-55(Pakistan)
hev037(India)
Morocco
Ch-T11(China)
Tw6310e(Taiwan)
Ch-T21(China)
Tw6196e(Taiwan)
Tw8e-2(Taiwan)
Tw2494e(Taiwan)
Tw5483e(Taiwan)
Tw32sw(Taiwan)
Tw74sw(Taiwan)
Ch-T1(China)
VH2(Spain)
E11(Spain)
VH1(Spain)
JRA1(Japan)
01-21160-1
01-19248-3
01-18934D1
01-18934C1
01-18356-1
HEV-US1(US)
UMC7B
UMC12B
UMC9A
UMC7A
01-16137-1
01-16138-2
01-16138-3
01-16139-2
01-16140-3
01-22171B1
01-19248-1
01-16139-1
01-9913
01-12116-2
Swine HEV(US)
UMC13A
HEV-US2(US)
01-22807-1
01-22807-2
01-18934D2
01-31692C1
01-15555A
01-15025-3
01-14185-1
Mexico
5 changes

Fig. 1. A phylogenetic tree based on the nucleotide sequences of a 304-bp region within the HEV ORF2 gene. The tree was constructed with the aid of the PAUP program. Heuristic search with 1,000 replicates of random adding and midpoint rooting option were used to construct the tree. A scale bar representing the numbers of character state changes is shown. The swine HEV isolates from this study are indicated in boldface. The GenBank database accession numbers of the sequences of HEV isolates used in the phylogenetic analyses are listed in the text.

Table 1. Detection of swine HEV RNA from fecal and serum samples of pigs of 2-4 months of ages from different herds in the United States

Herd ID	Specimen [a]	No. pigs Positive /Tested	Geographic location
01-23444B	F	0/1	Rogers, AR
01-15025	F	1/3	Story City, IA
01-14185-1	F	1/2	Remsen, IA
01-12886	F	0/2	Alden, IA
01-12116	F	1/2	Emmetsburg, IA
01-15555A	F	1/1	Ute, IA
01-15675	F	0/1	Stratford, IA
01-16137	F	1/2	Bloomfield, IA
01-16138	F	3/3	Bloomfield, IA
01-16139	F	2/2	Bloomfield, IA
01-16140	F	1/3	Bloomfield, IA
01-17912	F	0/11	Eldora, IA
01-18191	F	0/2	Buffalo center, IA
01-18356	F	2/3	Manson, IA
01-18934	F	5/6	Linn Grove, IA
01-19248	F	3/3	Alden, IA
01-21160	F	2/3	Cherokee, IA
01-22171	F	1/9	Everly, IA
01-22807	F	2/2	Dayton, IA
01-31692	F	1/3	Kamrar, IA
01-15552	F	0/1	Holland, MI
UMC 1 (A,B)	S	0/2	Marshall, MO
UMC 2 (A,B)	S	0/2	Monroe City, MO
UMC 3 (A,B)	S	0/2	Tipton, MO
UMC 4 (A,B)	S	0/2	Monroe City, MO
UMC 5 (A,B)	S	0/2	Columbia, MO
UMC 6 (A,B)	S	0/2	Shelbina. MO
UMC 7 (A,B)	S	2/2	Columbia, MO
UMC 8 (A,B)	S	0/2	Meta, MO
UMC 9 (A,B)	F	2/2	Columbia, MO
UMC 11(A,B,C)	F	0/3	Columbia, MO
UMC 12 (A,B,C)	F	1/3	Sweet Springs, MO
UMC 13 (A,B,C)	F	1/1	Pleasant Hill, MO
UMC 14	F	0/1	Columbia, MO
01-30609	F	0/3	Rose Hill, NC
01-14427	F	0/1	McLeansville, NC
01-9913	F	1/1 [b]	Holdenville, OK

[a] F, fecal sample; S: serum sample.
[b] A 7-month-old pig.

