Foodborne Transmission and Molecular Mechanism of Cross-species Infection of Hepatitis E Virus (HEV)

By

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ABSTRACT

Hepatitis E virus (HEV), the causative agent of hepatitis E, is an emerging virus of global distribution. At least four distinct genotypes of HEV exist worldwide: genotype 1 and 2 HEV strains are responsible for waterborne epidemics; genotype 3 and 4 HEV strains are responsible for sporadic occurrences of acute hepatitis E. Genotype 3 and 4 HEVs are zoonotic and have a more expanded host range than genotypes 1 and 2 which are restricted to humans. Genotype 3 and 4 HEV isolates obtained from animal tissues are genetically very similar, or identical in some cases, to human HEV recovered from hepatitis E patients. The objectives of this dissertation research were to assess the zoonotic foodborne transmission of HEV and elucidate the viral determinants of HEV host range. To determine the risk of HEV foodborne transmission, 127 packages of commercial pig liver were tested for HEV RNA. Eleven percent of them were positive for HEV RNA and the contaminating virus remained infectious. We also demonstrated that medium-to-rare cooking condition (56°C) does not completely inactivate HEV, although frying and boiling of the contaminated livers inactivated the virus. To reduce the risk of foodborne HEV transmission, commercial pig livers must be thoroughly cooked for consumption. To determine the host range of genotype 4 HEVs, pigs were inoculated with a genotype 4 human HEV. All pigs developed an active HEV infection indicating that genotype 4 human HEVs can cross species barriers and infect pigs. To identify viral determinant(s) of species tropism, ORF2 alone or in combination with its adjacent 5′ junction region (JR) and 3′ non-coding region (NCR), were swapped between genotypes

1 and 4, 3 and 4, and 1 and 3 to produce 5 chimeric viruses. Chimeric viruses containing ORF2 or JR+ORF2+3' NCR from genotype 4 human HEV in the backbone of genotype 3 swine HEV were viable *in vitro* and infectious *in vivo*. Chimeric viruses containing the JR+ORF2+3'NCR of genotypes 3 or 4 HEV in the backbone of genotype 1 human HEV were viable *in vitro* but non-infectious in pigs, suggesting that ORF1 may also be important for host range.

DEDICATION

I would like to dedicate this work to my wonderful family members for all of their love and support during my graduate training at Virginia Tech.

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ATTRIBUTION

Several individuals aided in the dissertation research discussed herein. Their organizational affiliation and contributions are described below.

Chapter 2: Detection and characterization of infectious hepatitis E virus from commercial pig livers sold in local grocery stores in the United States.

1. **Tanja Opriessnig, Ph.D.** (Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University): provided biosaftey level 2 (BSL-2) facility, assisted in the animal study, and sample collection.
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Chapter 3: Inactivation of infectious hepatitis E virus present in commercial pig livers sold in local grocery stores in the United States.

1. **Tanja Opriessnig, Ph.D.** (Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University): provided BSL-2 facility, assisted in the animal study, and sample collection.
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Chapter 5: Intergenotypic chimeric hepatitis E viruses (HEV) with the capsid gene of a genotype 4 human HEV cloned in the genomic backbone of a genotype 3 swine HEV are viable *in vitro* and infectious in pigs.

1. **Laura Cordoba, Ph.D.** (Department of Biomedical Sciences and Pathobiology, Virginia Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University): provided technical assistance.
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GENERAL INTRODUCTION

Hepatitis E virus (HEV) is an important human pathogen of public health concern (Meng, 2010a; Meng, 2010b). Infection by the small, non-enveloped RNA virus results in hepatitis E, a fecal-orally transmitted disease with a case fatality rate among infected pregnant women of 20-25%, although the overall rate is generally low (<1%) (Navaneethan et al., 2008**)**. At least four distinct genotypes of HEV are recognized (Meng, 2010b). Genotypes 1 and 2 are associated with epidemics, whereas genotypes 3 and 4 cause sporadic cases of hepatitis E. The contamination of public water supplies facilitates the spread of HEV infection with frequent outbreaks occurring in developing countries that lack proper sanitation conditions. Increasing evidence suggests that zoonotic transmission from animal reservoirs of HEV may be responsible for autochthonous hepatitis E reported among humans in industrialized countries.

Swine as reservoirs of HEV was proposed soon after the discovery of the first animal strain of HEV, swine HEV, from a pig in the United States (Meng et al., 1997). Swine HEV infection is common in various countries throughout the world with an infection rate of approximately 80-100% among 2-4 months old commercial pigs (Meng et al., 1997; Meng et al., 2003). Novel strains of HEV have also been identified from chickens, mongoose, sika deer, rats, and rabbits (Haqshenas et al., 2001; Nakamura et al., 2006; Zhao et al., 2009) and group into genotype 3 and 4 with the swine and human HEVs with the exception of the avian HEV and rat HEV. Cross-species infection of humans by swine and deer isolates of HEV has been demonstrated (Meng et al., 1998; Yazaki et al., 2003; Tei et al., 2003). Sharing only 50% sequence homology with mammalian HEVs, avian HEV isolated from chickens, however, failed to infect rhesus

monkeys (Huang et al., 2004) and it remains to be determined whether mongoose and rabbit strains of HEV can cross species barriers and infect humans. Humans and non-human primates are the only natural and experimental hosts, respectively, of genotype 1 and 2 HEVs that have a more restricted host range than genotypes 3 and 4.

The experimental infection of non-human primates with swine HEV strains coupled with the very high genetic similarity between human and swine HEV isolates suggests hepatitis E is a zoonosis. Genotype 3 and 4 human HEVs isolated from hepatitis E patients in the United States, Taiwan, and Japan share significant sequence identity with swine HEV recovered from pigs in the same area (Meng et al., 1997; Wang et al., 2002; Wu et al., 2002; Nishizawa et al., 2003; Takahashi et al., 2003). The identification of human HEV isolates from hepatitis E patients with a history of consuming raw or undercooked pig liver, wild boar, and deer meat that are very similar, or identical in a few cases, to strains isolated from commercially available pig liver or wild boar and deer meat suggests that zoonotic foodborne transmission is also possible (Yazaki et al., 2003; Tei et al., 2003; Tamada et al., 2004). The potential for HEV cross species transmission is a major public health concern. Therefore, it is important to characterize the risk of HEV infection from animal reservoirs commonly used as food and identify genomic elements permitting cross-species infection.

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CHAPTER 1

Literature Review

Hepatitis E

Hepatitis E is an enterically transmitted infectious disease that appeared in New Delhi, India as early as 1955 (Rakish Aggarwal and Sita Naik, 2009) and has since surfaced globally, occurring as large epidemics in developing regions of Africa, Asia, and Mexico and sporadically in the United States, Japan, and other industrialized countries (Meng, 2010). The symptoms of hepatitis E are clinically indistinguishable from other forms of hepatitis including jaundice, anorexia, abdominal pain, nausea, and vomiting. Infection is most common in young adults, aged 15-40, and the mortality rate in infected patients is generally low (<1%), but could reach as high as 25-30% in infected pregnant women (Navaneethan et al., 2008).

Hepatitis E Virus (HEV)

The causative agent of hepatitis E, HEV, is classified in a new family *Hepeviridae*, genus *Hepivirus* (Emerson et al., 2004). The genome of the small, non-enveloped virus is single-stranded, positive-sense RNA approximately 7.2 kb in length. The viral genome consists of three open reading frames (ORFs; ORF1-3) and short 5 and 3 noncoding regions (Tam et al., 1991; Tsarev et al., 1992; Emerson and Purcell, 2003) (Fig. 1A). At least one serotype and four genotypes of HEV exist worldwide (Meng, 2010b): genotype 1 (epidemic strains from Asia), genotype 2 (epidemic strains from Mexico and Africa), genotype 3 (strains from sporadic cases worldwide) and genotype 4 (strains from sporadic cases in China, Japan, and Taiwan).

HEV Replication Strategy

In the proposed model of HEV replication, the positve-sense RNA functions as a normal cellular messenger RNA (mRNA) and is immediately translated upon ribosome binding after entry into a permissive cell (Fig. 1B). It remains to be determined whether the resulting non-structural ORF1 polyprotein functions as a single protein with multiple functional domains or is proteolytically processed into individual proteins (Meng et al., 2010a). Using the positive strand RNA as a template, negative strand RNA synthesis is initiated by the binding of a cis-acting element in the 3' NCR to the RDRP (Agrawal et al., 2001). The negative strand RNA then serves as a template for the synthesis of two RNA transcripts. Initiation at the 3' end of the negative strand RNA template leads to the synthesis of full-length positive strand RNA. Initiation at the junction region (JR) leads to the formation of subgenomic RNA. The subgenomic RNA is subsequently translated into the structural capsid protein and the ORF3 protein (Panda et al., 2007).

HEV Genome

Open Reading Frame 1 (ORF1)

ORF1 polyprotein and associated domains.

The largest of the three ORFs, ORF1, encodes a nonstructural polyprotein of approximately 1700 amino acids (aa) in which putative methyltransferase, Y, papain-like cysteine protease, X, RNA helicase, and RNA-dependent RNA polymerase (RDRP) functional domains have been identified (Koonin et al. 1992, Panda et al, 2000). Functional activity of the HEV RDRP, helicase, and methyltransferase domains has been demonstrated (Thakaral et al., 2005; Karpe and Lole, 2010a; Karpe and Lole, 2010b). A region of high nucleotide and amino acid heterogeneity, the hypervariable region (HVR)

(Pudupakam et al. 2009, Reyes et al., 1991) exists within ORF1 between the putative papain-like cysteine protease and helicase functional domains ranging in length from 68 to 86 aa for genotypes 1-4 and avian HEV (Pudupakam et al., 2009). Deletions within the HVR do not affect viral viability, however, deleting larger portions of the HVR does result in an attenuated phenotype (Pudupakam et al., 2009).

Open Reading Frame 3 (ORF3)

Translation Start Site

The region between the stop codon of ORF1 and the start codon of open reading frame 2 (ORF2), termed the JR (Huang et al., 2007), contains three in-frame AUGs, each of which could serve as the start codon of ORF3 for genotype 1, 2, and 3 strains. Genotype 4 strains can only initiate translation of ORF3 at the third in-frame AUG due to a single nucleotide insertion between the second and third AUG. *In vitro* and *in vivo* studies demonstrated that the third in-frame AUG is also the genuine start of ORF3 translation for genotype 1 and 3 HEVs, and likely for genotype 2 as well (Huang et al., 2007; Graff et al., 2006, Yamada et al., 2009).

Subgenomic mRNA

The HEV ORF2 and ORF3 proteins are translated from the same subgenomic mRNA, initiating at nucleotide 5122 (Graff et al., 2006). The subgenomic RNA of genotype 3 and 4 HEVs also start at nucleotide 5122 (Ichiyama et al., 2009). Although Tam et al. (1991) reported the presence of two subgenomic RNAs (2.2 kb and 3.7 kb) in the liver of macaques infected with a genotype 1 HEV, Graff et al. (2006) and Ichiyama et al. (2009) only detected the presence of the 2.2 kb subgenomic RNA *in vitro*. The

initiation start site for genotype 4 HEVs has yet to be determined, however, the start site is presumed to be similar to genotype 1-3 HEVs.

ORF3 protein

Cell signaling. ORF3 encodes a small, 13kDa cytoskeleton-associated phosphoprotein (Zafrullah et al., 1997) with proline-rich PXXP motifs (Korkaya et al., 2001) in the C-terminal half of the ORF3 protein. Protein-protein interactions have been identified between regions of the ORF3 protein and cellular proteins involved in the ERK signaling cascade and the Cbl/CIN85 growth factor receptor (GFR) signaling pathway resulting in prolonged signal transduction (Korkaya et al., 2001;Chandra et al., 2008; Chandra et al., 2010). The expression of ORF3 protein has been shown to alter the behavior of cellular proteins involved in Jak/Stat signaling, the apoptotic pathway, and the glycolytic metabolic pathway (Chandra et al., 2008; Moin et al., 2007; Moin et al., 2009) to presumably induce a cellular environment more conducive to viral replication.

Virion transport. The ORF3 protein localizes to and increases the stability of microtubules (Zafrullah et al., 1997; Kannan et al., 2009). The interaction of the ORF3 protein with microtubules requires hydrophobic regions located in the N-terminal half of the protein and involvement of the motor protein dynein (Kannan et al, 2009). Association with the microtubule network may facilitate virion transport throughout the cell.

Replication and infectivity. The ORF3 protein is not required for HEV replication or the production of infectious particles *in vitro* (Emerson et al., 2004; Emerson et al., 2006). Virions produced in the absence of the ORF3 protein resemble wildtype virus in sedimentation rate, antigenicity, and thermostability and retain the

ability to infect naïve cells (Emerson et al., 2006). However, the HEV ORF3 protein is essential for viral replication *in vivo* (Graff et al., 2005; Huang et al., 2007).

ORF3 protein association with the ORF2 capsid protein. The ORF3 and ORF2 proteins co-localize to the cytoplasm and interact through a region mapped to aa 1-81 of the ORF3 protein (Tyagi et al., 2002). The aa 1-81 region contains the ORF3 protein phosphorylation site, serine at aa 80 (Ser^{80}), (Zafrullah et al., 1997); the presence and phosphorylation of the Ser^{80} site is critical for the ORF3 and ORF2 protein interaction. The ORF3 protein only interacts with full-length ORF2 protein, suggesting that the interaction occurs prior to the proteolytic processing of ORF2 protein (Tyagi et al., 2002). The ORF3 protein interacts with glycosylated and non-glycosylated forms of the ORF2 protein, although more frequently with the non-glycosylated form. The importance of the ORF2 protein glycosylation state in the ORF3-ORF2 protein interaction remains to be determined.

The ORF3 protein is detected on the surface of genotype 3 human HEV virions from serum, but not from fecal material, of infected patients and cell culture generated virions using anti-ORF3 mAb. Virions from fecal material, however, can be detected with anti-ORF2 mAb (Takahashi et al., 2008; Yamada et al., 2009). Treatment of cell culture generated virions with bile or non-ionic detergents diminishes binding to anti-ORF3 mAb but enhances binding to anti-ORF2 mAb, which suggests the ORF3 protein is associated with lipid on the virion surface (Yamada et al., 2009; Emerson et al., 2010).

ORF3 protein and viral egress. The HEV ORF3 protein is required for the release of genotype 3 infectious particles from infected cells (Yamada et al., 2009). Preventing the translation of ORF3 causes intracellular accumulation of HEV RNA with

little to no detection extracellularly (Yamada et al., 2009). Similar results were reported for a genotype 1 human HEV (Emerson et al., 2010). The ORF3 protein PXXP motif is involved in viral egress. The second proline within the PXXP motif is a critical amino acid. Changing the amino acid at this position will prevent the release of virions *in vitro* and infection *in vivo* (Emerson et al., 2010).

Cis-acting Elements

A cis-acting element was identified in a 12 nucleotide sequence, which includes the first and second in frame AUG in the JR, of the genotype 1 human HEV Sar-55 strain (Graff et al., 2005; Huang et al., 2007; Cao et al., 2010). This sequence is conserved among all mammalian HEVs and is critical for HEV replication. A cis-acting element was also identified in the conserved 3' end of the HEV genome. The 3' end contains two stem loop structures in the NCR and a poly (A) tail that are critical for the initiation of HEV negative strand synthesis (Agrawal et al., 2001).

Open Reading Frame 2 (ORF2)

ORF2 capsid protein

The HEV ORF2 encodes the 72 kDa capsid protein (Tam et al., 1991) which includes three potential glycosylation sites and a signal sequence in the N-terminal region (Jameel et al., 1996; Zafrullah et al., 1999). Expression from a recombinant baculovirus system produces a proteolytically cleaved (after aa 111), 56 kDa protein (Li et al., 1997; Robinson et al., 1998) capable of forming virus-like particles (VLPs) (Li et al., 1997).

ORF2 protein epitopes. Neturalizing epitopes of the HEV have been mapped to a 166 aa region (aa 452-617) of the ORF2 capsid protein (Meng, J.H. et al., 2001). Antibodies generated against this region are able to neutralize strains from different genotypes (Meng J. H. et al., 2001). A recombinant protein including aa 458-607 from a genotype 1, Sar-55 strain ORF2 protein is capable of detecting antibodies against all four genotypes (Zhou et al, 2004) and mAbs generated against aa 464-629 of ORF2 protein from a genotype 4, Chinese strain of HEV are capable of reacting with recombinant capsid proteins (aa 464-629) represented by all four genotypes of HEV (Zhang et al., 2009). All four genotypes appear to share a common epitope.

The truncated 56 kDa capsid protein, evaluated as a recombinant vaccine against hepatitis E, contains the epitopes needed to produce neutralizing antibodies and elicit protective immunity in monkeys when challenged with HEV (Tsarev et al., 1997; Zhang et al., 2002). The candidate vaccine consists of amino acids 112-607 of the ORF2 protein, a region more immunogenic in rhesus monkeys than recombinant polypeptides containing aa 1-111, aa 475-660, and aa 607-660 of the ORF2 protein and aa 91-123 of the ORF3 protein (Zhou et al., 2005), when antibody responses were compared.

ORF2 protein genome encapsidation and infectivity. The ORF2 protein is essential for the HEV encapsidation and infectivity. As the structural protein of HEV, the ORF2 capsid protein binds viral RNA during the encapsidation step of the viral life cycle. The ORF2 protein binds to the 5′end of HEV RNA (nt 1-250). The RNA binding region of the ORF2 protein is mapped to aa 112-660. A 76 nt region (nt 130-206) at the 5′end of HEV RNA (nt 1-910), was identified as the primary region required for binding to the

ORF2 capsid protein. However, a region 44 nt downstream, increases the strength of the binding (Surjit et al., 2004; Emerson et al., 2006).

Virus-like particles. A truncated form of the ORF2 capsid protein (aa 112-660) is capable of self-assembly and VLP formation (Li et al., 1997). Native HEV particles display a T=3 icosahedral symmetry. Cryo-electron microscopy of VLPs, resolved to 22 A°, revealed 60 copies of a single subunit forming 30 dimers in a T=1 icosahedral shell (Xing et al., 1999). Although smaller in size, the VLPs morphologically resemble native HEV particles (Li et al., 1997).

Crystal structure. A crystal structure of the HEV ORF2 recombinant protein (aa 112-608 from a genotype 3 strain), resolved to 3.5 A° by a cryoEM map, revealed structural details of the VLPs three domains (shell, aa 129-319; middle, aa 320-455; and protruding, aa 456-606), the location of three potential N-glycosylation sites (Asn-137-Leu-Ser, Asn-310-Leu-Thr, and Asn-562-Thr-Thr) (Zafrullah et al., 1999) on the VLP, and the identification of an amino acid, Tyr-288, essential for the formation of VLPs (Yamashita et al., 2009). Guu et al. (2009) also constructed a crystal structure of the HEV ORF2 recombinant protein (aa 112-608 from a Chinese, genotype 4 T1 strain) that revealed similar domains.

Some of the capsid proteins interact to form the base of the virus particle while others form homodimers, which protrude from the particle. Comparative analysis of the E2 (aa 394-606) (Zhang et al., 2001), E2a (aa 459-600) (Li et al., 2005), and p239 (aa 368-606) (Zhang et al., 2005) dimeric proteins resulted in the identification of a dimerization domain (aa 459-606) present in all 3 proteins. A crystal structure of the

dimerization domain of the recombinant protein E2 revealed an ORF2 capsid protein neutralization site dependent on dimer formation (Li et al., 2009).

N-glycosylation sites. Although the HEV ORF2 capsid protein has three potential N-glycosylation sites; Asn-137-Leu-Ser, Asn-310-Leu-Thr, and Asn-562-Thr-Thr (Zafrullah et al., 1999) that are present when expressed *in vitro*, the ORF2 capsid protein exists primarily in a non-glycosylated form. The N-glycosylation sites are not required for HEV replication *in vitro* (Graff et al., 2008). Although amino acid 562 is located in a more favorable location for N-glycosylation, amino acid 137 and 310 are not (Yamashita et al., 2009; Guu et al., 2009).

Virion transport. Heat shock protein 90 (Hsp90) was recently associated with virion transport (Zheng et al., 2010). The p239 bacterially expressed recombinant ORF2 protein containing aa 368-606 (Li et al., 2005) co-localizes with Hsp90 to the plasma membrane and cytoplasm (Zheng et al., 2010). An interaction between Hsp90 and p239 occurs *in vitro*, and inhibiting the activity of Hsp90 prevents the intracellular transport of p239 and inhibits viral replication (Zheng et al., 2010).

Cell Attachment. Cell surface heparan sulfate proteoglycans (HSPGs) mediate HEV attachment to and infection of cells (Kalia et al., 2009). A 56 kDa recombinant ORF2 protein (aa 458-607), capable of VLP formation, specifically bound transmembrane syndecan HSPGs with Syndecan-1 being the dominant proteoglycan. Sulfated heparin sulfate is critical for the binding of VLPs to cells and the pattern of proteoglycan sulfation (2-O, 6-O) is important in VLP biding; sulfation other than 6-O reduces VLP binding (Kalia et al., 2009). Kalia et al. (2009) proposes a two-step cell

entry mechanism that involves the non-specific HSPGs and a specific unidentified cell receptor.