TABLE 2. Pairwise comparison of the nucleotide sequences of the partial ORF2 gene of 27 swine HEV isolates identified in this study (in boldface) and other selected strains of HEV worldwide[a]

Isolate	% Identity																																				
	01-12116-2	**01-18356-1**	**UMC12B**	**01-18934D1**	**01-18934-D2**	**01-22807-1**	**01-22807-2**	**01-15555A**	**01-15025-3**	**01-19248-3**	**UMC7A**	**UMC7B**	**01-31692C1**	**01-16140-3**	**01-16137-1**	**MC13A**	**01-16139-1**	**01-16139-2**	**01-9913**	**01-14185-1**	**01-22171B1**	**UMC9A**	**01-19248-1**	**01-18934C1**	**01-16138-3**	**01-21160-1**	Swine HEV	JRA1	Tw6196e	Tw32sw	Mexico	US1	US2	Sar-55	Ch-T1	Morocco	Tw74sw	E11	Avian HEV
01-16138-2	92[b]	91	92	92	92	92	92	93	91	91	93	92	94	99	100	91	99	100	92	89	99	93	99	92	100	91	91	87	75	76	71	91	92	75	77	75	77	82	55
01-12116-2		94	90	92	92	90	90	92	93	91	90	92	92	92	92	91	93	92	96	91	93	90	92	89	92	92	98	87	75	74	73	92	90	76	77	75	76	86	55
01-18356-1			91	93	92	90	90	91	93	90	90	92	92	91	91	92	91	91	93	90	91	91	91	89	91	92	93	87	75	74	72	96	91	76	76	76	75	83	54
UMC12B				93	91	90	90	92	91	91	95	92	91	91	92	89	93	92	90	89	92	98	93	90	92	91	90	88	77	76	71	91	89	77	79	75	76	83	54
01-18934D1					92	90	90	93	92	94	92	93	92	91	92	91	92	92	92	90	92	92	92	92	92	94	92	87	78	75	73	93	90	75	78	76	76	83	54
01-18934-D2						93	93	91	92	91	91	93	92	92	92	95	93	92	91	91	92	90	92	91	92	91	92	86	76	75	72	91	94	76	78	76	76	82	55
01-22807-1							100	92	91	89	91	91	93	92	92	94	93	92	90	91	92	91	92	90	92	90	90	88	75	76	73	90	94	78	77	76	77	81	55
01-22807-2								92	91	89	91	91	93	92	92	94	93	92	90	91	92	91	92	90	92	90	90	88	75	76	73	90	94	78	77	76	77	81	55
01-15555A									93	91	91	92	94	92	93	93	93	93	92	91	94	93	94	91	93	91	91	86	77	75	72	92	92	75	77	76	77	85	54
01-15025-3										92	90	92	92	91	91	91	92	91	93	97	92	91	92	90	91	93	93	88	76	77	72	92	92	74	77	76	76	85	56
01-19248-3											91	91	90	90	91	89	92	91	91	90	91	90	91	90	91	93	91	85	77	75	72	91	89	74	77	76	77	82	54
UMC7A												93	91	92	93	90	93	93	90	88	93	96	93	89	93	91	89	87	75	75	70	91	90	76	77	74	76	82	53
UMC7B													92	91	92	92	93	92	92	91	93	92	92	91	92	93	92	87	76	75	72	93	91	76	78	75	76	83	55
01-31692C1														93	94	93	94	94	92	92	94	91	94	90	94	93	91	87	76	76	71	92	93	75	77	76	78	83	54
01-16140-3															99	91	98	99	92	89	98	92	98	92	99	90	91	86	75	76	72	90	92	76	76	75	76	82	55
01-16137-1																91	99	100	92	89	99	93	99	92	100	91	91	87	75	76	71	91	92	75	77	75	77	82	55
UMC13A																	92	91	90	90	91	90	92	91	91	91	90	87	76	75	73	90	96	76	77	75	76	83	54
01-16139-1																		99	93	90	99	94	99	92	99	92	92	88	75	76	72	92	93	75	78	75	77	83	55
01-16139-2																			92	89	99	93	99	92	100	91	91	87	75	76	71	91	92	75	77	75	77	82	55
01-9913																				90	92	90	92	91	92	92	96	86	76	74	74	92	91	75	77	75	76	85	54
01-14185-1																					90	89	90	89	89	92	90	87	77	78	73	89	91	76	77	78	77	84	56
01-22171B1																						93	99	92	99	91	92	87	75	75	72	91	92	75	77	75	77	83	54
UMC9A																							93	90	93	91	89	87	77	76	71	91	90	77	78	75	76	82	54
01-19248-1																								92	99	92	92	87	75	75	72	92	92	75	77	75	77	83	54
01-18934C1																									92	90	89	85	77	76	74	89	90	76	78	75	77	81	54
01-16138-3																										91	91	87	75	76	71	91	92	75	77	75	77	82	55
01-21160-1																											92	87	78	75	72	92	93	74	78	75	77	83	55
Swine HEV																												86	75	75	73	92	90	75	75	76	77	85	55
JRA1																													77	75	73	86	88	75	77	76	77	83	54
Tw6196e																														84	76	75	76	77	85	77	84	76	54
Tw32sw																															70	75	75	76	85	74	90	77	56
Mexico																																71	72	76	76	75	72	71	53
US1																																	90	76	77	76	77	83	54
US2																																		75	78	76	77	82	55
Sar-55																																			77	89	76	74	55
Ch-T1																																				76	86	78	54
Morocco																																					77	74	56
Tw74sw																																						78	54
E11																																							54