HEV Genotypes

Rules for assigning HEV genotypes and subtypes have not been formally defined. Recently, a nucleotide difference of 22.1%-26.7% was used to distinguish the four major HEV genotypes, a 6.2%–13.45% nucleotide difference to divide subtypes within genotypes 1 and 2, and a 12.1%-19.8% nucleotide difference to divide subtypes within genotypes 3 and 4 (Lu et al., 2006). However, the proposed subtype designations are not recognized by the HEV scientific community or accepted by the International Committee on Taxonomy of Viruses. Genotype 1 and 2 HEVs are predominantly human isolates that have been associated with waterborne epidemics of hepatitis E as a result of contaminated water supplies. The genotype 3 subtypes predominantly consist of isolates from the industrialized countries including the United States, Japan, and Europe (Argentina, Australia, Austria, Italy, New Zealand, and the Netherlands). Genotype 4 subytpes are represented, primarily, by isolates from Asian countries including China, Taiwan, India, and Japan (Lu et al., 2006; Panda et al., 2007). Geneotype 3 and 4 HEVs are linked to sporadic cases of hepatitis E ensuing from a potential zoonotic transmission.

Avian HEV, isolated from a chicken with hepatitis splenomegaly (HS) syndrome (Haqshenas et al., 2001), is comparable to mammalian HEVs in genomic organization and antigenic epitopes (Huang et al., 2004). Avian HEV is classified as a separate species within the *Hepeviridae* [http://talk.ictvonline.org/files/proposals/taxonomy_proposals_vertebrate1/m/vert01/2126.aspx], given that the avian HEV genome is approximately 6.6kb in length, 600 bp shorter

than mammalian HEVs and only shares 50-60% homology with the other four genotypes (Huang et al., 2004). The recent determination of the near full-length genome sequences of a European and Australian avian HEV and subsequent phylogenetic analysis revealed three distinct genotypes of avian HEV: genotype 1 consisting of Australian strains, genotype 2 consisting of American strains, and genotype 3 consisting of European strains of HEV (Bilic et al., 2009) provide further support of the classification of avian HEV into a separate species.

HEV Animal Reservoirs

Pigs

Pigs are reservoirs of HEV. Since the discovery of the first swine HEV isolate from a pig in the United States (Meng et al., 1997), HEV has been isolated from pigs in many other countries worldwide including Thailand, Japan, Canada, New Zealand, Austria, The Netherlands, China, and India (Hsieh et al., 1999; Garkavenko et al., 2001; Worm et al., 2000; Okamoto et al., 2001; Yoo et al., 20001; Van der Poel et al., 2001; Wang et al., 2002; Arankalle et al., 2002) where swine HEV infection is common (Meng et al, 1999; Chandler et al., 1999; Pina et al., 2000; Garkavenko et al., 2001; Arankalle et al., 2001). Thus far, all swine HEV isolates identified belong to either genotype 3 or 4 and are genetically closely related to genotype 3 and 4 human HEV strains responsible for sporadic cases of hepatitis E (Hsieh et al., 1999; Nishizawa et al., 2003; Takahashi et al., 2003, Yazaki et al., 2003). Human HEV isolates obtained from acute hepatitis E patients (with a history of consuming raw or undercooked pig liver) in Japan have a high nucleotide sequence identity, 100% in a few cases, to swine HEV isolated from commercial pig livers sold in local grocery stores (Yazaki et al., 2003). Genetic analysis

of HEV sequences obtained from hepatitis E patients with a history of consuming raw or undercooked wild boar meat revealed similar results (Matsuda et al., 2003; Tamada et al., 2004). The correlation between raw or undercooked pig liver/wild boar meat and sporadic cases of hepatitis E and the ability of genotype 3 and 4 swine HEVs to cross species barriers and infect humans (Meng et al., 1998; Arankalle et al., 2005) provides additional evidence in support of pigs as reservoirs of HEV. More recently, a case-control study was conducted in three families in France who consumed pig liver sausages "Figatellu" (Colson et al., 2010). It was shown that 7 of 13 individuals who ate raw figatellu and 0 of 5 individuals who did not eat raw figatellu became infected by HEV. HEV RNA of genotype 3 was detected from 7 of 12 figatelli purchased in supermarkets, further indicating zoonotic HEV transmission via contaminated food.

Other Animals

Similar to swine HEV, avian HEV infection of chickens is ubiquitous. Approximately 71% chicken flocks and 30% of chickens in the United States (Huang et al., 2002); and 89.7% of chicken flocks and 20-80% of chickens in Spain (Peralta et al., 2009) are thought to be positive for IgG anti-HEV antibodies.

HEV RNA and anti-HEV antibodies were detected in samples obtained from several acute hepatitis E patients who ingested raw or undercooked deer meat several weeks prior to the onset of disease (Tei et al., 2003). Human HEV isolated from the patients were 99.7-100% identical to isolates recovered from the deer meat (Tei et al., 2003) and clustered with other genotype 3 strains. Seroepidemiological studies are needed to assess the prevalence of HEV infection in deer.

Anti-HEV antibodies were detected in 44-60% of rabbits from two Chinese farms (Zhao et al., 2009). HEV RNA isolated from the farm rabbits has 73-79% nucleotide sequence identity with genotypes 1, 2 and 3. It remains to be determined whether rabbit HEV can cross-species barriers and infect humans.

Anti-HEV antibodies have been detected in 44-90% of United States rodents (Kabrane-Lazizi et al., 1999); 29-62% of cattle, 42-67% of sheep and goats in Somali, Tajikistan, and Turkmenistan (Favorov et al., 1998); and 11.1% of cats from Spain (Peralta et al., 2009). A rat HEV has recently been identified from rats caught in Germany (Johne et al., 2010), and sequence analysis revealed that the rat HEV is distinct from the 4 known mammalian genotypes of HEV. HEV sequences have also been reportedly detected from sheep and cattle in China (Wang and Ma, 2010; Hu and Ma, 2010), although independent confirmation of these findings are still lacking.

Cross-species Infection by HEV

The prototype genotype 3 swine HEV can cross species barriers and infect non-human primates and the genotype 3 US-2 strain of human HEV can cross species barriers and infect specific pathogen free (SPF) pigs (Meng et al., 1998). Similarly, a genotype 4 Indian swine HEV can infect rhesus monkeys (Arankalle et al., 2006) and the genotype 4 human HEVs can infect SPF pigs (Feagins et al, 2008). The genotype 1 human HEV Sar-55 strain and the genotype 2 human HEV Mex-14 strains failed to infected SPF pigs (Meng et al., 1998). Genotype 1 and 2 epidemic strains of HEV appear to have a more limited host range than the genotype 3 and 4 HEVs. Avian HEV, which is genetically and antigenically related to but distinct from human and swine HEV, failed to infect

rhesus monkeys (Huang et al., 2004). However, avian HEV can infect turkeys (Sun et al., 2004).

Vaccines and Antiviral Therapies

The 56 kDa ORF2 (aa 112-607) insect cell-expressed recombinant vaccine against hepatitis E, is highly immunogenic in rhesus monkeys (Zhang et al., 2002; Purcell et al., 2003), and yielded promising results in a human phase 3 efficacy trial in 2004. After two doses the vaccine was 85.7 % efficacious and after three doses the vaccine was 95.5 % effective at preventing hepatitis E (Shrestha et al., 2007). Alternatively, a bacterially expressed ORF2 recombinant polypeptide p239 (aa 368-606) was shown to protect rhesus monkeys (Li et al., 2005) and in a subsequent phase 2 clinical trial, 98-100% seroconversion (depending on dose and administering schedule) was achieved with minimal adverse effects (Zhang et al., 2009). The efficacy and safety of this bacterially-expressed recombinant hepatitis E vaccine in humans were recently reported in a large (>96,000 individuals) randomized, double-blind, placebo-controlled, phase 3 trial (Zhu et al., 2010). One year and 30 days after the third dose was administered, 15 individuals in the placebo group developed hepatitis E compared to zero in the vaccine group. Vaccine efficacy after three doses was 100% (Zhu et al., 2010).

The ORF3 protein was evaluated as a potential vaccine against hepatitis E (Ma et al., 2009). The ORF3 protein of a genotype 4 human HEV, fused to a interleukin (IL)-1β fragment at the N-terminus, expressed in bacteria induced anti-ORF3 antibodies in rhesus monkeys pre-challenge and reduced signs of infection after challenge (viremia and fecal virus shedding) with genotype 4 or 1 human HEV. Results were comparable between

groups challenged with a genotype 4 and genotype 1 strain. Fecal viral shedding was only prevented in one monkey.

The essential role of the HEV RdRp in viral replication makes it a promising target for anti-viral therapies. Small interfering RNA (siRNA), targeting the RDRP gene, is a potential therapeutic (Huang et al., 2009). Small interfering RNA reduced RDRP mRNA levels and ORF2 protein expression *in vitro* and prevented infection in an *in vivo* pig model (Huang et al., 2009). The ORF2 capsid protein is another potential candidate for siRNA therapy. Small interfering RNA targeting different regions of ORF2 from a Chinese genotype 4 swine HEV are effective in blocking ORF2 protein expression *in vitro* (Huang et al., 2010).

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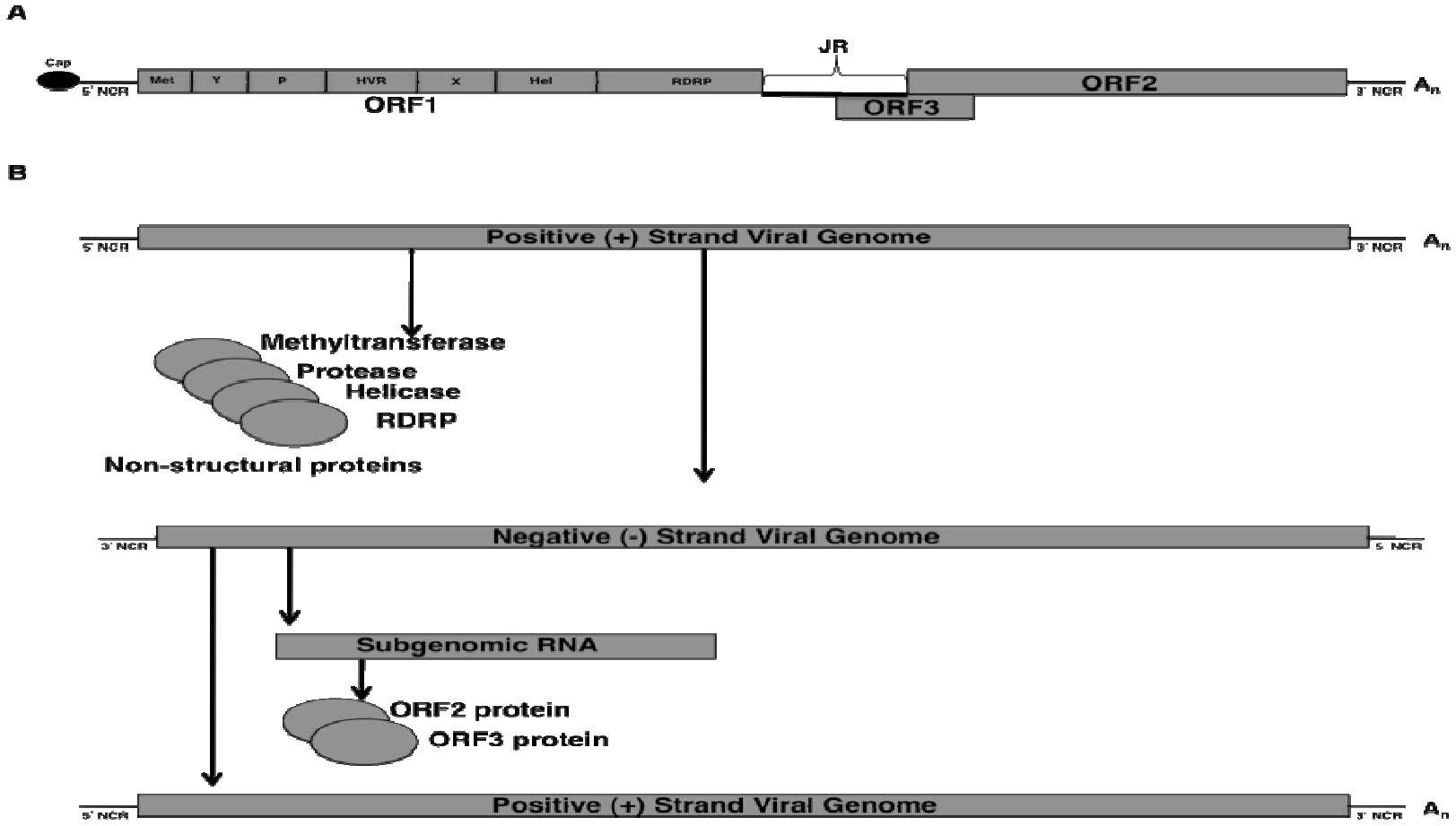
Figure legends

FIG. 1. Schematic diagram illustrating the genomic organization and replication strategy of HEV. **(A)** The HEV genome consists of three open reading frames (ORF1, ORF2, and ORF3), short 5' and 3' noncoding regions (NCR), junction region (JR), 5' cap structure, and 3' poly (A) tail. ORF1 putative functional domains include: Met, methyltransferase domain ; Y, Y domain; P, papain-like cysteine protease domain; X, X domain; Hel, Helicase domain, and RDRP, RNA dependent RNA polymerase. **(B)** The positve strand RNA is immediately translated into the non-structural polyprotein after entering a permissive cell. The positive strand RNA serves as a template for the synthesis of the negative strand RNA. The negative strand RNA serves as a template for the synthesis of two RNA transcripts: the full-length positive strand RNA and the subgenomic RNA. The subgenomic RNA is subsequently translated into the ORF2 capsid protein and the ORF3 protein.

FIG. 1


A
Cap
5' NCR
Met
Y
P
HVR
X
Hel
RDRP
ORF1
JR
ORF2
ORF3
3' NCR
An
B
5' NCR
Positive (+) Strand Viral Genome
3' NCR
An
Methyltransferase
Protease
Helicase
RDRP
Non-structural proteins
3' NCR
Negative (-) Strand Viral Genome
5' NCR
Subgenomic RNA
ORF2 protein
ORF3 protein
5' NCR
Positive (+) Strand Viral Genome
3' NCR
An

CHAPTER 2

Detection and characterization of infectious hepatitis E virus from commercial pig livers sold in local grocery stores in the United States

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ABSTRACT

Hepatitis E virus (HEV) is a zoonotic pathogen of which pigs are reservoirs. To determine the presence of HEV RNA in commercial pig livers sold in local grocery stores in the USA, 127 packages of commercial pig liver were purchased and tested by a universal RT-PCR assay capable of detecting all four known HEV genotypes. Among the 127 livers tested, 14 were positive for HEV RNA. Sequence and phylogenetic analyses revealed that the 14 isolates all belonged to genotype 3. An animal study was subsequently conducted in pigs to determine whether the PCR-positive pig livers still contained infectious virus. The results showed that pigs inoculated with two of the three PCR-positive pig-liver homogenates became infected, as evidenced by the detection of fecal virus shedding, viremia and seroconversion. The study demonstrated that commercial pig livers sold in grocery stores are contaminated by HEV and that the contaminating virus remains infectious, thus raising a public-health concern for food-borne HEV infection.

INTRODUCTION

Hepatitis E virus (HEV), the sole member of the genus *Hepevirus* (Emerson et al., 2004) in the family *Hepeviridae*, is a spherical, non-enveloped virus particle approximately 32–34 nm in diameter (Purcell, 1996). The genome of HEV is a single-stranded, positive-sense RNA molecule, approximately 7.2 kb in size. The viral genome consists of three open reading frames (ORFs 1, 2 and 3) and short 5' and 3' untranslated regions (Purcell, 1996; Wang et al., 2000; Emerson et al., 2001). Four major genotypes of HEV have been identified based on complete-genome sequence analyses (Emerson & Purcell, 2003; Meng, 2003): genotype 1 Asian and African strains, a genotype 2 Mexican strain, genotype 3 strains from industrialized countries and genotype 4 strains from sporadic cases in Asia (Tam et al., 1991; Huang et al., 1992, 2004; Schlauder et al., 1998).

The disease caused by HEV, hepatitis E, is a major public-health concern in developing countries (Arankalle et al., 1994; Purcell, 1996) where sanitation conditions are poor, but is also endemic in many industrialized countries, including the USA (Harrison, 1999; Meng, 2000a, b; Banks et al., 2004). Although the overall mortality rate is generally low (<1 %), it can reach up to 25 % in infected pregnant women (Hamid et al., 1996; Purcell, 1996). Only sporadic cases of acute hepatitis E were reported in the USA and other industrialized countries; however, a significant proportion of healthy individuals in these countries were found to be positive for HEV antibodies (Mast et al., 1997; Thomas et al., 1997; Meng et al., 2002). It has been hypothesized that an animal reservoir(s) exists for HEV (Meng, 2000a, b).

The first animal strain of HEV, swine HEV, was identified from a pig in the USA

and shown to be related antigenically and genetically to human HEV (Meng et al., 1997). More recently, another animal strain of HEV, avian HEV, was identified from chickens with hepatitis–splenomegaly syndrome in the USA (Haqshenas et al., 2001). Like swine HEV, avian HEV is also related genetically and antigenically to human HEV (Huang et al., 2004). Cross-species infections have been demonstrated: swine HEV infected non-human primates (Meng et al., 1998a), human HEV infected pigs (Meng et al., 1998a; Halbur et al., 2001) and avian HEV infected turkeys (Sun et al., 2004).

Recently, cluster cases of acute hepatitis E in Japan were linked epidemiologically and genetically to the consumption of undercooked pig livers and deer meat (Yazaki et al., 2003; Tei et al., 2003, 2004). Therefore, the objectives of this study were to detect and characterize HEV in commercial pig livers sold in local grocery stores in the USA and to determine whether the contaminating pig livers still contained infectious virus.

MATERIALS AND METHODS

In total, 127 packages of commercial pig livers were purchased weekly from three local grocery stores in Blacksburg, VA, USA (125 packages) and Ames, IA, USA (two packages) from September 2005 to March 2006. The packaging material of each pig liver consisted of a foam-insulated tray and a plastic wrap. Each package of pig liver was purchased frozen from the fresh-meat section of the grocery store and stored immediately in a –80 °C freezer until use. A portion of each pig liver was homogenized to a 10 % (w/v) suspension in sterile PBS. The liver homogenates were used for the detection of swine HEV RNA by a universal RT-PCR assay capable of detecting all four known genotypes of HEV essentially as described previously (Huang et al., 2002; Cooper et al., 2005). The expected size of the final product of the universal nested RT-PCR was 348 bp.

The amplified PCR products from each positive liver homogenate were separated in a 0.8 % agarose gel. The expected band was excised from the gel, purified by the glass-milk procedure with a GENECLEAN kit (Bio 101 Inc.) and sequenced for both strands at the Virginia Bioinformatics Institute (Blacksburg, VA, USA). The nucleotide sequences of the HEV isolates from the 14 positive pig livers were deposited in GenBank with accession numbers EF107626 –EF107639. Sequence analyses were conducted by using the MacVector computer program (Oxford Molecular Inc.). The HEV sequences amplified from commercial pig livers were compared with selected known human, swine and avian HEV strains. Phylogenetic analysis was performed by using the maximum-parsimony method in the PAUP program (Sinauer Associates Inc.). GenBank accession numbers and the geographical origins of the nucleotide sequences of the HEV strains used in the phylogenetic and sequence analyses are as follows: Arkell (AY115488, Canada), JRA1 (AP003430, Japan), SwJ570 (AB073912, Japan), JJT-Kan (AB091394, Japan), JKN-Sap (AB074918, Japan), US1 (AF060668, USA), US2 (AF060669, USA), swine (AF082843, USA), B1 (M73218, Burma), HeBei (M94177, China), pSK-HEV-3 (AF444003, Pakistan), Uigh 179 (D11093, China), Hev037 (X98292, India), Abb-2B (AF185822, Pakistan), TK15/92 (AF051830, Nepal), M1 (M74506, Mexico), T1 (AJ272108, China), IND-SW-00-01 (AY723745, India), swCH31 (DQ450072, China), JYI-ChiSai01C (AB197674, China), CCC220 (AB108537, China), JSN-Sap-FH (AB091395, Japan), JKK-Sap (AB074917, Japan), JAK-Sai (AB074915, Japan) and avian HEV (AY535004, USA).

To determine whether the pig livers positive by RT-PCR for HEV RNA still contained infectious HEV, an animal-transmission study was conducted in specific-

pathogen-free (SPF) pigs. Twenty-five, 4-week-old SPF pigs were purchased from a commercial source. Prior to inoculation, all pigs were confirmed to be negative for HEV antibodies by an ELISA (Meng et al., 1998a, b) with the exception of two pigs (1225 and 1227). These two pigs had very low, but detectable levels of maternal HEV antibodies (0.47 and 0.45 A_{405} values, respectively; cutoff value, 0.30) and were assigned to the negative-control group. The pigs were divided into five groups of five pigs each. The five pigs in group 1 were each inoculated intravenously (i.v.) with 2 ml liver homogenate from a PCR-negative commercial pig liver as negative controls. The five pigs in group 2 were each inoculated i.v. with 1 ml standard swine HEV infectious stock with an infectious titre of $5x10^{4.5}$ 50 % pig infectious doses (Meng et al., 1998a) as positive controls. Pigs in groups 3, 4 and 5 were each inoculated i.v. with 2 ml RT-PCR-positive liver homogenates from each of the three selected commercial pig livers. The rationale for selecting the three positive commercial livers for the pig-transmission study was based upon the sequence data of the 14 HEV isolates. To avoid testing the infectivity of positive pig livers that may originate from the same farm, we selected the three isolates that were genetically distinct from each other and had greater sequence divergence than the other 11 isolates when compared with the prototype swine HEV. The animals were monitored for 8 weeks for evidence of HEV infection. Serum samples from each pig were collected prior to inoculation and weekly thereafter for the detection of HEV viremia by RT-PCR as described above and for anti-HEV IgG by ELISA as described previously (Meng et al., 1997, 1998a, b). Fecal samples from each pig were collected prior to inoculation and weekly after inoculation for the detection of HEV RNA by RT-PCR.

RESULTS AND DISCUSSION

Commercial pig livers sold in local grocery stores in the United States are contaminated by genotype 3 HEV that are genetically closely related to human HEV. Of the 127 packages of commercial pig livers purchased from local grocery stores, 14 (11.0 %) tested positive for HEV RNA. Sequence analyses of the resulting 276 bp ORF2 sequence (excluding the PCR primer sequences) revealed that the 14 HEV isolates from pig livers in local grocery stores shared approximately 84–100 % nucleotide sequence identity with each other, 86–94 % identity with the prototype genotype 3 US swine HEV, 87–93 % identity with the two genotype 3 US strains of human HEV (US1 and US2), and 85–90 % identity with a Japanese strain of human HEV (JRA1) thought to be of swine origin. The 14 HEV isolates shared 74–81 % nucleotide sequence identity with genotype 1 HEV strains, 70–72 % identity with the genotype 2 HEV strain and 73–79 % identity with the genotype 4 HEV strains (data not shown). Phylogenetic analysis revealed that all of the 14 isolates identified from commercial pig livers in this study clustered in genotype 3, together with the US strains of human HEV and swine HEV (Fig. 1).

The HEV contaminated commercial pig livers from grocery stores in the United States still contain infectious virus. All negative-control group 1 pigs inoculated with a PCR-negative liver homogenate remained seronegative throughout the study and there was no detectable viremia or fecal virus shedding in group 1 pigs (Fig. 2; Table 1). As expected, all positive-control pigs in group 2 inoculated with a standard infectious stock of swine HEV seroconverted to IgG anti-HEV antibodies by 5 weeks post-inoculation (p.i.) and were still seropositive at the end of the 8 week study (Fig. 2).

Viremia and fecal virus shedding were detected in all five positive-control group 2 pigs (Table 1).

Evidence of active HEV infection was detected in two of the three groups of pigs (groups 3 and 4) inoculated with one of the three PCR-positive commercial liver homogenates, respectively. In group 3 pigs, seroconversion started at 3 weeks p.i. By the end of the 8 week study, all group 3 pigs had seroconverted to IgG anti-HEV antibodies (Fig. 2). Viremia and fecal virus shedding were detected variably in all five group 3 pigs (Table 1). Similarly, by 4 weeks p.i., all of the group 4 pigs had seroconverted to IgG anti-HEV antibodies (Fig. 2). Viremia and fecal virus shedding were also detected in all of the group 4 pigs (Table 1). However, the pigs in group 5, inoculated with another PCR-positive liver homogenate, showed no evidence of infection as there was no seroconversion, viremia or fecal virus shedding in group 5 pigs (Fig. 2; Table 1).

Viruses recovered from pigs 1250 (group 2), 1242 (group 3) and 1201 (group 4) were sequenced over the 276 bp ORF2 region. Sequence analyses confirmed that the viruses recovered from the infected pigs originated from the respective inocula.

HEV infection in pigs is ubiquitous worldwide (Meng, 2003). In some herds in the USA, approximately 60–100 % of pigs are infected (Meng et al., 1997). Swine HEV isolates identified from pigs worldwide belong to either genotype 3 or 4 (Hsieh et al., 1999; Okamoto et al., 2001; van der Poel et al., 2001; Huang et al., 2002; Takahashi et al., 2003; Meng, 2005), although recently, a genotype 1 HEV strain was reportedly detected in a pig in Cambodia (Caron et al., 2006). Genotype 3 and 4 HEV strains are primarily responsible for sporadic cases of hepatitis E in humans, whereas genotype 1 and 2 strains are mainly responsible for hepatitis E epidemics (Emerson & Purcell, 2003;

Meng, 2003, 2005). It has been demonstrated that a genotype 3 strain of human HEV (US2 strain), but not those of genotype 1 or 2, is readily transmissible to pigs (Meng et al., 1998a, b; Halbur et al., 2001). Conversely, genotype 3 swine HEV has been shown to infect non-human primates (Meng et al., 1998a). A genotype 4 swine HEV also infected non-human primates (Arankalle et al., 2006; Meng & Halbur, 2005).

It is now known that hepatitis E is a zoonotic disease and that pigs are reservoirs of HEV (Meng, 2000a, b, 2003, 2005; Meng & Halbur, 2005). In a large, well-controlled, seroepidemiological study involving 465 swine veterinarians, Meng et al. (2002) found that US swine veterinarians were 1.51 times more likely (using genotype 3 swine HEV antigen, P=0.03) to be positive for HEV antibodies than age- and geography-matched normal US blood donors. Similarly, Drobeniuc et al. (2001) reported that about 51 % of swine farmers from Moldova were positive for HEV antibodies, whereas only 25 % of control subjects were seropositive. In North Carolina, swine workers were shown to have a 4.5-fold higher anti-HEV IgG prevalence than control subjects (Withers et al., 2002). Therefore, humans who consume contaminated pork products, such as pig livers, or come into contact with pigs or contaminated pig manure are at potential risk of HEV infection (Matsuda et al., 2003; Tei et al., 2004).

Recently, swine HEV RNA was detected in approximately 2 % of raw pig livers sold in Japanese grocery stores (Yazaki et al., 2003). Unfortunately, it is not known whether the contaminated commercial pig livers from grocery stores in Japan would still contain infectious virus, and no study has been conducted to assess the prevalence of HEV contamination in commercial pig livers in the US. In the present study, we demonstrated that approximately 11 % of commercial pig livers sold in 3 local grocery

stores in the USA are contaminated by HEV. The 14 HEV isolates recovered from contaminated pig livers all clustered in genotype 3, together with the genotype 3 human HEV strains from patients in the USA, Japan and other countries. Our previous study found that the majority of pigs in the USA are infected at approximately 2–4 months of age (Meng et al., 1997). Therefore, viremia and virus shedding are expected to be cleared in the majority of market pigs at slaughterhouses, which are approximately 6 months of age. The detection of infectious HEV in the livers of a proportion of slaughtered pigs suggested that some pigs may have protracted viremia or may be infected beyond 4 months of age. In fact, HEV RNA has been detected in adult sows (Huang et al., 2002; Meng & Halbur, 2005).

The fecal–oral route of transmission indicates that HEV is resistant to inactivation by acidic and mild alkaline conditions in the intestinal tract. It has recently been shown that HEV is more heat-labile than Hepatitis A virus (HAV), another enterically transmitted hepatitis virus (Emerson et al., 2005). When fecal suspensions of the wild-type HM175 strain of HAV or the Sar-55 strain of human HEV were diluted in PBS buffer and compared in the same test by heating for 1 h at 45, 50, 56, 60, 66 or 70 °C, HAV was only 50 % inactivated at 60 °C, but was inactivated almost totally at 66 °C. In contrast, HEV was about 50 % inactivated at 56 °C and inactivated almost totally (96 %) at 60 °C. Therefore, the detection of HEV RNA by RT-PCR in commercial pig livers from grocery stores does not necessarily mean that the contaminating virus is still infectious.

In this study, we demonstrated that two of the three livers contaminated by HEV still harbored infectious virus, and the pigs inoculated with the PCR-positive liver

homogenate developed an active HEV infection indistinguishable from that in pigs infected experimentally with a standard infectious stock of swine HEV. However, pigs inoculated with a third PCR-positive liver homogenate (group 5) did not become infected, suggesting that not all contaminated pig livers contain infectious virus. The storage conditions (4 or –20 °C, or repeated freeze–thaw) in grocery stores probably inactivated the virus in the third PCR-positive pig liver. It is also possible that the infectious HEV titre in the third PCR-positive liver is too low to initiate an active HEV infection.

This is the first report demonstrating that commercial pig livers from grocery stores can contain infectious HEV. The results from this study raise additional public-health concerns over pork safety and the risk of HEV infection via the consumption of undercooked pork products. It remains to be determined whether cooking is effective at inactivating the virus in contaminated pig livers and whether other pork products, such as pig intestines, sold in grocery stores also contain infectious HEV. It is possible that the relatively high HEV antibody prevalence in normal blood donors in the USA and other countries may be a result of individuals consuming HEV-contaminated pork products. Even if cooking can destroy the infectivity of HEV in the contaminated commercial pig livers completely, repeated exposure to foreign HEV proteins from contaminated pork products could elicit the production of a low level of HEV antibodies.

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Figure legends

FIG. 1. A phylogenetic tree based on the nucleotide sequences of a 276 bp region within the ORF2 gene of the HEV genome. The tree was constructed with the PAUP program using the maximum-parsimony method with 100 bootstrap replicates. Avian HEV was included as an outgroup. A scale bar representing the number of character-state changes is shown; bootstrap values >75% are indicated above the major branches. GenBank accession numbers for the HEV sequences used in the phylogenetic tree are listed in the text. The 14 HEV isolates from commercial pig livers in this study are shown in bold. The four major genotypes (G1-G4) and the putative genotype 5 avian HEV (G5?) are indicated. Superscript letters indicate the species from which the strain was isolated: [a]human; [b]swine; [c]chicken.

FIG. 2. Seroconversion to IgG anti-HEV in inoculated and control pigs. **Panel A**, pigs inoculated with a liver homogenate negative for HEV RNA by RT-PCR as negative controls (group 1); **Panel B**, pigs inoculated with an standard infectious stock of swine HEV as positive controls (group 2); **Panels C, D and E**, pigs inoculated with each of the 3 selected pig liver homogenates positive for HEV RNA by RT-PCR (groups 3, 4 and 5, respectively). The ELISA cutoff A_{405} value is 0.30.

FIG. 1

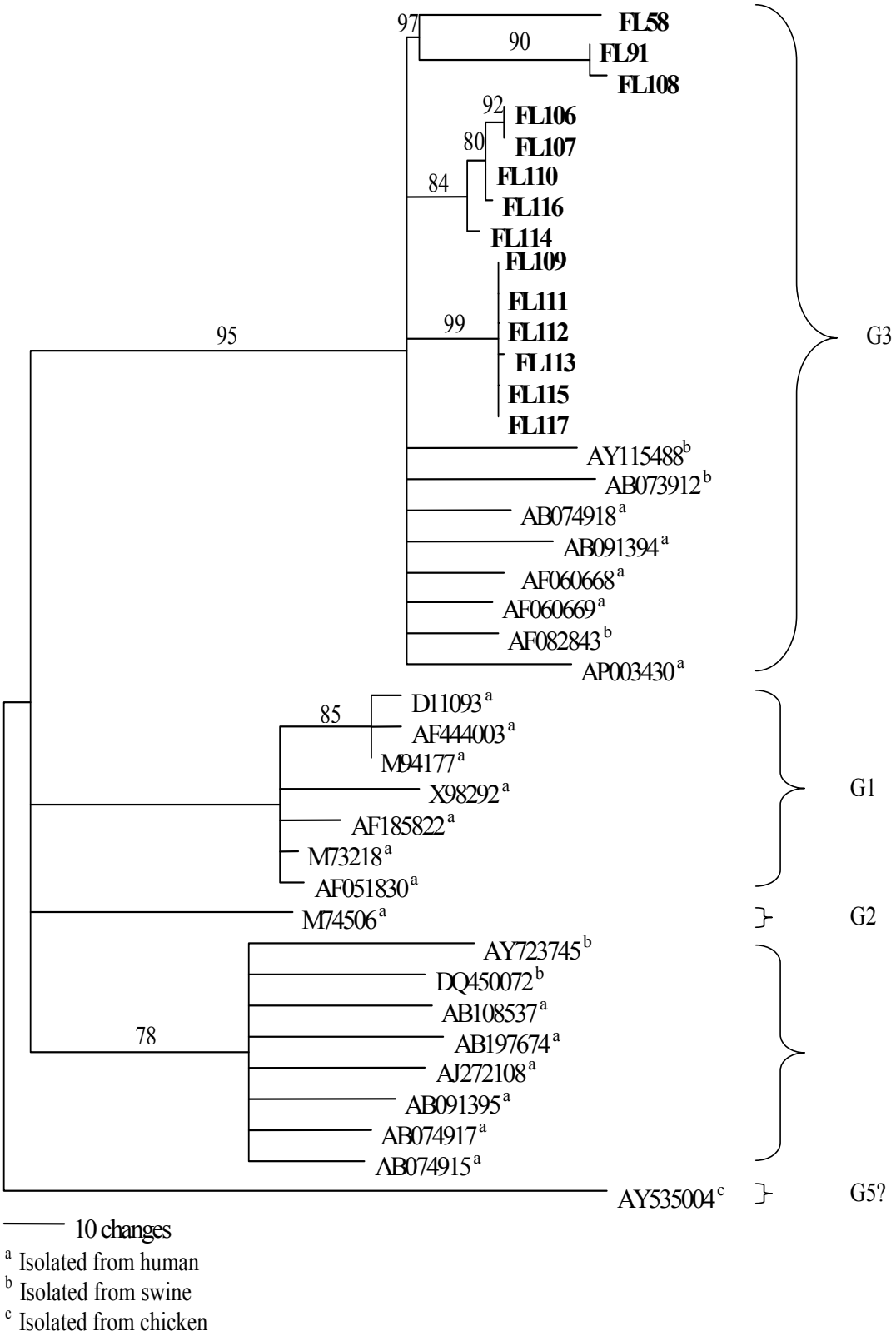
97
90
FL58
FL91
FL108
92
FL106
80
FL107
84
FL110
FL116
FL114
FL109
FL111
99
FL112
95
FL113
FL115
FL117
AY115488[b]
AB073912[b]
AB074918[a]
AB091394[a]
AF060668[a]
AF060669[a]
AF082843[b]
AP003430[a]
G3
85
D11093[a]
AF444003[a]
M94177[a]
X98292[a]
AF185822[a]
M73218[a]
AF051830[a]
G1
M74506[a]
G2
AY723745[b]
DQ450072[b]
AB108537[a]
78
AB197674[a]
AJ272108[a]
AB091395[a]
AB074917[a]
AB074915[a]
AY535004[c]
G5?
10 changes
[a] Isolated from human
[b] Isolated from swine
[c] Isolated from chicken

FIG. 2

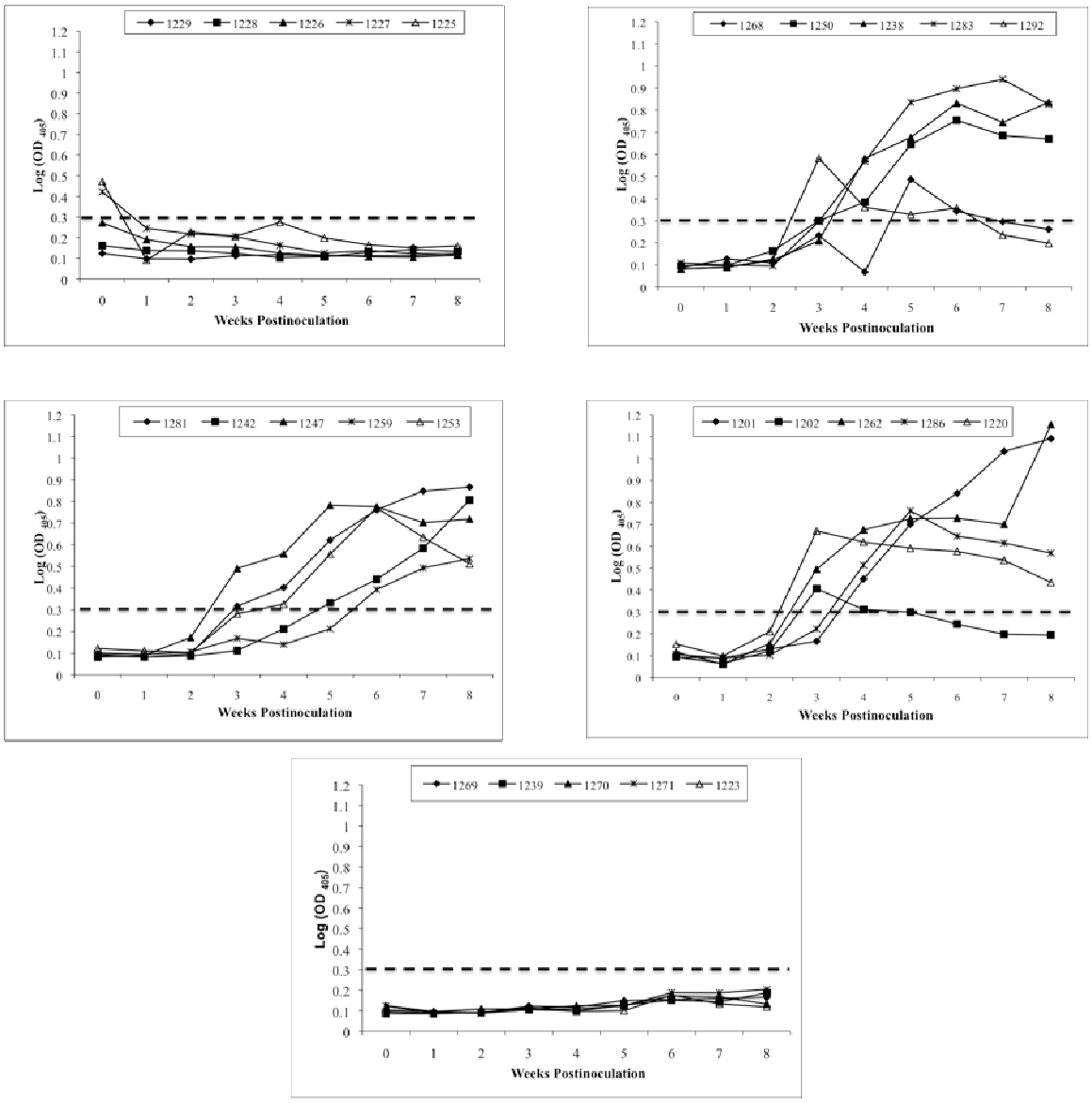
1229 1228 1226 1227 1225
1268 1250 1238 1283 1292
1281 1242 1247 1259 1253
1201 1202 1262 1286 1220
1269 1239 1270 1271 1223
Log (OD 405)
Weeks Postinoculation

TABLE 1. Detection of HEV RNA in weekly samples (fecal/serum) collected from pigs inoculated with liver homogenates positive or negative for HEV RNA by RT-PCR

Group	Pig ID	positive (+) or negative (-) in fecal / serum samples at indicated wpi								
		0	1	2	3	4	5	6	7	8
1[a]	1229	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	1228	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	1226	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	1227	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	1225	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
2[b]	1268	-/-	+/+	+/+	+/-	-/-	-/-	-/-	-/-	-/-
	1250	-/-	+/+	+/-	+/-	-/-	-/-	-/-	-/-	-/-
	1238	-/-	+/-	+/+	+/-	-/-	-/-	-/-	-/-	-/-
	1283	-/-	+/-	+/+	+/-	-/-	+/-	+/-	-/-	-/-
	1292	-/-	+/+	+/+	+/-	-/-	-/-	-/-	-/-	-/-
3[c]	1281	-/-	+/+	+/+	+/+	+/+	+/-	-/-	+/-	-/-
	1242	-/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
	1247	-/-	+/+	+/+	+/+	+/+	+/+	-/-	+/-	-/-
	1259	-/-	+/+	+/-	+/-	+/+	+/-	+/-	+/-	-/-
	1253	-/-	+/+	+/+	+/-	+/-	-/-	-/-	+/-	-/+
4[d]	1201	-/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
	1202	-/-	+/+	+/+	+/-	+/-	+/-	-/-	-/-	-/-
	1262	-/-	+/-	+/+	-/+	+/-	-/-	+/-	+/-	-/-
	1286	-/-	+/+	+/+	+/+	+/+	-/-	+/-	+/-	-/-
	1220	-/-	+/+	+/+	+/-	+/-	-/-	-/-	+/-	-/-
5[e]	1269	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	1239	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	1270	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	1271	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	1223	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-

a – inoculated with a liver homogenate FL1, negative for HEV RNA by RT-PCR (negative controls)
b – inoculated with a swine HEV infectious stock (positive controls)
c – inoculated with a liver homogenate FL58, positive for HEV RNA by RT-PCR
d – inoculated with a liver homogenate FL91, positive for HEV RNA by RT-PCR
e – inoculated with a liver homogenate FL114, positive for HEV RNA by RT-PCR

CHAPTER 3

Inactivation of infectious hepatitis E virus present in commercial pig livers sold in local grocery stores in the United States

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ABSTRACT

Hepatitis E virus (HEV) is a zoonotic pathogen and pigs are a known reservoir. Recently, we showed that approximately 11% of commercial pig livers sold in local U.S. grocery stores for food consumption are contaminated by infectious HEV. In this study, a swine bioassay was used to determine if the infectious HEV in contaminated commercial pig livers could be inactivated by traditional cooking methods. Group 1 pigs (n = 5) were each inoculated intravenously (I.V.) with a HEV-negative liver homogenate as negative controls, group 2 pigs (n = 5) were each inoculated I.V. with a pool of two HEV-positive pig liver homogenates as positive controls, groups 3, 4 and 5 pigs (n = 5, each group) were each inoculated I.V. with a pool of homogenates of two HEV-positive livers incubated at 56 °C for 1 h, stir-fried at 191 °C (internal temperature of 71 °C) for 5 min or boiled in water for 5 min, respectively. As expected, the group 2 positive control pigs all became infected whereas the group 1 negative control pigs remained negative. Four of the five pigs inoculated with HEV-positive liver homogenates incubated at 56 °C for 1 h also became infected. However, pigs in groups 4 and 5 did not become infected. The results indicated that HEV in contaminated commercial pig livers can be effectively

inactivated if cooked properly, although incubation at 56 °C for 1 h cannot inactivate the virus. Thus, to reduce the risk of food-borne HEV transmission, pig livers must be thoroughly cooked.