[a] References for other HEV strains used in the comparisons: the prototype U.S. strain of swine HEV, swine HEV (21); a Japanese strain of human HEV, JRA1 (36); Taiwanese strains of swine HEV (Tw32sw and Tw74sw) and human HEV (Tw6196e) (10, 43); U.S. strains of human HEV (US1 and US2) (4, 32); the Pakistani strain of human HEV, Sar-55 (38); a variant Chinese strain of human HEV, Ch-T1 (40, 41); a Spanish strain of HEV of possible swine origin, E11 (29); and the avian strain of HEV (9).

General conclusions

In this dissertation, avian HEV and swine HEV have been genetically characterized. Our results showed that, like human HEV, isolates of swine HEV and avian HEV from different geographic regions are heterogeneic. Swine HEV and avian HEV are enzootic in pig herds and chicken flocks, respectively, in the United States, and the prevalence is age-specific. Swine HEV can cross-species and infect non-human primates. However, unlike swine HEV, avian HEV failed to experimentally infect two rhesus monkeys, thus may not infect humans either. The full-length genomic sequence of avian HEV was determined and characterized. Compared to mammalian HEVs, avian HEV shares similar genomic organization and conserved putative functional motifs and domains in ORF1, supporting the conclusion that avian HEV is a member of the genus *Hepevirus*. With the availability of the consensus full-length genomic sequence of avian HEV, three infectious cDNA clones have been successfully constructed, biologically and experimentally characterized in a chicken liver cell line and in SPF chickens. The availability of the infectious clones for a chicken strain of HEV should be very useful for the study of HEV replication, pathogenesis and cross-species infection.

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REFERRED PUBLICATIONS

1. **Huang F.F.**, F.W. Pierson, T.E. Toth, and X.J. Meng. Construction and characterization of infectious cDNA clones of an animal strain of hepatitis E virus (HEV), avian HEV. *Journal of Virology*. To be submitted.
2. Billam P., **F.F. Huang,** Z.F. Sun, D.K. Guenette, F.W. Pierson, T.E. Toth, R. Duncan, F. Elvinger, T.E. Toth and X.J. Meng. Systematic pathogenesis and replication of a strain of the hepatitis E virus (HEV) in its natural host: avian HEV infections in specific-pathogen-free adult chickens. *Journal of Virology*. In press.
3. Cooper K., **F.F. Huang,** L. Batista, C.D. Rayo, J.C. Bezanilla, T.E. Toth, and X.J. Meng. Identification of genotype 3 hepatitis E virus (HEV) isolates from pigs in Thailand and Mexico where genotype 1 or 2 HEV strains are prevalent in humans. *Journal of Clinical Microbiology*. Submitted.
4. Kasorndorkbua C., D.K. Guenette, **F.F. Huang**, P.J. Thomas, X.J. Meng, and P.G. Halbur. Routes of transmission of swine hepatitis E virus in pigs. 2004. *Journal of Clinical Microbiology.* Submitted.
5. Sun Z.F., C.T. Larsen, **F.F. Huang**, P. Billam, F.W. Pierson, T.E. Toth, and X.J. Meng. Generation and infectivity titration of an infectious stock of avian hepatitis E virus in chickens and cross-species infection of turkeys with avian HEV. *Journal of Clinical Microbiology.* 2004. 42:2658-2562.
6. **Huang F.F.**, Z.F. Sun, S.U. Emerson, R.H. Purcell, H.L. Shivaprasad, F.W. Pierson, T.E. Toth, and X.J. Meng. Determination and analysis of the complete genomic sequence of avian hepatitis E virus (avian HEV) and attempts to infect rhesus monkeys with avian HEV. *Journal of General Virology.* 2004. 85:1609-1618.
7. Sun Z.F., C.T. Larsen, A. Dunlop, **F.F. Huang**, F.W. Pierson, T.E. Toth, and X.J. Meng. Genetic identification of avian hepatitis E virus (HEV) from healthy chicken flocks and characterization of the capsid gene of 14 avian HEV isolates from chickens with hepatitis-splenomegaly syndrome in different geographical regions of the United States. *Journal of General Virology.* 2004. 85:693-700.
8. Sun Z.F., **F.F. Huang**, P.G. Halbur, S.K. Schommer, F.W. Pierson, T.E. Toth, and X.J. Meng. Use of heteroduplex mobility assays (HMA) for pre-sequencing screening and identification of variant strains of swine and avian hepatitis E viruses. *Veterinary Microbiology.* 2003. 96: 165-176.
9. **Huang F.F.**, G. Haqshenas, H.L. Shivaprasad, D.K. Guenette, P.R. Woolcock, C.T. Larsen, F.W. Pierson, F. Elvinger, T.E. Toth, and X.J. Meng. Heterogeneity

and seroprevalence of a newly identified avian hepatitis E virus from chickens in the United States. *Journal of Clinical Microbiology.* 2002. 40:4197-4202.

10. Haqshenas G., **F.F. Huang**, M. Fenaux, D.K. Guenette, F.W. Pierson, C.T. Larsen, H.L. Shivaprasad, T.E. Toth, and X.J. Meng. The putative capsid protein of the newly identified avian hepatitis E virus shares antigenic epitopes with that of swine and human hepatitis E viruses and chicken big liver and spleen disease virus. *Journal of General Virology.* 2002. 83:2201-2209.
11. **Huang F.F.**, G. Haqshenas, D.K. Guenette, P.G. Halbur, S.K. Schommer, F.W. Pierson, T.E. Toth, and X.J. Meng. Detection by reverse transcription-PCR and genetic characterization of field isolates of swine hepatitis E virus from pigs in different geographic regions of the United States. *Journal of Clinical Microbiology.* 2002. 40:1326-1332.