INTRODUCTION

Hepatitis E, an enterically transmitted form of viral hepatitis, is a disease of major public health concern in many developing countries of Asia and Africa (Emerson and Purcell, 2003; Meng, 2003) where the sanitation conditions are usually substandard. However, sporadic cases of acute hepatitis E were also reported in individuals from the United States, Japan, and other industrialized countries (Hsieh et al., 1999; van der Poel et al., 2001; Clemente-Casares et al., 2003; Yazaki et al., 2003; Mizuo et al., 2005; Takahashi et al., 2003). The mortality rate in individuals infected with hepatitis E virus (HEV) is generally low (< 1%), but it can be as high as 25–30% in infected pregnant women (Hussaini et al., 1997). There are at least four major genotypes of HEV worldwide: genotype 1 (epidemic strains from Asia and Africa), genotype 2 (a single epidemic strain from Mexico), genotype 3 (human and swine strains from industrialized countries), and genotype 4 (human and swine strains from sporadic cases in Asia) (Tam et al., 1991; Huang et al., 1992; Schlauder et al., 1998; Nishizawa et al., 2003' Huang et al., 2007).

Human hepatitis E is not frequently reported in industrialized countries despite the relatively high level of HEV antibody prevalence in healthy individuals from these countries (Mast et al., 1997; Thomas et al., 1997; Meng et al., 2002). It has been hypothesized that hepatitis E is a zoonotic disease and that there are animal reservoir(s) for HEV (Meng et al., 1998; Meng, 2000). The discovery of animal strains of HEV, swine HEV from pigs (Meng et al., 1997) and avian HEV from chickens with hepatitis-splenomegaly syndrome (Haqshenas et al., 2001; Huang et al., 2004), as well as their demonstrated ability to infect across species (Meng et al., 1998; Halbur et al., 2001; Sun

et al., 2004) provide strong support for this hypothesis.

Zoonotic foodborne transmissions of HEV from undercooked pig liver and deer meat to humans have been reported in Japan (Tei et al., 2003; Yazaki et al., 2003; Tamada et al. 2004). It has been shown that commercial pig livers purchased from local grocery stores as food in Japan and the United States (Yazaki et al., 2003; Feagins et al., 2007) are contaminated by HEV and that some of the HEV-contaminated commercial pig livers still contain infectious virus (Feagins et al., 2007). The risk of HEV infection via the consumption of HEV-contaminated pig livers raises further public health concern since it is not clear whether cooking conditions will be effective in inactivating the virus present in the contaminated pig livers. The objective of this study was to determine if traditional cooking methods are effective in inactivating infectious HEV present in contaminated commercial pig livers.

MATERIALS AND METHODS

Source of commercial pig livers. Two packages of commercial pig livers (FL58 and FL91), previously found to contain genotype 3 infectious HEV (Feagins et al., 2007), and one package of commercial pig liver (FL1) negative for HEV (Feagins et al., 2007), were purchased from a local grocery stores in Blacksburg, VA, and were used in this study. The livers were stored in a −80°C freezer until use.

Inactivation of HEV present in contaminated commercial pig livers by different cooking methods and preparation of inocula. A portion of pig livers FL58 and FL91 were separately homogenized in 10% (w/v) sterile phosphate-buffered saline (PBS). Liver homogenates were clarified by centrifugation at 3,000 rpm for 15 minutes at 4°C. Half of FL58 homogenate was pooled with half of FL91 homogenate, untreated and

utilized as the positive control inoculum. The remaining half of FL58 and FL91 homogenates was pooled into a 50 ml conical tube and treated with a heat inactivation step by incubating in a 56°C water bath for 1 hour (the pooled homogenate in the tube was mixed every 10 minutes). A portion of HEV RNA-negative FL1 liver was homogenized in 10% PBS (w/v) and utilized as the negative control inoculum.

Consumers of pig livers typically cook them by either stir-frying or boiling. Therefore, equal amounts of FL58 and FL91 HEV-contaminated commercial pig livers were cut into 0.5 to 1.0 cm^2 cubes, pooled (a total of 24 g), and stir-fried at 191°C for 5 min in 15 ml of pure canola cooking oil. The stir-fried pig livers were then homogenized in 10% (w/v) PBS and used as an inoculum. Similarly, equal amount of FL58 and FL91 HEV-contaminated commercial pig livers were cut into 0.5 to 1.0 cm^2 cubes, pooled (a total of 24 g), and boiled for 5 minutes in 1,420 ml of water. The boiled pig livers were then homogenized in 10% (w/v) PBS and used as an inoculum. A Rival 12" skillet with adjustable temperature control was utilized to stir-fry as well as boil the pig livers. A Taylor TruTemp instant read compact digital thermometer was used to ensure the internal temperature of the stir-fried and boiled pig livers reached at least 71°C.

Swine bioassay to assess the effect of different cooking and inactivation methods on virus infectivity. To determine the effects of different cooking and inactivation methods on virus infectivity, a swine bioassay (Feagins et al., 2007) was conducted since a reliable cell culture for HEV propagation is not available. The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC). Briefly, twenty-five, four-week-old, specific-pathogen-free (SPF) pigs were purchased from a commercial source. Prior to inoculation, all pigs were confirmed to be

negative for HEV antibodies by an enzyme-linked immunosorbent assay (ELISA) (Meng et al., 1998; Halbur et al., 2001). The pigs were divided into 5 groups of 5 pigs each. The 5 pigs in group 1 were each inoculated intravenously (I.V.) with 2 ml of liver homogenate from a PCR-negative commercial pig liver, FL1, as negative controls. The 5 pigs in group 2 were each inoculated I.V. with 2 ml of a pooled homogenate of two HEV-contaminated livers (FL58 and FL91) as positive controls. Pigs in groups 3, 4, and 5 were each inoculated I.V. with 2 ml of a pool of two HEV-contaminated liver homogenates (FL58 and FL91) incubated at 56°C for 1 hr, a pooled homogenate of two HEV-positive livers (FL58 and FL91) stir-fried at 191°C for 5 min or boiled in water for 5 min, respectively. The animals were monitored for a total of 8 weeks for evidence of HEV infection as previously described (Feagins et al., 2007).

Serum samples from each pig were collected prior to inoculation and weekly thereafter for the detection of HEV viremia by reverse transcription-polymerase chain reaction (RT-PCR) and for anti-HEV IgG by ELISA as described previously (Meng et al., 1997, 1998; Halbur et al., 2001). Fecal samples from each pig were collected prior to inoculation and weekly after inoculation for the detection of HEV RNA by RT-PCR.

RT-PCR to detect HEV RNA. To detect HEV RNA in fecal and serum samples of inoculated pigs, a universal RT-PCR assay was performed as previously described (Huang et al., 2002; Cooper et al., 2005). Briefly, total RNAs were extracted by the use of Trizol Reagent (GIBCO-BRL) from 100 µl of the 10% fecal suspension or serum. The total RNA was resuspended in 11.0 µl of DNase, RNase-, and proteinase-free water (Invitrogen). Reverse transcription was performed at 42°C for 60 min with 1 µl of the reverse primer 3157N (5'- CCCTTA(G)TCC(T)TGCTGA(C)GCATTCTC-3'), 1 µl of

Superscript II reverse transcriptase (Invitrogen), 1 µl of 0.1 M dithiothreitol, 4 µl of 5 × RT buffer, 0.5 µl of RNase inhibitor (Promega), and 1 µl of 10mM deoxynucleoside triphosphates. Ten microliters of the resulting cDNA was amplified in a 50 µl PCR reaction with Ampli*Taq* Gold DNA polymerase (Applied Biosystems).

The nested RT-PCR assay amplifies a region within the ORF2 capsid gene (Huang et al., 2002; Cooper et al., 2005). The first round PCR was performed with a set of degenerate HEV primers: 3156N (forward, 5'-AATTATGCC(T)CAGTAC(T)CGG(A)GTTG- 3') and 3157N (reverse, 5'-CCCTTA(G)TCC(T)TGCTGA(C)GCATTCTC-3'). The second round PCR was performed with another set of degenerate HEV primers using the first round PCR product as the template: 3158N (forward, 5'- GTT(A)ATGCTT(C)TGCATA(T)CATGGCT-3') and 3159N (reverse, 5'- AGCCGACGAAATCAATTCTGTC-3'). The PCR parameters for the first and second round PCR 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 min at 42°C, extension for 1 min at 72°C, and a final extension at 72°C for 7 min. The expected final product of the nested RT-PCR was 348 bp.

Samples found to be positive using the universal RT-PCR assay were further confirmed by a RT-PCR assay specific for the FL58 and FL91 HEV strains. The degenerate primers for the specific RT-PCR assay were based upon the published sequences of FL58 and FL91 strains (Feagins et al., 2007): external primer set CL P1 (forward, 5'-CTGGA(T)GCATTGGGGCTT(C)CTTGA-3') and CL P2 (reverse, 5'-A(T)AGAGCA(T)ATACCT(G)CGG(A)CCCACC-3') (first round) and internal primer set CL P3 (forward, 5'-GACACCCGGGAACACTAACACC-3') and CL P4 (reverse, 5'-

TGGTAAGCTCA(T)GCGGTCCCATC-3') (second round). Reverse transcription was performed at 57.5°C for 60 minutes with 1 µl of the reverse primer CL P2 (5'-A(T)AGAGCA(T)ATACCT(G)CGG(A)CCCACC-3'). PCR amplification was performed essentially as described above except that the annealing was 1 min at 57.5°C.

DNA sequencing and sequence analyses. The amplified PCR products from each positive sample were separated in a 0.8% agarose gel. The expected band was excised from the gel and purified by the glassmilk procedure with a GENECLEAN kit (Bio 101 Inc., Carlsbad, CA). The purified PCR products were sequenced for both strands at the Virginia Bioinformatics Institute (Blacksburg, VA). Sequence analyses were conducted using the MacVector computer program (Oxford Molecular Inc.).

RESULTS AND DISCUSSIONS

As expected, all pigs in the group 1 inoculated with a HEV-negative liver homogenate remained seronegative throughout the study, and there was no detectable viremia, fecal virus shedding, or seroconversion in group 1 pigs (Fig. 1, Table 1). All pigs in the group 2 inoculated with a pooled homogenate of two HEV-positive commercial pig livers became infected by HEV, as evidenced by seroconversion to IgG anti-HEV as early as 3 weeks postinoculation (wpi) (Fig. 1), viremia, and fecal virus shedding in 4 of 5 group 2 pigs (Table 1). The infected pigs remain clinically normal, which is consistent with HEV infection in pigs. In general, fecal virus shedding appears prior to viremia, generally at 1 to 2 weeks post inoculation followed by a transient or no viremia. The disappearance of viremia or fecal virus shedding is generally followed by seroconversion to HEV antibodies (Meng et al., 1998). The course of infection, viremia and fecal virus shedding pattern is consistent with acute HEV infection in pigs (Meng et al., 1998).

Interestingly, four of the five pigs in group 3 inoculated with a pool of two HEV-positive liver homogenates incubated at 56°C for 1 hr developed an active HEV infection. Seroconversion started at 2 wpi, by 4 wpi all but one pig seroconverted to IgG anti-HEV and remained seropositive at the end of the 8-week study (Fig. 1). Viremia and fecal virus shedding were detected variably in 4 of the 5 group 3 pigs (Table 1).

The pigs in group 4 inoculated with a pooled homogenate of two HEV-positive livers stir-fried at 191°C for 5 min had no evidence of infection as there was no seroconversion, viremia, or fecal virus shedding in any of the inoculated pigs. Similarly, the pigs in group 5 inoculated with a pooled homogenate of two HEV-positive livers boiled in water for 5 min also showed no evidence of HEV infection (Fig. 1, Table 1). All pigs from each group were necropsied at 8 wpi. Samples tested positive for HEV RNA by the specific RT-PCR assay were sequenced for the ORF2 capsid gene region. Sequence analyses confirmed that the viruses recovered from the experimentally infected pigs originated from the respective inoculum (data not shown).

HEV transmission in industrialized countries is not well understood. It remains to be determined why only sporadic cases of hepatitis E occur in industrialized countries even though a high level of anti-HEV antibody prevalence has been detected in the population (Mast et al., 1997; Thomas et al., 1997; Meng et al., 2002). Because HEV antibodies have been detected in a number of animal species including chickens, swine, cattle, sheep, and goats (Meng, 2000), it has been suggested that HEV transmission is zoonotic. Recently, Yazaki et al. (2003) reported direct evidence of zoonotic HEV transmission via the consumption of grilled or undercooked commercial pig liver purchased from local grocery stores in Japan. The sequences of seven swine HEV isolates

recovered from commercial pig livers from local grocery stores were closely related, or identical in a few cases, to the viruses recovered from the Japanese human hepatitis E patients. The majority of the patients in that study had a history of consuming undercooked pig livers prior to the onset of the disease, indicating that consumption of pig livers is a risk factor for hepatitis E.

In a previous study, we found that approximately 11% of pig livers purchased from local grocery stores in the United States are contaminated by HEV (Feagins et al., 2007). Most importantly, we demonstrated that two of the three contaminated pig livers still contain infectious virus that is transmissible to pigs, indicating that individuals who consume inadequately cooked pig livers may be at an increased risk of acquiring zoonotic HEV infection. Yazaki et al. (2003) suggested that the degree of cooking may affect the risk of acquiring an infection. Unfortunately, no such study has been done to assess whether the current pork safety cooking regulations are sufficient in inactivating HEV present in commercial pig livers. Only limited information is available regarding HEV resistance to inactivation by physical or chemical means. As a fecal-orally transmitted virus, HEV is likely resistant to inactivation by the acidic conditions of the stomach. The ability of HEV to survive harsh or extreme environments can be attributed to its non-enveloped viral structure. Emerson et al. (2005) recently demonstrated that HEV is more heat labile in comparison to hepatitis A virus, also an enterically transmitted hepatitis virus.

The United States Department of Agriculture (USDA) recommends a cooking method for fresh pork that will result in a minimum internal cooking temperature of 71°C (http://www.fsis.usda.gov/is_it_done_yet/, accessed on March 15, 2007). The United

States National Pork Board (NPB) also recommends cooking pork to a minimum internal temperature of 71°C on an instant-read meat thermometer (http://www.pork.org/NewsAndInformation/QuickFacts/default.html, accessed on March 15, 2007). While internal cooking temperatures are used by industry for consistency when different cooking methods are used, many of the recipes do not specify a minimum cooking temperature. Instead, a time stipulation is suggested based on the level of heat. Stir-frying and boiling are the two most widely used and accepted methods for cooking pig livers by consumers. We therefore evaluated if stir-frying and boiling of HEV-contaminated pig livers can effectively inactivate the virus by using a swine bioassay to determine the virus infectivity. Pig livers were stir-fried at 191°C since, under the condition we used in the study, this was the cooking temperature that produced an internal cooking temperature closer to the recommended 71°C without burning the tissue. We demonstrated that pigs inoculated with the homogenates of HEV-positive livers that were stir-fried at 191°C for 5 min or boiled for 5 min (with a minimal internal temperature of 71°C) did not develop HEV infection. Therefore, these two cooking methods were effective in inactivating infectious HEV in contaminated pig livers purchased from local grocery stores. However, it is important to note that the time required to inactivate HEV in pig liver pieces larger than the 0.5 to 1.0 cm^2 cubes used in this study may need to be extended.

By using an *in vitro* system, Emerson et al. (2005) reported that HEV is approximately 50% inactivated when heated at 56°C for 1 hr, therefore a third experimental group was added to determine whether the heating of HEV-contaminated liver homogenate at 56°C for 1 hr would be sufficient to inactivate the virus by using the

swine bioassay. We demonstrated that incubation of homogenates of the contaminated pig livers in a 56°C water bath for 1 hr did not inactivate the virus, as four of the five inoculated pigs developed an active HEV infection, thus supporting the *in vitro* results of Emerson et al (2005).

This is the first report demonstrating that adequate cooking of HEV-contaminated commercial pig livers will inactivate HEV in the tissue. This is also the first report that partially inactivated HEV (heat at 56°C for 1 hr) is still able to initiate an active infection *in vivo*. The results from this study indicate that commercial pig livers purchased from grocery stores for food consumptions should be thoroughly cooked to ensure proper inactivation of infectious HEV present in the livers, and to decrease the risk of potential food-borne HEV transmission.

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Figure legends

FIG. 1. Seroconversion to IgG anti-HEV in inoculated and control pigs. **Panel A**, pigs inoculated with a liver homogenate negative for HEV as negative controls (group 1); **Panel B**, pigs inoculated with a pool of two HEV-positive pig liver homogenate as positive controls (group 2); **Panels C, D and E**, each with a pool of two HEV-positive liver homogenates incubated at 56°C for 1 hr, stir-fried at 191°C for 5 min or boiled in water for 5 min (groups 3, 4 and 5, respectively). The ELISA cutoff OD value is 0.30.

FIG. 1

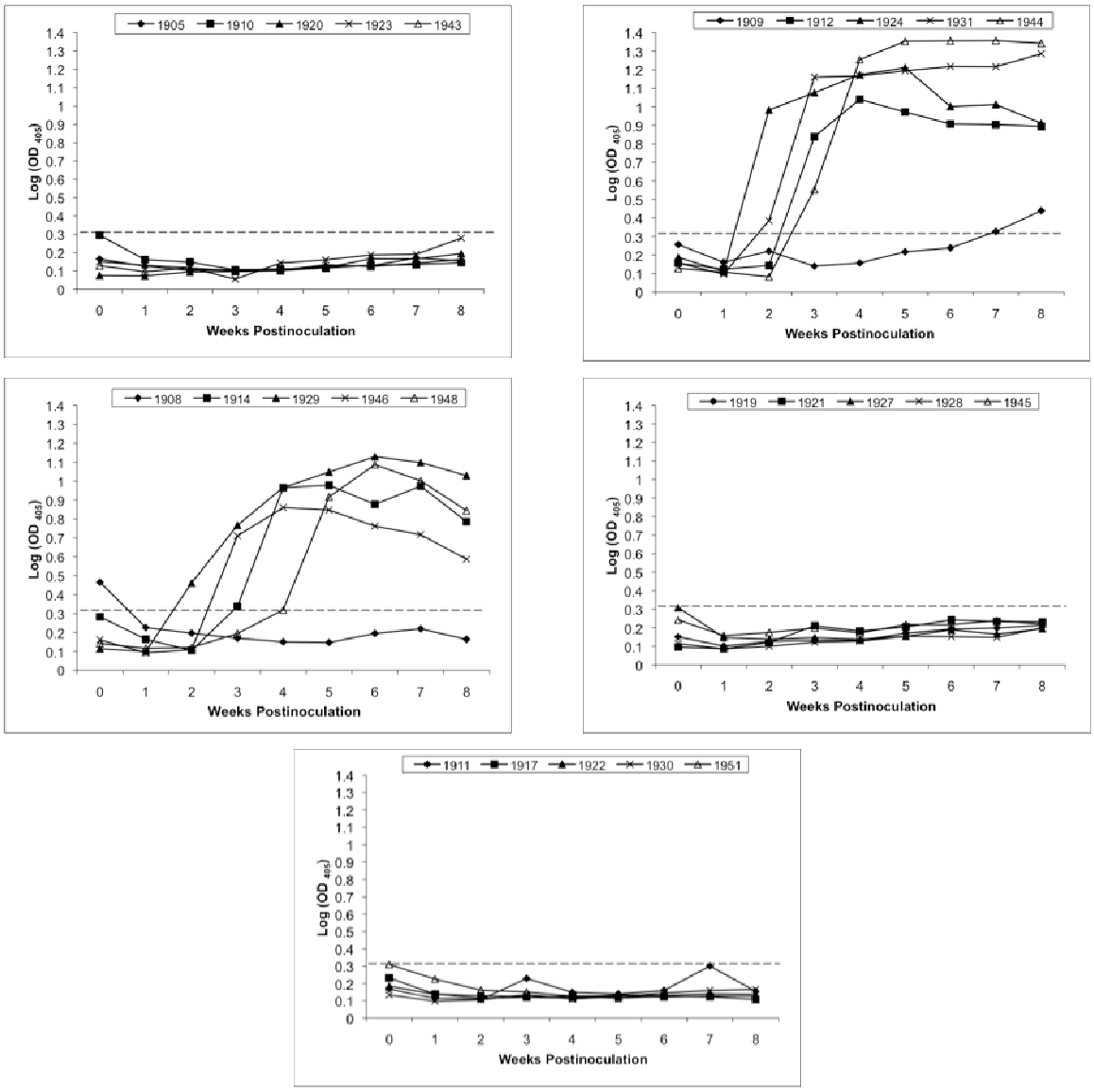
1905 1910 1920 1923 1943
1909 1912 1924 1931 1944
1908 1914 1929 1946 1948
1919 1921 1927 1928 1945
1911 1917 1922 1930 1951
Log (OD 405)
Weeks Postinoculation

TABLE 1. Detection of HEV RNA in samples (fecal/serum) collected weekly from pigs inoculated with commercial pig liver homogenates positive or negative for HEV

Group	Pig ID	positive (+) or negative (-) in fecal / serum samples at indicated wpi								
		0	1	2	3	4	5	6	7	8
1[a]	1905	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
	1910	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
	1920	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
	1923	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
	1943	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
2[b]	1909	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
	1912	- / -	+ / -	+ / -	- / -	- / -	- / -	- / -	- / -	- / -
	1924	- / -	+ / +	+ / +	- / -	- / -	- / -	- / -	- / -	- / -
	1931	- / -	+ / -	- / +	- / -	- / -	- / -	- / -	- / -	- / -
	1944	- / -	+ / -	- / -	- / -	- / -	+ / -	- / -	- / -	- / -
3[c]	1908	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
	1914	- / -	+ / -	+ / -	- / -	- / -	- / -	- / -	- / -	- / -
	1929	- / -	+ / -	- / +	- / -	- / -	- / -	- / -	- / -	- / -
	1946	- / -	+ / -	+ / -	- / -	- / -	- / -	- / -	- / -	- / -
	1948	- / -	+ / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
4[d]	1919	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
	1921	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
	1927	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
	1928	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
	1945	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
5[e]	1911	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
	1917	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
	1922	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
	1930	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
	1951	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -

a – inoculated with a liver homogenate FL1 negative for HEV (negative controls)
b – inoculated with a pool of two HEV-positive pig liver homogenates (positive controls)
c – inoculated with a pool of two HEV-positive liver homogenates heat-treated by incubating at 56°C for 1 hr
d – inoculated with a pool of homogenates of two HEV-positive livers stir-fried at 191°C for 5 min
e – inoculated with a pool of homogenates of two HEV-positive livers boiled in water for 5 min

CHAPTER 4

Cross-species infection of specific-pathogen-free pigs by a genotype 4 strain of human hepatitis E virus

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ABSTRACT

Hepatitis E virus (HEV) is an important pathogen. The animal strain of HEV, swine HEV, is related to human HEV. The genotype 3 swine HEVcan infect humans and genotype 3 human HEV can infect pigs. The genotype 4 swine and human HEV strains are genetically related, but it is unknown whether genotype 4 human HEV can infect pigs. A swine bioassay was utilized in this study to determine whether genotype 4 human HEV can infect pigs. Fifteen, 4-week-old, specific pathogen- free pigs were divided into three groups of five each. Group 1 pigs were each inoculated intravenously with PBS buffer as negative controls, group 2 pigs similarly with genotype 3 human HEV (strain US-2), and group 3 pigs similarly with genotype 4 human HEV (strain TW6196E). Serum and fecal samples were collected at 0, 7, 14, 21, 28, 35, 42, 49, and 56 days postinoculation (dpi) and tested for evidence of HEV infection. All pigs were necropsied at 56 dpi. As expected, the negative control pigs remained negative. The positive control pigs inoculated with genotype 3 human HEV all became infected as evidenced by detection of HEV antibodies, viremia and fecal virus shedding. All five pigs in group 3 inoculated with genotype 4 human HEV also became infected:

fecal virus shedding and viremia were detected variably from 7 to 56 dpi, and seroconversion occurred by 28 dpi. The data indicate that genotype 4 human HEV has an expanded host range, and the results have important implications for understanding the natural history and zoonosis of HEV.