NON-REFERRED PUBLICATIONS

1. Kasorndorkbua C., **F.F. Huang**, D.K. Guenette, P.J. Thomas, X.J. Meng, and P.G. Halbur. Hepatitis E virus in swine manure storage facilities is infectious to pigs. *Proceedings of the 18th International Pig Veterinary Society.* June 27th-July 1st, 2004, Hamburg, Germany.
2. Martin M., J. Segales, **F.F. Huang,** and X.J. Meng. Detection of hepatitis E virus in serum samples of naturally porcine circovirus type 2 infected pigs with and without lesions of hepatitis. *Proceedings of the 18th International Pig Veterinary Society.* June 27th-July 1st, 2004, Hamburg, Germany.
3. **Huang F.F.**, F.W. Pierson, T.E. Toth, and X.J. Meng. Construction and characterization of infectious cDNA clones of an animal strain of hepatitis E virus (HEV), avian HEV. *Proceedings of the 16th annual VMRCVM research symposium.* June 17-18, 2004. Blacksburg, VA.
4. Cooper K., **F.F. Huang,** L. Batista, C.D. Rayo, J.C. Bezanilla, T.E. Toth, and X.J. Meng. Molecular ad serological epidemiology of swine hepatitis E virus isolates from pigs in two countries. *Proceedings of the 16th annual VMRCVM research symposium.* June 17-18, 2004. Blacksburg, VA.
5. Billam P., **F.F. Huang,** Z.F. Sun, D.K. Guenette, F.W. Pierson, T.E. Toth, R. Duncan, F. Elvinger, T.E. Toth and X.J. Meng. Systematic pathogenesis and replication of a strain of the hepatitis E virus (HEV) in its natural host: avian HEV infections in specific-pathogen-free adult chickens. *Proceedings of the 16th annual VMRCVM research symposium.* June 17-18, 2004. Blacksburg, VA.
6. Sun Z.F., C.T. Larsen, **F.F. Huang**, P. Billam, F.W. Pierson, T.E. Toth, and X.J. Meng. Cross-species infection of turkeys with avian hepatitis E virus (avian HEV). *Proceedings of the 16th annual VMRCVM research symposium.* June 17-18, 2004. Blacksburg, VA.
7. **Huang F.F.**, Z.F. Sun, H.L. Shivaprasad, S.U. Emerson, R.H. Purcell, F.W. Pierson, T.E. Toth, and X.J. Meng. Characterization and analysis of the complete genomic sequence of avian hepatitis E virus (avian HEV). *Proceedings of the 20th Annual Research Symposium of Virginia Tech*, March 23th, 2004. Blacksburg VA.
8. Billam P., **F.F. Huang,** Z.F. Sun, D.K. Guenette, F.W. Pierson, T.E. Toth, R. Duncan, and X.J. Meng. Systematic pathogenesis and replication of a strain of the

hepatitis E virus (HEV) in its natural host: avian HEV infections in specific-pathogen-free adult chickens. *Proceedings of the 20th Annual Research Symposium of Virginia Tech*, March 23th, 2004. Blacksburg VA.

9. Sun Z.F., C.T. Larsen, **F.F. Huang**, P. Billam, F.W. Pierson, T.E. Toth, and X.J. Meng. Generation and infectivity titration of an infectious stock of avian hepatitis E virus (HEV) in chickens and attempt to experimentally infect turkeys with avian HEV. *Proceedings of the 20th Annual Research Symposium of Virginia Tech*, March 23th, 2004. Blacksburg VA.
10. **Huang F.F.**, Z.F. Sun, H.L. Shivaprasad, S.U. Emerson, R.H. Purcell, F.W. Pierson, T.E. Toth, and X.J. Meng. Molecular and experimental characterization of the avian hepatitis virus (avian HEV). *Proceedings of the 84th annual conference of research workers in animal diseases.* No. 168. Nov. 9-11th, 2003. Chicago, IL.
11. Billam P., **F.F. Huang,** Z.F. Sun, D.K. Guenette, F.W. Pierson, T.E. Toth, R. Duncan, and X.J. Meng. Systematic pathogenesis and replication of a strain of the hepatitis E virus (HEV) in its natural host: avian HEV infections in specific-pathogen-free adult chickens. *Proceedings of the 84th annual conference of research workers in animal diseases.* No. 170. Nov. 9-11th, 2003. Chicago, IL.
12. Sun Z.F., C.T. Larsen, **F.F. Huang**, P. Billam, F.W. Pierson, T.E. Toth, and X.J. Meng. Generation and infectivity titration of an infectious stock of avian hepatitis E virus (HEV) in chickens and attempt to experimentally infect turkeys with avian HEV. *Proceedings of the 84th annual conference of research workers in animal diseases.* No. 169. Nov. 9-11th, 2003. Chicago, IL.
13. Haqshenas G., C. Kasorndorkbua, P.G. Halbur, D.K. Guenette, **F.F. Huang,** M. Fenaux, Y.W. Huang, X.J. Meng. Generation of an infectious but attenuated cDNA clone of swine hepatitis E virus. *Proceedings of 4th International Conferences on Emerging Zoonoses.* pp42. Sept. 18-21st, 2003. Ames, IA.
14. Haqshenas G., **F.F. Huang**, M. Fenaux, Z.F. Sun, D.K. Guenette, F.W. Pierson, C.T. Larsen, H.L. Shivaprasad, T.E. Toth, and X.J. Meng. The newly identified avian hepatitis E virus shares common antigenic epitopes in the capsid protein with that of swine and human hepatitis E viruses. *Proceedings of 4th International Conferences on Emerging Zoonoses.* pp42. Sept. 18-21st, 2003. Ames, IA.
15. **Huang F.F.**, Z.F. Sun, F.W. Pierson, T.E. Toth, and X.J. Meng. Complete genomic sequence analyses of the newly discovered avian hepatitis E virus (avian HEV) reveal that avian HEV is genetically related to but distinct from swine and human HEVs. *Proceedings of the 15th annual VMRCVM research symposium.* June 5-6, 2003. Blacksburg, VA.
16. **Huang F.F.**, G. Haqshenas, H.L. Shivaprasad, D.K. Guenette, P.R. Woolcock, C.T. Larsen, F.W. Pierson, F. Elvinger, T.E. Toth, and X.J. Meng. Heterogeneity and seroprevalence of the newly identified avian hepatitis E virus from chickens in the United States. *Proceedings of the 19th Annual Research Symposium of Virginia Tech,* March 26th, 2003. Blacksburg, VA.
17. **Huang FF**, G. Haqshenas, H.L. Shivaprasad, D.K. Guenette, P.R. Woolcock, C.T. Larsen, F.W. Pierson, F. Elvinger, T.E. Toth, and X.J. Meng. Heterogeneity and seroprevalence of the newly identified avian hepatitis E virus from chickens in the

United States. *Proceedings of the 83rd annual conference of research workers in animal diseases*. Nov. 10-12, 2002. St. Louis, MO.

18. Sun Z.F., **F.F. Huang**, P.G. Halbur, S.K. Schommer, F.W. Pierson, T.E. Toth, and X.J. Meng. Use of heteroduplex mobility assays (HMA) for pre-sequencing screening and identification of variant strains of swine and avian hepatitis E viruses. *Proceedings of the 83rd annual conference of research workers in animal diseases*. Nov. 10-12, 2002. St. Louis, MO.
19. **Huang F.F.**, G. Haqshenas, H.L. Shivaprasad, D.K. Guenette, P.R. Woolcock, C.T. Larsen, F.W. Pierson, F. Elvinger, T.E. Toth, and X.J. Meng. Heterogeneity and seroprevalence of the newly identified avian hepatitis E virus from chickens in the United States. *Proceedings of the 14th annual VMRCVM research symposium*. June 6-7, 2002. Blacksburg, VA.
20. **Huang F.F.**, G. Haqshenas, D.K. Guenette, P.G. Halbur, S.K. Schommer, F.W. Pierson, T.E. Toth, and X.J. Meng. Field isolates of swine hepatitis E virus from pigs in different geographic regions of the United States are genetically heterogenic. *Proceedings of the 18th Annual Research Symposium of Virginia Tech,* April 2-3, 2002. Blacksburg, VA.
21. **Huang F.F.**, G. Haqshenas, D.K. Guenette, P.G. Halbur, S.K. Schommer, F.W. Pierson, T.E. Toth, and X.J. Meng. Molecular characterization of swine hepatitis E virus isolates from pigs in the different regions of the United States. *Proceedings of the 82nd annual conference of research workers in animal diseases*. Nov. 11-13, 2001. St. Louis, MO.