INTRODUCTION

Hepatitis E, caused by hepatitis E virus (HEV), occurs predominately in Asia, Africa, and other developing countries (Emerson and Purcell, 2003; Meng, 2003), but has also been reported in industrialized countries as sporadic cases (Hsieh et al., 1999; van der Poel et al., 2001; Clemente-Casares et al., 2003; Takahashi et al., 2003; Yazaki et al., 2003; Mizuo et al., 2005; Haagsman et al., 2007; Lewis et al., 2008; Mansuy et al., 2008). The mortality rate associated with HEV infection is generally low (<1%), but it can reach as high as 25– 30% in infected pregnant women (Hussaini et al., 1997). Transmission is primarily via the fecal-oral route (Emerson and Purcell, 2003), and in countries with less than optimal sanitation conditions, contaminated water has been the source of major epidemics. However, increasing evidence suggests that zoonotic transmission may be responsible for the spread of hepatitis E as well, especially for the sporadic cases (Tei et al., 2003).

HEV is a single-stranded, positive-sense RNA molecule (Purcell, 1996). The approximately 7.2 kb viral genome consists of three open reading frames (ORFs 1, 2, and 3) and short 5' and 3' untranslated regions (UTR) (Purcell, 1996; Wang et al., 2000; Huang et al., 2007). Currently, HEV isolates can be classified into one of four major genotypes (Emerson and Purcell, 2003; Meng, 2003). Many of the epidemic HEV infections occurring in Asia and Africa are caused by genotype 1 strains whereas an epidemic occurring in Mexico in 1987 was caused by a genotype 2 strain. Genotype 3 and 4 strains are responsible for sporadic cases of HEV infections worldwide (Tam et al., 1991; Huang et al., 1992; Schlauder et al., 1998; Takahashi et al., 2003, 2004; Lewis et al., 2008). The recently identified avian hepatitis E virus (avian HEV) is genetically and

antigenically related to mammalian HEV strains (Haqshenas et al., 2001; Huang et al., 2004; Guo et al., 2006, 2008) but it remains to be determined whether avian HEV belongs to a new 5th genotype or a separate genus.

A significant proportion of healthy individuals from industrialized countries are found positive for HEV antibodies (Mast et al., 1997; Thomas et al., 1997; Meng et al., 2002). The detection of genotypes 3 and 4 strains of HEV in humans that are genetically closely-related, or identical in some cases, to HEV strains from pigs (Nishizawa et al., 2003; Yazaki et al., 2003) suggested that hepatitis E is a zoonotic disease and pigs and maybe other animal species are reservoir(s) for HEV (Meng, 2003). Thus far, HEV has been detected in domestic and wild pigs (Meng et al., 1997), chickens (Haqshenas et al., 2001; Huang et al., 2004), and deer (Takahashi et al., 2004). Meng et al. (1997) isolated the first animal strain of HEV, swine HEV, from a pig in the United States. Since then, swine HEV has been isolated from pigs in many other countries (van der Poel et al., 2001; Wang et al., 2002; Yazaki et al., 2003). Swine HEV isolates obtained from countries worldwide belong to either genotype 3 or 4 (Huang et al., 2002; Cooper et al., 2005).

Genotype 3 swine HEV, which is related both antigenically and genetically to human HEV, has been shown to infect nonhuman primates. Similarly, the US-2 strain of human HEV has been shown to infect pigs (Meng et al., 1998b; Halbur et al., 2001). Compared to genotype 3 HEV, genotype 4 strains of human and swine HEVs have not been well characterized experimentally. The objective of this study was to determine if a genotype 4 strain of human HEV has the ability to cross species barriers and infect pigs.

MATERIALS AND METHODS

Virus Stocks. The infectious titers of the genotype 3 human HEV stock (US-2 strain) and the genotype 4 human HEV stock (TW6196E strain) used in this study have been determined: $10^{5.3}$ 50% monkey infectious dose (MID_{50}) per ml for the US-2 strain (Meng et al., 1998b), and 2 x 10^3 MID_{50} per ml for the TW6196E strain (Huang et al., 2002; Wu et al., 2002).

Pigs. Fifteen, 2-week-old, specific-pathogen-free (SPF) pigs were purchased from a commercial source (Geneticporc Inc., Alexandria, MN). Prior to inoculation, all pigs were confirmed to be negative for IgG anti-HEV antibodies by an enzyme-linked immunosorbent assay (ELISA) (Meng et al., 1998a,b; Halbur et al., 2001).

Experimental design for the swine bioassay. The infectious virus stocks were appropriately diluted to adjust the titer to the same for both the genotypes 3 and 4 virus stocks. The pigs were randomly divided into 3 groups of 5 pigs each. Group 1 pigs were each inoculated intravenously (IV) with 1 ml of sterile phosphate buffered saline (PBS) as negative controls, group 2 pigs were each inoculated IV with 1 ml (10^3 MID_{50}) of the genotype 3 human HEV (strain US-2) as positive controls, and group 3 pigs were each inoculated IV with 1 ml (10^3 MID_{50}) of the genotype 4 human HEV (strain TW6196E) as the experimental group. The pigs were monitored for evidence of HEVinfections for a total of 8 weeks, and all animals were necropsied at 56 dpi.

Sample collection and processing. Serum samples and fecal swabs were collected weekly from each pig at 0, 7, 14, 21, 28, 35, 42, 49, and 56 days postinoculation (dpi). Serum samples were tested for HEV viremia by a universal reverse transcription-polymerase chain reaction (RT-PCR) (Huang et al., 2002; Cooper et al., 2005) and for

anti-HEV IgG by ELISA as described previously (Meng et al., 1997, 1998a,b; Halbur et al., 2001). Fecal swabs (10% suspension in PBS buffer) were also tested for HEV RNA by RT-PCR.

In addition, samples of feces were collected from each pig in group 2 and group 3 every 3 days for the first 4 weeks to investigate the level of HEV viral RNA shedding in the feces during the acute stage of infection. A portion of the fecal material was made into 10% fecal suspension in sterile PBS buffer and used for the quantification of HEV RNA by a real-time RT-PCR. Samples of feces were also tested for HEV RNA by the universal RT-PCR as described previously (Huang et al., 2002; Cooper et al., 2005) for comparison purpose.

During necropsy, samples of serum, feces, bile, and liver tissue were collected and stored at -80ºC until use. A portion of the liver tissue samples were homogenized in 10% (w/v) sterile PBS buffer. The liver homogenates were clarified by centrifugation at 3,000 rpm for 15 min at 4ºC (Eppendorf centrifuge 5810) and then used for the detection of HEV RNA by RT-PCR.

ELISA to detect anti-HEV antibodies. A purified, 56 kDa truncated form of the HEV capsid protein, expressed from a recombinant baculovirus containing the ORF2 of the genotype 1 Sar-55 human strain of HEV, was used as the antigen for the ELISA as described previously (Meng et al., 1997, 1998a,b).

RT-PCR to detect HEV RNA. To detect HEV RNA in serum samples, fecal swabs, feces, bile, and liver tissue homogenates of inoculated pigs, a universal RT-PCR assay was performed as described previously (Huang et al., 2002). The universal RT-PCR assay is capable of detecting all 4 known genotypes of HEV (Huang et al., 2002;

Cooper et al., 2005). Briefly, total RNAs were extracted by the use of Trizol Reagent (GIBCO-BRL) from 100 µl of the serum, fecal suspension, bile, or 10% liver homogenate. The total RNA was resuspended in 11 µl of DNase, RNase-, and proteinase-free water (Invitrogen). Reverse transcription was performed at 42ºC for 60 min with 1 µl (10 mM) of the reverse primer 3157N [5'-CCCTTA(G)TCC(T)TGCTGA(C)GCATTCTC-3'], 1 ml (200 U/ml) of Superscript II reverse transcriptase (Invitrogen), 1 µl of 0.1 M dithiothreitol, 4 µl of 5 x RT buffer, 0.5 µl (40 U/ml) of RNase inhibitor (Promega), and 1 µl of 10 mM deoxynucleoside triphosphates. Ten microliters of the resulting cDNA was subsequently amplified in a 50 µl PCR reaction with AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA).

The nested universal RT-PCR assay amplifies a region within the ORF2 capsid gene (Huang et al., 2002; Cooper et al., 2005). The first round PCR was performed with a set of degenerate HEVprimers: 3156N [forward, 5'-AATTATGCC(T)CAGTAC(T)CGG(A)GTTG- 3'] and 3157N [reverse, 5'-CCCTTA(G)TCC(T)TGCTGA(C)GCATTCTC-3']. The second round PCR was performed with another set of degenerate HEV primers using the first round PCR product as the template: 3158N [forward, 5'-GTT(A)ATGCTT(C)TGCATA(T)-

CATGGCT-3'] and 3159N [reverse, 5'-AGCCGACGAAATCAATTCTGTC-3']. The PCR parameters for the first and second round PCR were similar with an initial denaturation step at 95ºC for 9 min, followed by 39 cycles of denaturation for 1 min at 94ºC, annealing for 1 min at 42ºC, extension for 1 min at 72ºC, and a final extension at 72ºCfor 7 min. The expected final product of the universal nested RT-PCR was 348 bp.

Real-time RT-PCR to quantify HEV RNA in feces. Quantification of HEV RNA in the feces (10% feces suspension in PBS buffer) during the first 4 weeks of infection was performed essentially as described previously (Jothikumar et al., 2006) with a few modifications. Briefly, to generate a HEV RNA standard, a plasmid containing a region of ORF3 was constructed from the infectious cDNA clone pSHEV-3 of a genotype 3 swine HEV (Huang et al., 2005, 2007) using the TA Cloning Kit (Invitrogen) pCR12.1 vector and the following primers: [forward, 5'-ATGCTGCCCGCGCCACCG- 3'] and [reverse, 5'-AGGGGTTGGTTGGATGAA-3']. Plasmid DNA was purified using the GenEluteTM Plasmid Miniprep Kit (Sigma–Aldrich, St. Louis, MO) and quantified using the Nanodrop ND-1000 according to the manufacturer's instructions. The mMESSAGE mMACHINE1 High Yield Capped RNA Transcription Kit (Ambion, Austin, TX) was used to generate an *in vitro* RNA transcript from the plasmid DNA template containing a T7 promoter according to the manufacturer's protocols. Plasmid DNA was removed completely with DNase I treatment and the integrity of the RNA transcript was checked by gel electrophoresis.

A standard curve was generated from 10-fold serial dilutions of the in vitro-synthesized HEV RNA standard and used for real-time RT-PCR with the QuantiTect Probe RT-PCR Kit (Qiagen, Valencia, CA) on an iCycler (Bio-Rad, Hercules, CA). The primers used for the realtime PCR are: forward [5'-GGTGGTTTCTGGGGTGAC-3'] and reverse [5'-GGTTGGTTGGATGAATATAGGG-3']. The probe [5'-TGATTCTCAGCCCTTCGC-3'] contained a 5' 6-carboxy fluorescein fluorophore (FAM) and a 3' black hole quencher (BHQ-1). The primers (Invitrogen, Carlsbad, CA) and the probe (MWG Biotech, Huntsville, AL) were commercially

synthesized. HEV RNA was extracted from 10% fecal suspensions of group 2 and group 3 pigs collected at different time points during the first 4 weeks post-inoculation. In brief, 100 µl of fecal suspension was mixed with 1 ml of Trizol Reagent and 200 µl of chloroform. The aqueous phase was added to the RNeasy Mini Kit spin column (Qiagen) for RNA extraction and purification using on-column DNase digestion according to the manufacturer's instructions and the RNA was stored at -80ºC until use. Reverse transcription was carried out at 50ºC for 30 min, followed by denaturation at 95ºC for 15 min. Real-time PCR amplification was performed with 50 cycles at 95ºC for 10 sec, 55ºC for 20 sec, and 72ºC for 15 sec. The reproducibility of the assay was determined by testing each sample in triplicate and the mean value was calculated.

RESULTS

As expected, all pigs in the negative control group 1 inoculated with sterile PBS buffer remained seronegative throughout the study, and there was no detectable HEV viremia, fecal virus shedding, or seroconversion in group 1 pigs (Fig. 1, Table 1). All pigs in the positive control group 2 inoculated with genotype 3 human HEV (strain US-2) became infected, as evidenced by seroconversion to IgG anti-HEV as early as 2 weeks postinoculation (wpi), viremia, and fecal virus shedding (Fig. 1, Table 1).

All five pigs in the experimental group 3 inoculated with the genotype 4 human HEV strain TW6196E also became infected. Seroconversion started at 2 wpi, and by 4 wpi all pigs had seroconverted to IgG anti-HEV and remained seropositive at the end of the 8-week study (Fig. 1). Viremia and fecal virus shedding were also detected variably in group 3 pigs (Table 1). Viremia is transient and of very short duration in both genotypes 3 and 4 human HEV-infected pigs (Table 1). Fecal virus shedding in the group

2 pigs inoculated with the genotype 3 human HEV lasted approximately 4 weeks: by weeks 5 and 6, there is only one pig that still shed virus in feces. In group 3 pigs inoculated with the genotype 4 human HEV, fecal virus shedding appears to last longer: 4/5 pigs at 6 wpi and 3/5 pigs at 8 wpi still shed virus in feces (Table 1).

In general, fecal virus shedding in pigs infected by swine HEV or genotype 3 human HEV occur in the first 4 to 5 wpi [Meng et al., 1998a,b; Halbur et al., 2001]. Therefore, to evaluate the onset and pattern of fecal virus shedding between genotype 3 and genotype 4 human HEV-infected pigs, the amounts of fecal viral shedding during the first 4 weeks of the infection were determined by a real-time PCR. HEV RNA was detected in fecal samples from group 2 and group 3 pigs as early as 3 dpi. In group 2 pigs infected with genotype 3 human HEV, viral RNA levels in feces ranged from 3.49 to 9.10 log copies per mg of feces during the first 4 weeks. For group 2 pigs, the virus titers peaked in the feces of most pigs at approximately 12–18 dpi (Table 2, Fig. 2). Fecal virus shedding disappeared in one pig (ID 32) at 24 dpi, whereas another pig (ID 44) stopped shedding virus at 27 dpi. The remaining 3 pigs in group 2 still shed virus in feces at the end of the 4 week observation period (Table 2). In group 3 pigs infected by the genotype 4 human HEV, viral RNA levels in feces ranged from 4.21 to 9.26 log copies per mg of feces (Table 2). The fecal virus titers of most group 3 pigs peaked at approximately 9–21 dpi (Table 2, Fig. 2). Samples with detectable HEV RNA in feces by real-time RT-PCR (Table 2) were also determined to be positive by the universal RT-PCR (data not shown). All pigs from each group were necropsied at 8 wpi. The PCR products amplified from serum, fecal swabs, bile, and liver samples from two selected pigs from group 2 and group 3 were sequenced for the ORF2 gene region. Sequence

analyses confirmed that the viruses recovered from the experimentally infected pigs originated from the respective inoculum (data not shown).

DISCUSSION

The rare incidence of human-to-human HEV transmission in endemic regions suggests that animal reservoirs of HEV may exist (Meng, 2003). Human HEVstrains (US-1 and US-2) recovered from hepatitis E patients in the United States are closely related to the genotype 3 swine HEV strain isolated from a pig in the same geographical area (Schlauder et al., 1998; Meng et al., 1998b). Similarly, genotype 4 human HEV isolates obtained from hepatitis E patients in China and Taiwan (Hsieh et al., 1999;Wanget al., 2002;Wuet al., 2002) are very similar to swine HEV isolates obtained from pigs in the same area. These findings strongly suggest that pigs are reservoirs for genotype 3 and genotype 4 strains of HEV.

Meng et al. (1998b) demonstrated that the genotype 3 swine HEV was able to cross species barriers and infect rhesus monkeys and a chimpanzee and, in a reciprocal experiment, that SPF pigs were experimentally infected with the US-2 strain of genotype 3 human HEV. However, a genotype 1 human HEV strain (Sar-55) and the genotype 2 human HEV strain (Mex-14) were not able to infect SPF pigs under experimental conditions (Meng et al., 1998a). The failure to infect pigs with the Sar-55 (genotype 1) and Mex-14 (genotype 2) HEV strains suggest that the transmissibility of human HEV to other species such as pigs varies from HEV genotype to genotype. Therefore, it is important to determine whether or not the genotype 4 human HEV strain identified recently has the ability to infect across species barriers.

The genomes of genotype 4 human HEV strains have been shown to be closely

related to genotype 4 swine HEV strains obtained from the same geographic region (Nishizawa et al., 2003; Takahashi et al., 2003). A genotype 4 swine HEV strain isolated from a pig on a swine farm in Hokkaido, Japan was found to have 99% nucleotide sequence identity with a genotype 4 human HEV isolate obtained from a patient with sporadic acute hepatitis E in Hokkaido (Nishizawa et al., 2003). Similar results have also been reported with genotype 4 swine and genotype 4 human HEV strains in Bali, Indonesia (Wibawa et al., 2007). In addition, Yazaki et al. (2003) found that a genotype 4 swine HEV isolate obtained from pig livers purchased in local grocery stores in Hokkaido, Japan shared 97.8–100% nucleotide sequence identity with genotype 4 human HEV strains obtained from patients with sporadic acute or fulminant hepatitis E who had consumed undercooked pig livers prior to the onset of the disease. The prevalence of anti-HEV IgG, when tested with a genotype 4 HEV capsid protein as the antigen, was found to be 58% in pigs from commercial herds in Hokkaido (Takahashi et al., 2003). These data strongly suggest that, similar to genotype 3 HEV, genotype 4 HEV may also have an expanded host range. In fact, recently Arankalle et al. (2006) were able to infect rhesus monkeys with a genotype 4 Indian strain of swine HEV.

In this present study, we demonstrated that SPF pigs experimentally inoculated with genotype 4 human HEV (strain TW6196E) (Wu et al., 2002) became infected. Fecal virus shedding and viremia were detected variably from 7 to 56 dpi, seroconversion occurred by 28 dpi. There are a few minor discrepancies in HEV RNA positivity detected in the weekly fecal swabs by RT-PCR (Table 1) and in the feces samples collected every 3 days during the first 4 weeks (Table 2). For example, the fecal swabs from weeks 1 and 2 in pig number 28 were tested negative for HEV RNA (Table 1), whereas the same pig

was tested positive for HEV RNA during the first 2 weeks when the feces samples (instead of swabs) were tested (Table 2). This is not surprising since the amounts of fecal materials collected weekly by fecal swabs are variable (Meng et al., 1998a) and sometimes only a small amount of fecal materials can be obtained by swabs in some pigs, and thus likely reflecting the minor discrepancies of the results between fecal swabs (Table 1) and feces (Table 2). The fecal virus shedding in pigs infected with genotype 4 human HEV appears to last longer than pigs infected with the genotype 3 human HEV. With the exception of one pig, the fecal virus shedding in all other 4 genotype 3 HEV-infected pigs disappeared after 4 wpi (Table 1). In contrast, three of the 5 genotype 4 HEV-infected pigs still shed virus at the end of the 8-week study. However, the titers of viral RNA levels quantified by real-time PCR were comparable in pigs infected with genotype 3 human HEV and genotype 4 human HEV, and there is no significant difference in virus RNA titers in feces (Fig. 2). It remains to be determined whether there exist virulence differences between genotype 3 and genotype 4 human HEV strains. Since both genotypes 3 and 4 human HEV strains are now shown to infect pigs, future experiments to compare the pathogenicity of these strains in pigs can now be performed.

This is the first report of a genotype 4 human HEV strain crossing species barriers and infecting pigs, thereby supporting field observations that genotypes 1 and 2 HEV strains are restricted to humans, whereas genotypes 3 and 4 HEV strains have a broader host range that include both humans and swine. The results from this study have important implications for understanding the natural history and zoonosis of HEV. The ubiquitous nature of genotype 3 and 4 swine HEV worldwide (Wang et al., 2002; Yazaki et al., 2003; Banks et al., 2004; Cooper et al., 2005) and the demonstrated ability to infect

across species raises potential public health concerns. Approximately 60–80% of swine herds in the United States are infected with swine HEV and swine veterinarians in the United States have an increased risk for zoonotic infection (Meng et al., 2002). Similar results have also been reported amongst pig handlers in Moldova (Drobeniuc et al., 2001) and Taiwan (Hsieh et al., 1999). Sporadic cases of hepatitis E have been linked to the consumption of raw or undercooked pig livers (Yazaki et al., 2003). Therefore, individuals who consume raw or undercooked pig livers are also at an increased risk for zoonotic HEV infection (Yazaki et al., 2003; Feagins et al., 2007). On the other hand, since pigs can be infected by both genotypes 3 and 4 human HEV, pigs may play an important role in the survival and transmission of genotypes 3 and 4 human HEV in human populations in endemic areas. Future studies are warranted to determine the mechanism of cross-species infection by genotypes 3 and 4 HEV strains.

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Figure legends

FIG. 1. Seroconversion to IgG anti-HEV in HEV-inoculated and control pigs. **Panel A**, pigs inoculated with sterile phosphate buffered saline (PBS) as negative controls (group 1); **Panel B**, pigs inoculated with a genotype 3 human HEV (strain US-2) as positive controls (group 2); **Panel C**, pigs inoculated with a genotype 4 human HEV (strain TW6196E) as the experimental group (group 3). The ELISA cutoff OD value is 0.30.

FIG. 2. Average titers of HEV RNA in feces (expressed as log copies per mg of feces) from all five pigs in each group at the indicated days post-inoculation (dpi) during the first 4 weeks of infection. The open bars represent pigs inoculated with the genotype 3 human HEV (Strain US-2) and the filled bars represent pigs inoculated with genotype 4 human HEV (Strain TW6196E). The error bars represent ± standard deviation.

FIG. 1

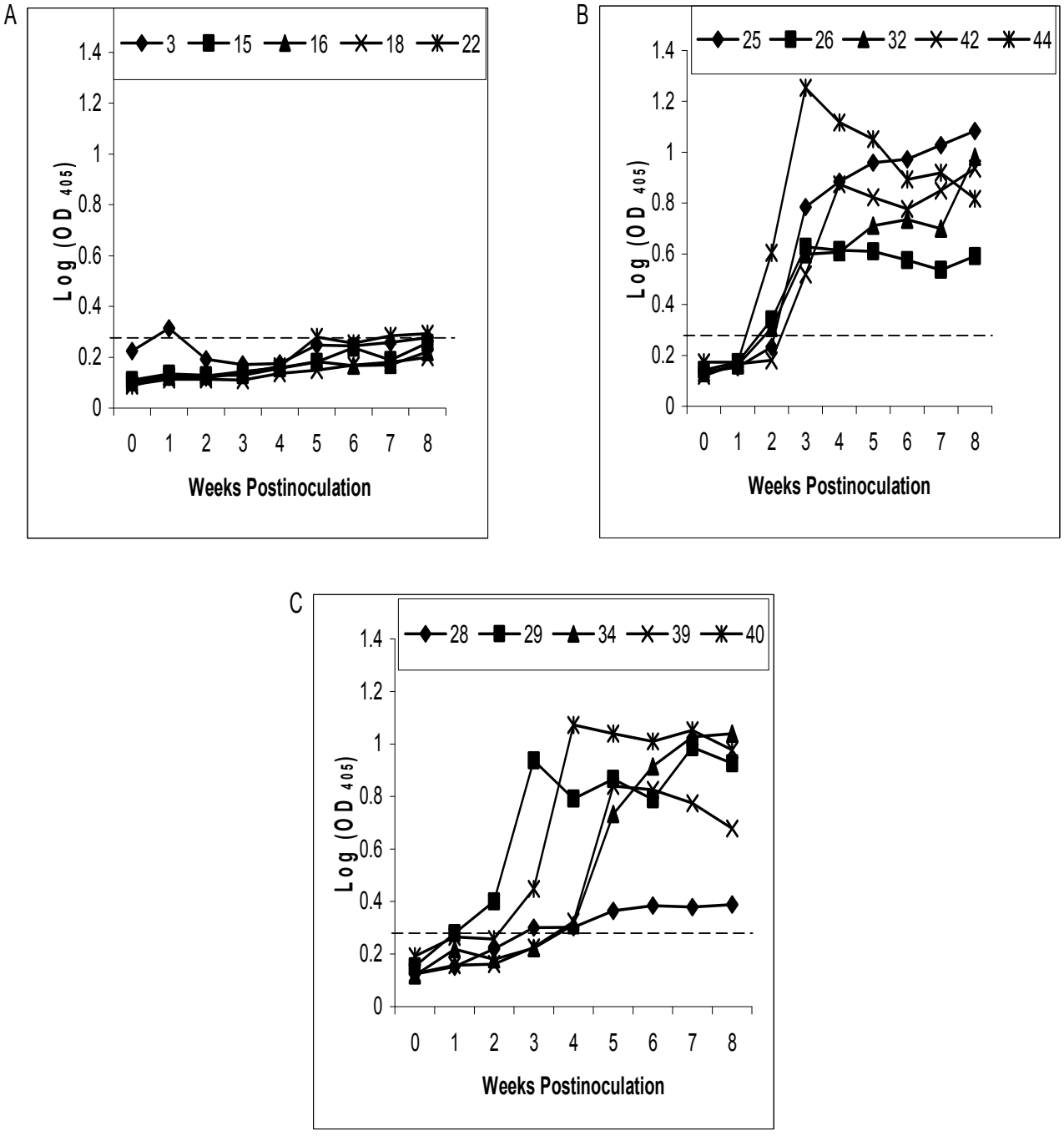
A
3
15
16
18
22
Log (OD 405)
Weeks Postinoculation
B
25
26
32
42
44
Log (OD 405)
Weeks Postinoculation
C
28
29
34
39
40
Log (OD 405)
Weeks Postinoculation

FIG. 2

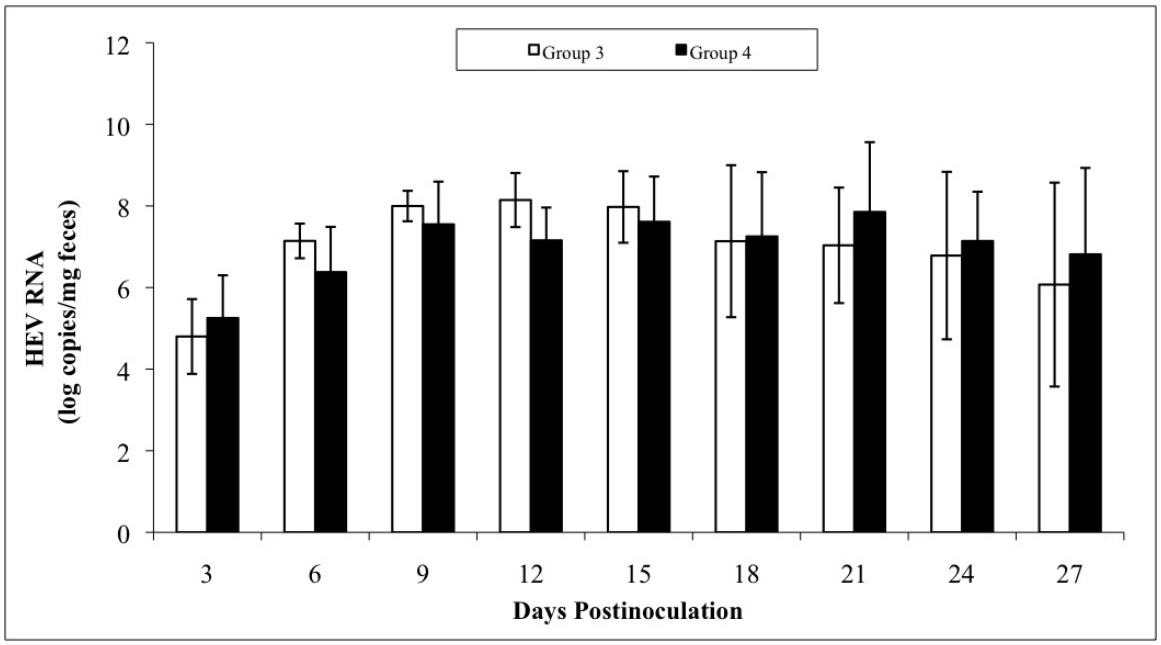
Group 3
Group 4
HEV RNA
(log copies/mg feces)
12
10
8
6
4
2
0
3
6
9
12
15
18
21
24
27
Days Postinoculation

TABLE 1. Detection of HEV RNA by RT-PCR in samples (fecal/serum) collected weekly from pigs inoculated with PBS buffer, with a genotype 3 human HEV, and a genotype 4 human HEV

Group	Pig ID	positive (+) or negative (-) in fecal / serum samples at indicated wpi							
		1	2	3	4	5	6	7	8
1[a]	3	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	15	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	16	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	18	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	22	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
2[b]	25	+/-	+/+	+/+	+/+	+/-	+/-	-/-	-/-
	26	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-
	32	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-
	42	-/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-
	44	-/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-
3[c]	28	-/-	-/-	+/-	+/-	+/-	-/-	-/-	-/-
	29	+/-	-/+	+/+	+/-	-/-	+/-	+/-	+/-
	34	+/-	-/-	+/+	+/+	+/+	+/+	+/+	+/+
	39	-/-	-/-	+/-	+/-	-/-	+/-	+/-	-/-
	40	+/-	-/-	+/+	+/-	+/-	+/-	-/-	+/-

a – inoculated with sterile phosphate buffered saline, PBS (negative controls)
b – inoculated with a genotype 3 human HEV strain US-2 (positive controls)
c – inoculated with a genotype 4 human HEV strain TW6196E (experimental group)

TABLE 2. Quantification by real-time RT-PCR of HEV RNA in fecal samples collected every three days from pigs inoculated with a genotype 3 (G3, group 2) and a genotype 4 (G4, group 3) human HEV between 3 and 27 days post-inoculation

Days postinoculation	Group 2 - G3 US-2 Strain HEV RNA in fecal samples (log copies/mg)				
	pig 25	pig 26	pig 32	pig 42	pig 44
3	3.49	5.21	4.96	4.40	5.94
6	7.56	7.55	7.01	6.55	7.04
9	7.93	8.54	7.77	7.58	8.17
12	8.86	8.56	8.05	7.11	8.14
15	9.00	6.58	7.98	8.20	8.11
18	8.85	4.49	6.51	8.99	6.84
21	8.93	5.45	6.50	8.04	6.25
24	9.10	5.20	-[a]	6.05	8.86
27	8.95	4.81	-	4.46	-

Days postinoculation	Group 3 - G4 TW6196E Strain HEV RNA in fecal samples (log copies/mg)				
	pig 28	pig 29	pig 34	pig 39	pig 40
3	5.15	5.70	4.21	4.40	6.79
6	6.71	7.52	5.83	4.74	7.09
9	7.82	8.62	7.25	5.90	8.16
12	7.33	8.34	7.31	6.45	6.35
15	6.29	8.95	8.50	7.46	6.86
18	4.57	8.34	7.99	7.13	8.23
21	4.80	8.75	8.71	8.52	8.48
24	5.04	7.53	7.97	7.91	7.25
27	4.99	8.51	9.26	6.89	4.42

[a], not detected

CHAPTER 5

Intergenotypic chimeric hepatitis E viruses (HEV) with the capsid gene of a genotype 4 human HEV cloned in the genomic backbone of a genotype 3 swine HEV are viable *in vitro* and infectious in pigs

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Submitted to Virus Research

ABSTRACT

There exists at least four major genotypes of mammalian hepatitis E virus (HEV): genotypes 1 and 2 HEV infect only humans whereas genotypes 3 and 4 HEV infect both humans and pigs. To evaluate the mechanism of cross-species HEV infection between humans and swine, in this study we constructed five intergenotypic chimeric viruses and tested for their infectivity *in vitro* and in pigs. We demonstrated that chimeric viruses containing the ORF2 capsid gene either alone or in combination with its adjacent 5' junction region (JR) and 3' noncoding region (NCR) from a genotype 4 human HEV in the backbone of a genotype 3 swine HEV are replication-competent in Huh7 cells and infectious in HepG2/C3A cells and pigs, thus supporting the hypothesis that genotypes 3 and 4 human HEV strains are of swine origin. However, chimeric viruses containing the JR+ORF2+3' NCR of genotypes 3 or 4 HEV in the backbone of genotype 1 human HEV failed to infect pigs, suggesting that other genomic regions such as the 5' NCR and ORF1 may also be involved in HEV cross-species infection. The results from this study provide the first experimental evidence of the exchangeability of the capsid gene between

genotype 3 swine HEV and genotype 4 human HEV, and have important implications for understanding the mechanism of HEV cross-species infection.

INTRODUCTION

Hepatitis E virus (HEV) is an important but extremely understudied pathogen (Meng, 2010a; Meng, 2010b). The genome is a single-stranded, positive-sense RNA molecule of approximately 7.2 kb in length with three open reading frames (ORFs), and 5′ and 3′ non-coding regions (NCR) (Li et al., 2000; Emerson et al., 2004, 2010). ORF1 encodes nonstructural proteins (Pudupakam et al., 2009). ORF2 encodes the capsid protein which contains immunodominant epitopes (Li et al., 2000, Riddell et al., 2000; Schofield et al., 2000), forms virus-like particles (Guu et al., 2009), and binds to viral RNA (Surjit et al., 2004). Translation of ORF2 is essential for production of infectious virions (Emerson et al., 2006). The ORF3 encodes a small multifunctional protein (Tyagi et al., 2002; Kar-Roy et al., 2004; Moin et al., 2007; Chandra et al., 2008, 2010; Takahashi et al., 2008; Yamada et al., 2009; Emerson et al., 2010). The junction region (JR) between ORF1 and ORF2 contains a double stem-loop structure that may be important for virus replication (Huang et al., 2007). The 3′ NCR contains a *cis*-acting element that can bind to RdRp for replication initiation (Agrawal et al., 2001; Emerson et al., 2001).

At least four genotypes of mammalian HEV have been identified: genotype 1 and 2 strains are restricted to humans, whereas genotype 3 and 4 strains infect both humans and pigs (Meng et al., 1997; Okamoto, 2007; Yazaki et al., 2003; Meng et al., 2010b). Under experimental conditions, genotype 3 and 4 swine HEV strains infected rhesus monkeys and conversely, genotype 3 and 4 human HEV strains infected pigs (Meng et al., 1998b; Arankalle et al; 2006; Feagins et al., 2008). However, genotype 1 and 2 human HEV strains failed to infect pigs (Meng et al., 1998a), indicating that HEV

genotypes 1 and 2 have a limited host range. To evaluate viral determinant(s) for species tropism, the ORF2 capsid gene, either alone or in combination with its adjacent JR and 3′ NCR, were swapped between genotypes 1 and 4, genotypes 3 and 4, and genotypes 1 and 3 to produce 5 chimeric viruses, and their infectivity *in vitro* and in pigs was determined.

MATERIALS AND METHODS

By using the genotype 1 human HEV infectious clone pSK-HEV-2 (Emerson et al., 2001) as the genomic backbone, we first constructed three chimeric viruses: chimera rAB4-1h with the JR+ORF2 region of genotype 4 human HEV replacing that of genotype 1 human HEV; chimera rABC4-1h with the JR+ORF2+3'NCR region of genotype 4 human HEV replacing that of genotype 1 human HEV; and chimera rABC3-1h with the JR+ORF2+3'NCR region of genotype 3 swine HEV replacing that of genotype 1 human HEV (Fig. 1). The complete sequence of genotype 4 human HEV (strain TW6196E) (Wu et al., 2000; Feagins et al., 2008) was determined in this study. By using the genotype 3 swine HEV infectious cDNA clone pSHEV-3 (Huang et al., 2005) as the genomic backbone, two additional chimeric viruses were constructed: chimera rA4-3sw with the ORF2 gene of genotype 4 human HEV replacing that of genotype 3 swine HEV; and chimera rABC4-3sw with the JR+ORF2+3'NCR of genotype 4 human HEV replacing that of genotype 3 swine HEV (Fig. 1). Standard and fusion PCRs with primers PF5130/PR7089 (rA4-3sw), P14510-P47173 (rABC4-3sw), P1A-P4C (rAB4-1h), P1A-P4A (rABC4-1h), and P1-P4 (rABC3-1h) (Table 1) were used to produce the final fragments, which were then cloned in the respective genotype 1 or genotype 3 HEV infectious clone backbone. The genome of each chimera was completely sequenced to verify that no mutation was introduced.

To determine the *in vitro* replication competency of the 5 chimeric viruses, the plasmid DNAs from each clone were linearized with XbaI (pSHEV-3, rA4-3sw, rABC4-3sw) or AclI (rAB4-1h, rABC4-1h, rABC3-1h) and *in vitro*-transcribed to produce capped RNA transcripts (Emerson et al., 2004, 2006; Huang et al., 2005; Pudupakam et al., 2009). Twenty-five microliters of each transcription reaction were used to transfect a T25 flask of Huh7 cells (Emerson et al., 2004; Emerson et al., 2006). Three days post-transfection, the cells were trypsinized and plated on an 8-chamber Lab-Tek^{TM} slide (Thermo Scientific). The remaining cells were transferred to a T75 flask to produce a virus stock. After incubation at 34.5°C for 3 more days, cells were washed with phosphate-buffered saline, fixed with acetone and stained with a chimpanzee 1313 anti-HEV antibody by an immunofluorescence assay (IFA) (Emerson et al., 2004).

To generate virus stocks for *in vitro* and *in vivo* infectivity assays, Huh7 cells transfected with each chimeric clone in T75 flasks were trypsinized at 9 days post-transfection, the cells were pelleted by centrifugation and the pellets were resuspended in approximately 0.9 ml of water. After freezing (-80°C) and thawing 3 times, the cell lysates were centrifuged for 10 min at 3,400 rpm at 4°C, and the supernatants were used to inoculate pigs and HepG2/C3A cells. The HepG2/C3A cell line was chosen for the *in vitro* infectivity assay since a HEV infectivity assay has been established for HepG2/C3A cells (Emerson et al., 2010). To determine the infectivity of the chimeric viruses *in vitro*, a 100-µl cell lysate was added to a well of HepG2/C3A cells grown in eight-well glass chamber slides (Emerson et al., 2010). After incubation at 34.5°C for 5 h, approximately 0.4 ml of growth medium was added to each well, and incubated for 5 days at 34.5°C.

The cells were fixed with acetone and stained by IFA, and cells positive for ORF2 protein were counted.

A swine bioassay was used to determine the infectivity of the chimeric viruses in pigs (Meng et al., 1998a; Halbur et al., 2001). Fourteen, 8-week-old, specific-pathogen-free pigs were divided into 7 groups of 2 pigs each (Table 2). Pigs in each group were inoculated intravenously with 3 ml of the respective inoculum, one of 5 chimeric viruses, the wildtype pSHEV-3 virus (positive control), and culture medium (negative control). The animals were monitored for 9 weeks for evidence of HEV infection. Fecal swabs and serum samples were collected prior to inoculation and weekly thereafter. At necropsy at 63 days post-inoculation (dpi), samples of serum, bile, liver, and intestinal contents were collected. A nested RT-PCR assay (Huang et al., 2002) was modified to detect the ORF2 gene of genotype 4 human HEV or genotype 3 swine HEV. For genotype 4 human HEV, primer 6959 was used for cDNA synthesis followed by a nested PCR with primers 5911 and 6959 (first round), and 6205 and 6636 (second round, Table 1). For genotype 3 swine HEV, primer 6915 was used for cDNA synthesis followed by a nested PCR with primers 5734 and 6915 (first round), and 6309 and 6647 (second round, Table 1). An enzyme-linked immunosorbent assay (ELISA) was used to detect anti-HEVIgG in the weekly sera (Meng et al., 1997, 1998a, 1998b).

RESULTS AND DISCUSSION

The chimeric viruses are replication-competent in Huh7 cells. As expected, positive fluorescent signals were detected in cells transfected with capped RNAs from the wildtype pSHEV-3 clone. Also, positive IFA signals were detected in Huh7 cells transfected with capped RNAs from all 5 chimeric virus clones, indicating that each

chimeric virus was replication-competent in Huh7 liver cells. A fluorescent signal was not detected in the negative control cells (Fig. 2).

The two intergenotypic chimeric viruses containing the ORF2 (chimera rA4-3sw) or JR+ORF2+3'NCR (chimera rABC4-3sw) from the genotype 4 human HEV in the backbone of genotype 3 swine HEV are infectious in HepG2/C3A cells and in pigs. Chimeras rA4-3sw and rABC4-3sw produced infectious particles in Huh7 cells, as did the wildtype pSHEV-3, as positive IFA signals were detected in HepG2/C3A cells (Table 2), indicating that both chimeras rA4-3sw and rABC4-3sw are infectious *in vitro*. As expected, both pigs (ID# 360, 362) in the negative control group remained negative. The two pigs (ID# 357, 361) in the positive control group seroconverted at 2-3 weeks post-inoculation (wpi) (Fig. 2). Fecal virus shedding was detected in both pigs from 1-2 wpi, and viremia was detected in pig 361 at 2 wpi. The two pigs (ID# 364, 366) inoculated with the chimera rA4-3sw had fecal virus shedding at 2 wpi (pig 366) and 3 wpi (pig 364, 366), and a transient viremia at 1 wpi for pig 366, and seroconversion at 6 wpi (pig 364) (Fig. 3). The two pigs (ID# 358, 369) inoculated with chimera rABC4-3sw seroconverted to anti-HEV at 2-3 wpi, developed a transient viremia at 1 wpi, and shed virus in feces from 1-2 wpi (Fig. 3). The positive PCR products from all samples were sequenced, and sequence analyses confirmed that the viruses recovered from the infected pigs originated from their respective inoculum (data not shown). The patterns of viremia, fecal virus shedding and seroconversion of the chimeric genotype 3 swine HEV rABC-4-3sw containing the JR+ORF2+3'NCR of genotype 4 human HEV are indistinguishable from the wildtype genotype 3 swine HEV, although the chimera rA4-3sw containing only the ORF2 from genotype 4 human HEV had a lower level of antibody response in

infected pigs (Fig. 3). The results indicated that the ORF2 capsid gene, either alone or in combination with its adjacent JR and 3'NCR, between the genotype 3 swine HEV and genotype 4 human HEV is fully exchangeable. The transient viremia and shorter duration of fecal virus shedding likely reflect the low titers of chimeric viruses in the inocula since HEV infection is dose-dependent (18, 33).

The three intergenotypic chimeric viruses containing the ORF2 and its adjacent region (JR+3'NCR) from genotypes 3 and 4 HEV in the backbone of a genotype 1 human HEV failed to infect pigs, although one chimera (rABC4-1h) did produce infectious virus particles in Huh7 cells. Since genotype 1 HEV does not infect pigs whereas genotypes 3 and 4 HEV infect both pigs and humans, three chimeric viruses containing the ORF2 and its adjacent region from genotypes 3 or 4 HEV in the backbone of genotype 1 human HEV were subsequently constructed to determine if the chimeric viruses will gain the ability to infect pigs (Table 2). One (pig 368) of the two pigs inoculated with chimeric virus rAB4-1h had a low level seroconversion with OD values slightly above the 0.30 cutoff at 8 (OD=0.343) and 9 (OD=0.332) wpi. However, neither pig had detectable viremia or fecal virus shedding. In the absence of viremia or fecal virus shedding, the significance of a delayed low level seroconversion in pig 368 is unclear. The pigs inoculated with chimeras rABC4-1h (ID# 359, 365) and rABC3-1h (ID# 363, 367) had no detectable viremia, fecal virus shedding or seroconversion (Table 2), although chimera rABC4-1h, but not chimeras rABC3-1h and rAB4-1h, did produce infectious virus particles in Huh7 cells (Table 2). The results showed that the JR+ORF2+3'NCR region from a genotype 3 or 4 HEV could not confer infectivity in pigs to the human genotype 1-based chimeric viruses.

Genotype 3 and 4 swine HEV strains have been identified from pigs in essentially all major swine-producing countries worldwide (Meng et al., 2003; Yazaki et al., 2003; Takahashi et al., 2002; Nishizawa et al., 2003; Bouwknegt et al., 2008; de Deus et al., 2008). Recent sequence and phylogenetic analyses (Xia et al., 2010) along with demonstrable cross-species infection between genotypes 3 and 4 swine and human HEV strains (Meng et al., 1998b; Arankalle et al., 2006; Feagins et al., 2008; Meng et al., 2010) suggest that genotypes 3 and 4 HEV are of swine origin. We have previously shown that the genotype 4 human HEV TW6196E strain was able to infect pigs (Feagins et al., 2008). In this study, we are now able to demonstrate, for the first time, that chimeric viruses generated by swapping the genomic regions of a genotype 3 swine HEV with the same regions from the genotype 4 human HEV TW6196E strain produced an infection in pigs that is comparable to the wild type genotype 3 swine HEV, thus lending further credence to the idea that genotypes 3 and 4 HEV strains originated from pigs.

Given its critical role in cell attachment and infection, the capsid protein of HEV is presumed to be an important determinant of HEV host range (He et al., 2008; Kalia et al., 2009). However, the inability of a genotype 1 HEV strain to infect pigs after exchanging the ORF2 capsid gene and its adjacent 5' JR and 3' NCR with that of a genotype 3 or 4 HEV strain indicates that the 5'NCR and the ORF1 polyprotein may also be important for cross-species infection. The HEV ORF1 gene contains several functional motifs characteristic of methyltransferases, papain-like cysteine proteases, helicases, RNA dependent RNA polymerases (Koonin et al. 1992, Panda et al, 2000) and a proline-rich hypervariable region (HVR) that is dispensable for viral replication (Pudupakam et

al., 2009). Future studies to explore the role of different ORF1 domains in cross-species infection are warranted.

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Figure Legends

FIG. 1. Construction of intergenotypic chimeric viruses using the genomic backbone of a genotype 3 swine HEV (pSHEV-2) and a genotype 1 human HEV (pSK-HEV-3) infectious cDNA clone. (A). In chimeric virus rA4-3sw, pSHEV-2 ORF2 (AUG4→TAA) was swapped with ORF2 of genotype 4 human HEV (TW6196E strain). For rABC4-3sw, pSHEV-2 ORF2, JR and 3′ NCR (AUG1→NCR) were swapped with the corresponding genomic regions of TW6196E. **(B).** In chimeric virus rAB4-1h, pSK-HEV-2 ORF2 and JR (AUG1→TAG) were swapped with ORF2 and JR of TW6196E. For rABC4-1h, ORF2, JR, and 3′ NCR (AUG1→NCR) were swapped with the corresponding genomic regions of TW6196E. For rABC3-1h, pSK-HEV-2 ORF2, JR, and 3′ NCR (AUG1→NCR) were swapped with the corresponding regions of pSHEV-3.

FIG. 2. Immunofluorescence staining with a chimpanzee anti-HEV antibody of a subclone of Huh7 cells transfected with capped full-length RNA transcripts from chimeric viruses. (A) Mock transfected; **(B)** wildtype genotype 3 swine HEV pSHEV-3; **(C)** chimera rA4-3sw; **(D)** chimera rABC4-3sw; (E) chimera rAB4-1h; **(F)** chimera rABC4-1h; and **(G)** chimera rABC3-1h.

FIG. 3. Experimental infection of specific-pathogen-free pigs with 2 intergenic chimeric viruses containing the genotype 3 swine HEV backbone but not with chimeric viruses containing the genotype 1 human HEV backbone. (A) Pigs #360 and #362 inoculated with cell culture media DMEM (negative control); **(B)** Pigs #357 and #361 inoculated with wildtype genotype 3 swine HEV infectious clone pSHEV-3

(positive control); **(C)** Pigs #364 and #366 inoculated with chimera rA4-3sw; **(D)** Pigs #358 and #369 inoculated with chimera rABC4-3sw. Since evidence of HEV infection was lacking in pigs inoculated with chimeras rAB4-1h (pig ID# 356, 368), rABC4-1h (pig ID# 359, 365), and rABC3-1h (pig ID# 363, 367), and the results of viremia, fecal virus shedding and antibody response in these three groups of pigs were not shown here. IgG anti-HEV antibodies are plotted as ELISA OD values (cut-off value: 0.30). Presence (▪= detected in both pigs in each group, ▪= detected in the pig represented by the symbol in the chart figure legend) or absence (▫) of HEV RNA in fecal and serum samples tested by nested RT-PCR.

FIG. 1

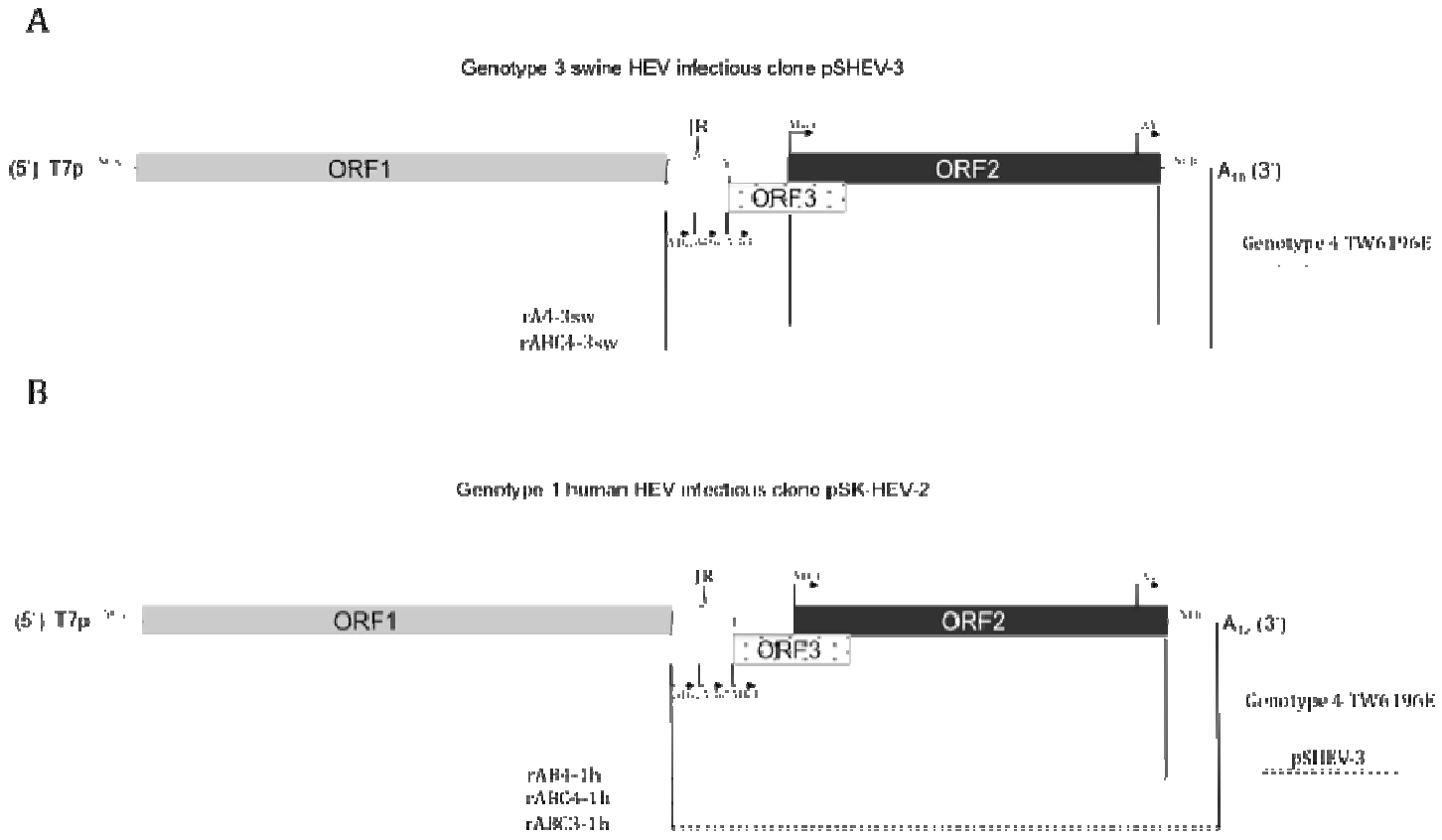
A
Genotype 3 swine HEV infectious clone pSHEV-3
(5') T7p
ORF1
ORF2
ORF3
A10 (3')
Genotype 4 TW6196E
rAH3sw
rAHC4-3sw
B
Genotype 1 human HEV infectious clone pSK-HEV-2
(5') T7p
ORF1
ORF2
ORF3
A10 (3')
Genotype 4 TW6196E
pSHEV-3
rAH4-1h
rAHC4-1h
rAHC3-1h

FIG. 2

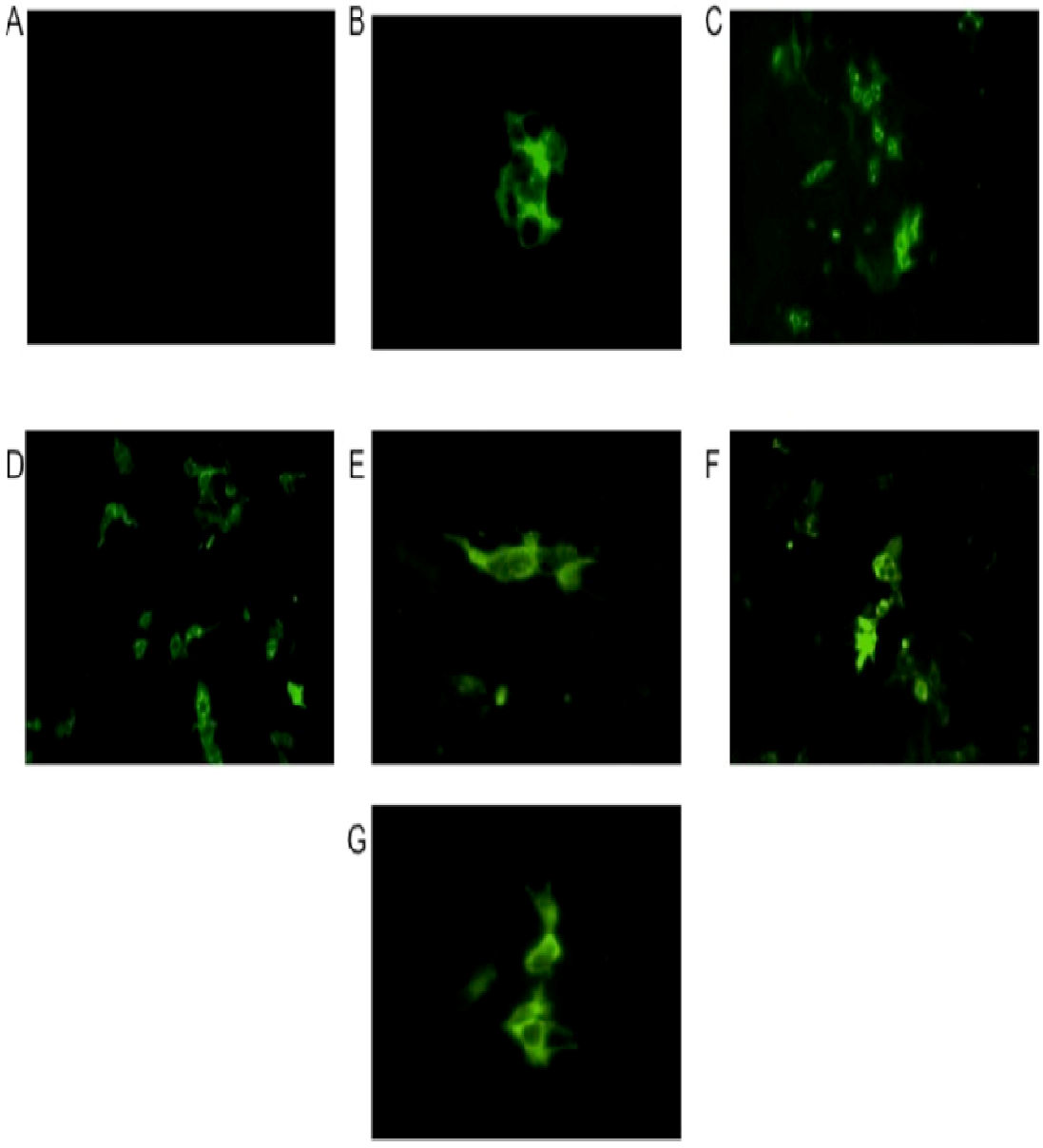

FIG. 3

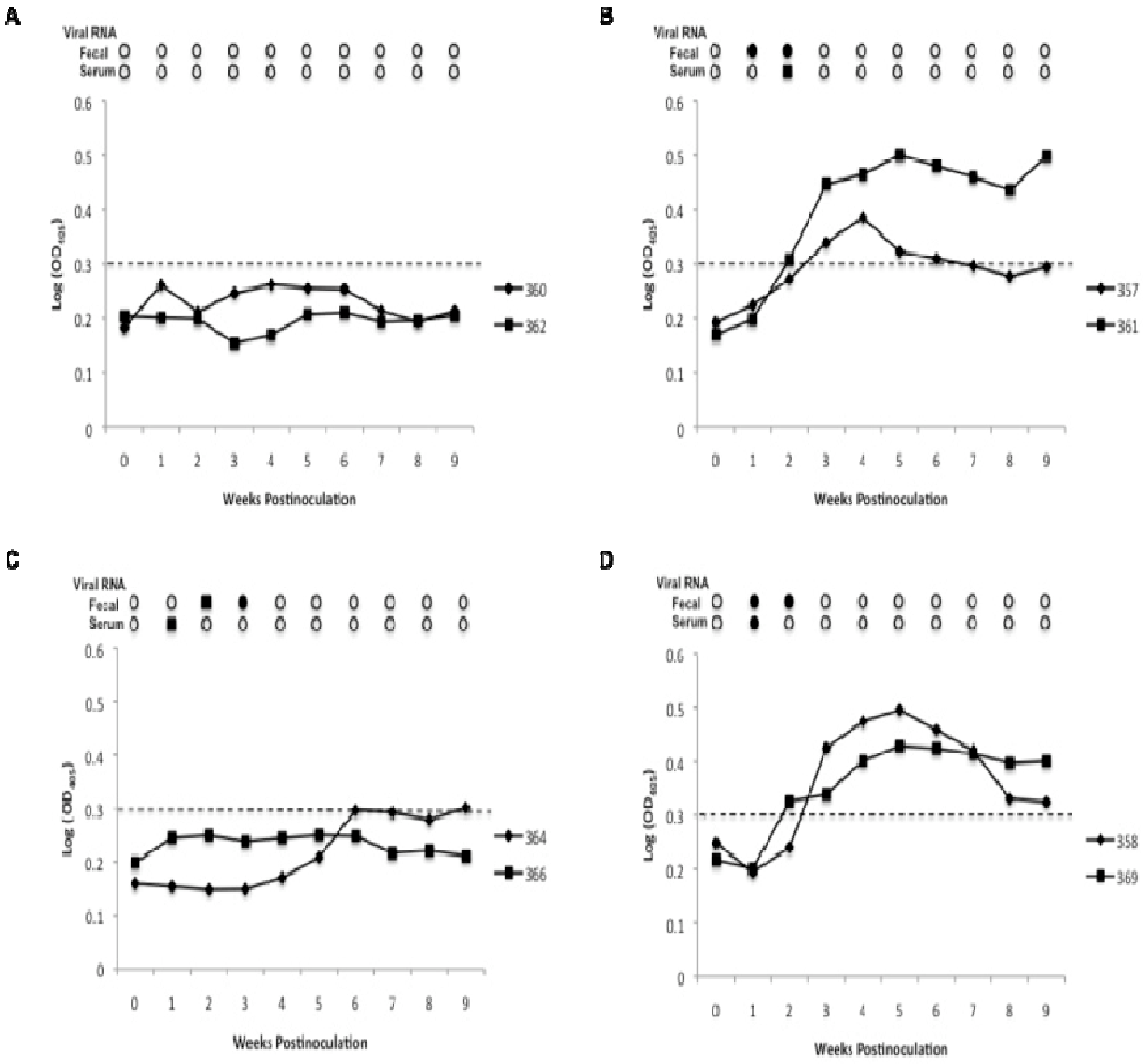
A
Viral RNA
Fecal
Serum
Log (OD405)
Weeks Postinoculation
360
362
B
Viral RNA
Fecal
Serum
Log (OD405)
Weeks Postinoculation
357
361
C
Viral RNA
Fecal
Serum
Log (OD405)
Weeks Postinoculation
364
366
D
Viral RNA
Fecal
Serum
Log (OD405)
Weeks Postinoculation
358
369

TABLE 1. Oligo primers used for the construction of the 5 intergenotypic chimeric hepatitis E viruses and for the detection of the chimeric viruses from infected pigs

Chimeric virus construction		
	Primer ID	**Primer sequences (5'→3')**
rA4-3sw	PF5130	ATACCTAGGGCTCTTCTGTTTTTGC, AvrII
	PR7089	GAATTAATTAATACTCCCGGGTTTTACC, PacI
rABC4-3sw	P14510	ATACTTAAGGGTTTCTGGAAGAACATT, AflII
	P25067	GAACATGTTATTCATTCCACCCGCTGTATG
	P35067	CATACAGCGGGTGGAATGAATAACATGTTC
	P47173	TATTCTAGATTTTTTTTTTTTTTTTTTTTTTTTCCAGGGA, XbaI
rAB4-1h	P1A	TAGGCATGCTACAGGCTGTTGCTGA, SphI
	P2A	GAACATGTTATTCATTCCACCCGACACAG
	P3A	CTGTGTCGGGTGGAATGAATAACATGTTC
	P5A	TACCAGGTGTCAAATTACGAAACTCAAGC
	P6A	GCTTGAGTTTCGTAATTTGACACCTGGTA
	P2C	GGCACAAGCAAATAAATCAATACTCCC
	P3C	CCCGGGAGTATTGATTTATTTGCTTGT
	P4C	TATGATATCAACGTTTTTTTTTTTTTCAGGGAGCG, EcoRV
rABC4-1h	P1A	same as above
	P2A	same as above
	P3A	same as above
	P5A	same as above
	P6A	GCTTGAGTTTCGTAATTTGACACCTGGTA
	P4A	TATGATATCAACGTTTTTTTTTTTTTTTTTTTTTTTTCCAGGGAG, EcoRV
rABC3-1h	P1	TTGGCATGCTACAGGCTGTTGCTGATG, SphI
	P2	AAAGACATGTTATTCATTCCACCCGACACAG
	P3	CTGTGTCGGGTGGAATGAATAACATGTCTTT
	P4	TATGATATCAACGTTTTTTTTTTTTTTTTTCCAGGGAGCGCG, EcoRV
HEV RNA detection		
	Primer ID	**Primer sequences (5'→3')**
G4 human HEV		
1st round	F5911*	CGAGTTAGTGATCCCCAGTGAG
1st round	R6959†	CGACAGCAGAAATAGAAACAGG
2nd round	F6205	GCTAACTACTACTGCTGCTACACG
2nd round	R6636	GGATTGGTAGAAGAGCCGTAGG
G3 swine HEV		
1st round	F5734	GCTCAGTATCGGGTTGTTCG
1st round	R6915	GGTAGAGATAGCAACACGGTGG
2nd round	F6309	CCTTGCTGATACGCTTCTTGGTGG
2nd round	R6647	GGAGACATACATAGGGTTGGTGGACG

*F, forward primer

†R, reverse primer

Table 2. Replication competency in Huh7 liver cells, and infectivity in HepG2/C3A liver cells and in pigs of intergenotypic chimeric hepatitis E viruses

Group (pig ID#)	Inocula	Description*	Replication competency in Huh7 cells	Infectivity in HepG2/C3A cells	Infectivity in pigs
1 (360, 362)	DMEM media	Negative control	-	-	-
2 (357, 361)	pSHEV-3	Wildtype genotype 3 swine HEV (positive control)	+	+	+
3 (364, 366)	Chimera rA4-3sw	Genotype 3 swine HEV backbone containing the ORF2 of genotype 4 human HEV	+	+	+
4 (358, 369)	Chimera rABC4-3sw	Genotype 3 swine HEV backbone containing JR¶+ORF2+3'NCR* of genotype 4 human HEV	+	+	+
5 (356, 368)	Chimera rAB4-1h	Genotype 1 human HEV backbone containing JR+ORF2 of genotype 4 human HEV	+	-	-†
6 (359, 365)	Chimera rABC4-1h	Genotype 1 human HEV backbone containing JR+ORF2+3'NCR of genotype 4 human HEV	+	+	-
7 (363, 367)	Chimera rABC3-1h	Genotype 1 human HEV backbone containing JR+ORF2+3'NCR of genotype 3 swine HEV	+	-	-

*3'NCR: 3' non-coding region

¶JR: ORF1/ORF2 junction region

†a delayed seroconversion at 8 and 9 weeks post-inoculation with low level of antibody in one pig (pig ID 368).

CHAPTER 6

Construction and in vitro characterization of a genotype 4 human hepatitis E virus infectious cDNA clone

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To be submitted

ABSTRACT

Hepatitis E is an important human disease endemic to many developing and industrialized countries worldwide. Hepatitis E virus (HEV), the etiological agent of Hepatitis E, has been isolated from several mammalian and non-mammalian animal species. Animal strains of HEV are very closely related to human HEV isolates and cross-species infection has been demonstrated. We have previously demonstrated that the genotype 4 human HEV TW6196E strain infected pigs. In this study, the full-length genomic sequence of a genotype 4 human HEV (strain TW6196E) was first determined. Subsequently, two full-length cDNA clones of the genotype 4 human HEV were constructed and tested for its *in vitro* viability in liver cell line Huh7. The results showed that Huh7 cells transfected with capped RNA transcripts of each clone were replication-competent, as the ORF2 capsid protein was detected by immunofluoresence assay (IFA) in approximately 10-25% of transfected cells. The availability of this genotype 4 human HEV infectious cDNA clone will facilitate future studies to understand the molecular mechanism of cross-species infection by genotype 4 HEV.

INTRODUCTION

Hepatitis E virus (HEV), the causative agent of hepatitis E, is a non-enveloped, single-stranded, positive-sense RNA virus primarily transmitted via the fecal oral route although foodborne transmission also occurs (Emerson and Purcell, 2003). The 7.2kb genome contains three open reading frames (ORF): ORF1 encodes nonstructural proteins required for viral replication, ORF2 encodes the structural capsid protein, and ORF3 encodes a multi-functional phosphoprotein (Tam et al., 1991; Tsarev et al., 1992; Emerson and Purcell, 2003). Classified in the genus *Hepevirus* within the family *Hepeviridae*, HEV strains group into at least four major genotypes: genotype 1 and 2 HEVs cause waterborne epidemics in developing countries, whereas genotype 3 and 4 HEVs cause sporadic cases of hepatitis E worldwide (Emerson et al., 2004; Meng, 2010b). A low mortality rate is associated with hepatitis E in the general population, however the mortality rate among infected pregnant women can reach up to 25% (Navaneethan et al., 2008).

In the developing countries of Asia, Africa, and Mexico, hepatitis E is endemic with outbreaks occurring from the contamination of water and lack of proper sanitation conditions. Hepatitis E is a potential public health concern in industrialized countries where hepatitis E is also endemic (Meng, 2010b). The occurrence of autochthonous cases of human Hepatitis E in Europe, the United States, and Japan has been associated with animal reservoirs of HEV (Meng et al., 2010a). Although humans and non-human primates are the only natural hosts of genotype 1 and 2 HEVs, genotype 3 and 4 HEVs have been recovered from swine, deer, mongooses, and rabbits (Meng et al., 1997; Takahashi et al., 2004; Zhao et al., 2009; Nakamura et al., 2006). Animal HEVs isolated

from swine, wild boar, and deer are closely related, or identical, to human HEV recovered from patients suggesting hepatitis E is a zoonosis (Meng, 2010a; Meng, 2010b). Cross-species infection of non-human primates with genotype 3 and 4 swine HEVs, and pigs with genotype 3 HEVs provide additional evidence in support of the hypothesis (Meng et al., 1998; Arankalle et al., 2006; Feagins et al., 2008).

The discovery of avian HEV, a separate species within the *Hepevirdiae* family, from chickens with hepatitis-splenomegaly (HS) syndrome recognized chickens as a host for HEV (Haqshenas et al., 2001), although avian HEV is likely not transmissible to humans (Huang et al., 2004). Since the identification of avian and swine HEVs, chickens and swine have been characterized as useful animal models for HEV. Together, animal models, cell lines that support limited HEV replication, and several infectious cDNA clones provide the tools necessary to investigate HEV replication, pathogenesis, and cross-species infection. Currently, infectious clones are available for avian HEV, genotype 1 human HEV, genotype 3 human HEV, and genotype 3 swine HEV (Huang et al., 2005; Emerson et al., 2001; Huang et al., 2005; Yamada et al., 2009; Kwon et al., 2010). The objective of this study was to construct and characterize an infectious cDNA clone of a genotype 4 human HEV strain.

MATERIALS AND METHODS

Virus and cells. A standard infectious stock of the genotype 4 human HEV TW6196E strain was prepared as a 10% suspension of fecal material collected from experimentally infected specific-pathogen-free (SPF) pigs (Feagins et al., 2008). A subclone of Huh7 cells (human hepatoma cell line), kindly provided by Dr. S.U. Emerson of NIAID, NIH, Bethesda, MD, was maintained in Dulbecco's Modified Eagle's Medium

(DMEM; Invitrogen) containing 10% fetal bovine serum (FBS) at 37˚C with 5% CO_2 (Emerson et al., 2004; Emerson et al., 2006).

RNA Extraction. Total RNA was extracted from the genotype 4 human HEV TW6196E virus stock with Trizol Reagent (MRC) according to the manufacturer's protocol, resuspended in 30 µl of DNase, RNase, and proteinase free water (Invitrogen), and stored at -80˚C until use (Huang et al., 2002; Cooper et al., 2005).

Determination of the full-length TW6196E genomic sequence. A 346 bp region of TW6196E ORF2 was published and available from NCBI database (GenBank Accession Number: AF117278). Based on this short published sequence, specific primers were designed and used with the 3' RACE System for Rapid Amplification of cDNA ends (RACE, Invitrogen) to amplify the 3' end of the HEV genome, according to the manufacturer's protocol. Degenerate primers were subsequently designed based on other available genotype 4 HEV sequences from the GenBank along with specific primers based on the resulting 3'end sequence of the TW6196E strain to amplify a 1-2 kb fragment upstream of the 3' end product using Superscript III One-step RT-PCR System with Platinum Taq High Fidelity kit (Invitrogen), according to the manufacturer's protocol. PCR products with expected sizes were gel-purified (Geneclean II Kit, QBiogene), cloned into an appropriate vector (TOPO TA Cloning Kit, Invitrogen), and sequenced. The complete genome of strain TW6196E, excluding the extreme 5' end, was determined by primer walking along the viral genome towards the 5' end. A 5' RACE System for Rapid Amplification of cDNA ends (Invitrogen) was used to determine the genomic sequence of the extreme 5' end following the manufacturer's instructions. The

full-length sequence of the TW6196E strain was deposited in GenBank (accession number HQ634346).

Construction of full-length cDNA clones of TW6196E. Three overlapping genomic fragments were amplified using the Superscript III One-step RT-PCR System with Platinum Taq High Fidelity kit (Invitrogen) using three sets of PCR primers (Table 1). Fragment Ia, representing the 5' end of the genome, was amplified with the primers F12 and R1. The forward primer 5' – GGG**TCTAGA**TAATACGACTCACTATAggcagaccacgtatgtggtc –3' contained an engineered XbaI restriction enzyme site (bold) and a T7 promoter sequence (underlined) followed by the extreme 5' end of the viral genome (lowercase). Fragment Ib was the same as fragment I except that only one G was inserted between the promoter and viral sequence. Fragment II, representing the middle portion of the viral genome, was amplified with primers F2 and R3 that partially overlapped fragments Ia (or Ib) and II. Fragment III, representing the 3' end of the viral genome, was amplified with primers F4 and R42. The reverse primer, R42, introduced 18 adenosine and a ClaI restriction site at the 3' end of the genome. Fragment Ia (Ib) and II were simultaneously ligated into pGEM-7zf (-) vector between the XbaI and ClaI sites in a three-way ligation reaction. Fragment III was subsequently ligated into the pGEM-7zf (-) vector using the EcoRI restriction site partially overlapping fragment II and the ClaI restriction site located within the pGEM-7zf (-) vector. The full-length cDNA clones assembled with fragments Ia, II, and III was designated Clone A; and the clone assembled with fragments Ib, II, and III was designated Clone B (Fig. 1). QuikChange Site-Directed Mutagenesis kit (Stratagene) was subsequently used to delete the built-in T7 promoter sequence in Clone

A and Clone B and a SpeI restriction site was added to each clone for linearization between the l8th adenosine and ClaI restriction site to produce Clone A2 and Clone B3. Sequence analysis revealed 4 non-silent mutations in Clone A2 and 5 non-silent mutations in clone B3 when compared to the TW6196E wildtype sequence. Subsequently, QuikChange Multi Site-Directed Mutagenesis kit (Stratagene) was used to correct these non-silent mutations in Clone A2 and B3 to produce final clones pHEV-4TWa and pHEV-4TWb, respectively.

***In vitro* transcription.** The pHEV-TWa and pHEV-TWb clones were linearized by digestion with SpeI and purified by phenol chloroform extraction. pSHEV-3, the genotype 3 swine HEV infectious cDNA clone, was linearized with XbaI and served as a positive control. Capped RNA transcripts were generated using the Riboprobe System-T7 (Promega). Briefly, 26 µl reaction mixtures containing 1 µg of linearized DNA, 5 µl of 5X buffer, 2.5 µl of DTT, 0.5 µl of RNasin, 5 µl of nucleotides, 2.5 µl of Cap analog, and 0.5 µl of T7 polymerase were incubated at 37˚C for 1.5 hrs (Huang et al., 2007). To assess the quality of the RNA transcripts, 1 µl of the transcription reactions were analyzed on a 0.8% agarose gel. RNA transcripts of each clone were immediately used for the transfection of Huh7 cells.

***In vitro* transfection and immunofluorescence assay (IFA).** Huh7 cells growing in T25 flasks were washed with phosphate-buffered saline (PBS; Invitrogen) and transfected with a mixture containing 25 µl of each transcription reaction, 1ml of Opti-MEM (Invitrogen), and 20 µl DMRIE-C transfection reagent (Invitrogen) as previously described (Emerson et al., 2006; Huang et al., 2007). Huh7 cells transfected with a mixture containing only Opti-MEM and DMRIE-C served as a negative control. After 5

hrs of incubation at 34.5°C, the mixture was aspirated and 5ml DMEM containing 10% FBS was added to each flask. Three days post-transfection, the cells were trypsinized and plated on an 8 chamber Lab-Tek™ slide (Thermo Scientific). Six days post-transfection, cells were washed with PBS and fixed with acetone. Chimpanzee 1313 anti-HEV convalescent serum, specific for the ORF2 protein, was added to fixed cells. After incubation at room termperature, the slides were washed with PBS and overlaid with Alexa fluor 488-conjugated goat anti-human IgG (Molecular Probes). The slides were washed with PBS after incubation at room temperature and Vectashield (Vector Laboratoreis) was added for viewing by fluorescence microscopy.

RESULTS AND DISCUSSION

Construction of two cDNA clones of genotype 4 human HEV TW6196E. The complete genomic sequence of TW6196E was determined using 5' RACE, 3' RACE, and the primer walking method. The complete genomic sequence determined in this study was used to construct two-full length cDNA clones by the ligation of three overlapping fragments representing the entire genome of TW6196E. Clone pHEV-4TWa differed from pHEV-4TWb by an extra non-viral nucleotide G between the T7 promoter and 5'end of the viral sequence. It has been reported that transcription efficiency is enhanced by the extra guanosine.

Capped RNA transcripts for each of the two clones were replication-competent when transfected into Huh7 cells. The additional G did not significantly affect i*n vitro* transcription as similar amounts of capped RNA transcript were generated for pHEV-4TWa and pHEV-4TWb (data not shown). Huh7 cells were transfected with the capped RNA transcripts of each clone to determine the *in vitro* viability. A

fluorescent signal with HEV capsid protein-specific antibody was detected for pSHEV-3, pHEV-4TWa, and pHEV-4TWb. Aproximately 10-15% and 20-25% of the cells transfected with pHEV-4TWa and pHEV-4TWb, respectively, were positive by IFA. No fluorescence signal was detected for the mock-transfected cells, and as expected approximately 10-15% of cells transfected with genotype 3 HEV infectious clone pSHEV-3 were positive (Fig. 2). It will now be critical to characterize the infectivity of pHEV-4TWa and pHEV-4TWb clones *in vivo.*

The availability of a genotype 4 human HEV infectious cDNA clone will facilitate future studies to understand the molecular mechanism of HEV host range through the construction of intergenotypic chimeric viruses.

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Figure Legends

FIG. 1. Construction of full-length cDNA clones of genotype 4 human HEV TW6196E strain. BamHI and EcoRI are restriction sites naturally present in the TW6196E strain that were used to facilitate the construction of each cDNA clone. An XbaI restriction site, T7 promoter sequence, and two guanosines were introduced at the 5' end of fragment Ia. For fragment Ib, one guanosine was introduced. Eighteen adenosines, SpeI, and ClaI restriction sites were introduced at the 3' end of the genome. Fragments Ia, II, and III were ligated into the pGEM-7zf (-) vector to generate pHEV-4TWa. Similarly, fragments Ib, II, and III were ligated to generate pHEV-4TWb.

FIG. 2. Immunofluorescence staining with an ORF2 anti-HEV antibody of a subclone of Huh7 cells transfected with capped full-length RNA transcripts from each cDNA clone. (A) Mock transfected; **(B)** wildtype genotype 3 swine HEV pSHEV-3; **(C)** cDNA clone pHEV-4TWa; and **(D)** cDNA clone pHEV-4TWb.

FIG. 1

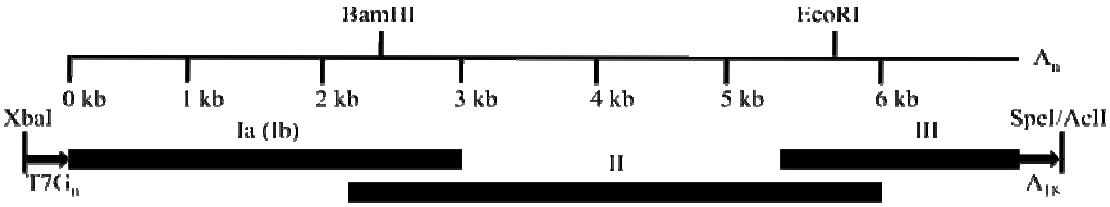
BamHI
EcoRI
0 kb
1 kb
2 kb
3 kb
4 kb
5 kb
6 kb
XbaI
Ia (Ib)
III
SpeI/AclI
II

FIG. 2

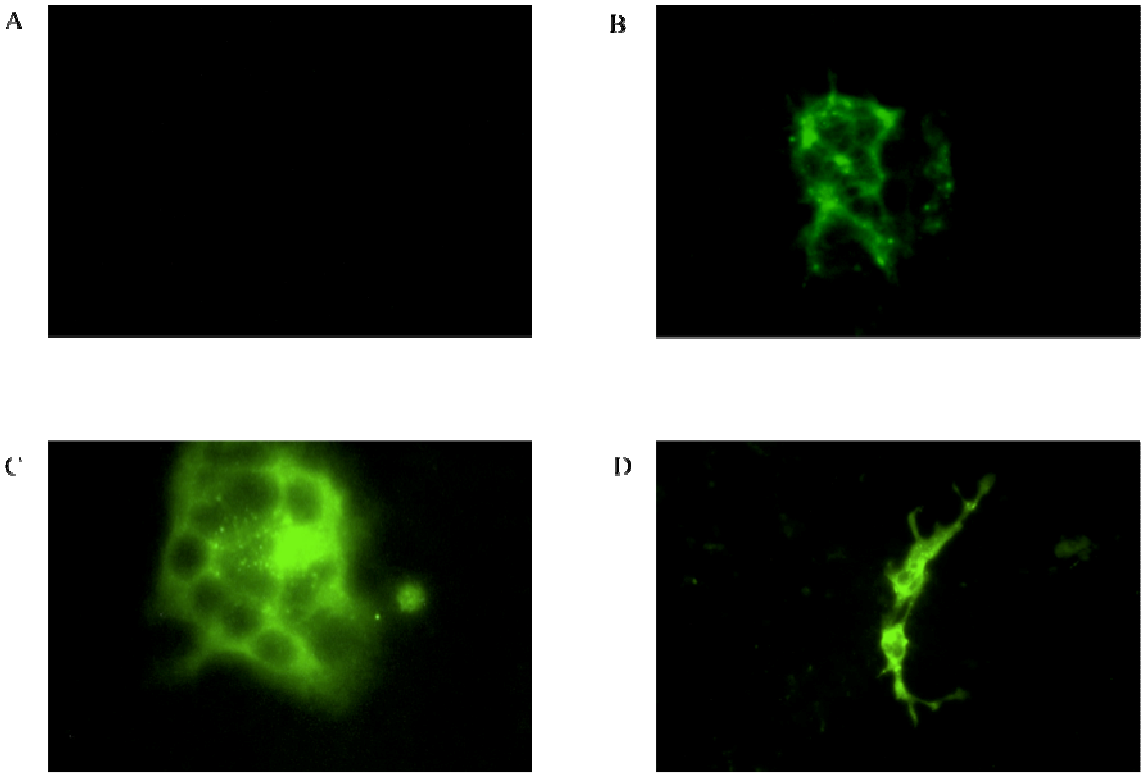

TABLE 1. Oligonucleotide primers used in the construction of genotype 4 human HEV TW6196E infectious cDNA clones

Primer ID	Sequence (5' – 3')†
F12	**GGGTCTAGATAATACGACTCACTATAGgcagaccacgtatg**
R1	**AAGGGATCCggcatacacttttgagccatcgg**
F2	**GCCGGATCCctttttgagtcc**
R3	**ATGGAATTCatatccacggacgtcgg**
F4	**TATGAATTCcatcacttccaccgat**
R42	**GTGATCGATttttttttttttttttttccag**

† - lower case letters represent viral sequence

CHAPTER 7

General Conclusions

In this dissertation, zoonotic foodborne transmission and host range of HEV have been investigated. Our results show that pig livers sold in local grocery stores in the United States are contaminated by HEV and the contaminating virus retains the ability to cause infection. This finding is extremely important, and has major public health implication. The dissertation research also showed that different cooking methods and temperatures have different effects on the inactivation of HEV in contaminated pig livers. Regulatory agencies can use this data to implement safer cooking protocols for consumers of pig liver and other pork products. Our results also show that the host range of genotype 4 human HEVs includes pigs. We demonstrated that the ORF2 capsid gene is interchangeable between genotypes 3 and 4 strains of HEV, however the mechanism of cross-species infection may involve genomic regions in ORF1. Finally, the availability of an infectious clone for the genotype 4 human HEV will aid future studies on HEV host range and cross-species infection.