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Intergenotypic chimeric hepatitis E viruses (HEV) with the genotype 4 human HEV capsid gene in the backbone of genotype 3 swine HEV are infectious in pigs

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Abstract

Genotypes 1 and 2 hepatitis E virus (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 in pigs, and thus supporting the hypothesis that genotypes 3 and 4 human HEV 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 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.

Keywords

Hepatitis E virus (HEV); Chimeric viruses; Cross-species infection; Pigs; Host range

Hepatitis E virus (HEV) is an important but extremely understudied pathogen (Meng, 2010). 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) (Koonin et al., 1992; Emerson et al., 2004) (Fig. 1A). ORF1 encodes nonstructural proteins (Pudupakam et al., 2009). ORF2 encodes the capsid protein which contains

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immunodominant epitopes (Riddell et al., 2000), forms virus-like particles (Guo 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; 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*-reacting 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: genotypes 1 and 2 strains are restricted to humans whereas genotypes 3 and 4 strains infect both humans and pigs (Meng et al., 2010). Under experimental conditions, genotypes 3 and 4 swine HEV infected rhesus monkeys and conversely, genotypes 3 and 4 human HEV infected pigs (Meng et al., 1998b; Arankalle et al., 2006; Feagins et al., 2008). However, genotypes 1 and 2 human HEV failed to infect pigs (Meng et al., 1998a), indicating that genotypes 1 and 2 HEV 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.

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 (Fig. 1B): 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. The complete sequence of genotype 4 human HEV (strain TW6196E; GenBank accession number HQ634346) (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 (Fig. 1C): 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. 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) (Supplementary 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 (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). Three days post-transfection, the cells were trypsinized and plated on an 8-chamber Lab-Tek™ 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). 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 (Table 1). A fluorescent signal was not detected in the negative control cells (Fig. 2).

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*, 100 µl of 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. The results showed that chimeras rA4-3sw, rABC4-3sw, and rABC4-1h produced infectious particles in Huh7 cells, as did the wildtype pSHEV-3, as positive IFA signals were detected in HepG2/C3A cells (Table 1). The infectivity titers of the cell lysates were not determined due to the inefficient HepG2/C3A cell culture system for HEV infection (Emerson et al., 2004; Graff et al., 2005).

A swine bioassay was used to determine the infectivity of the chimeric viruses in pigs (Meng et al., 1998a). Fourteen, 8-week-old, specific-pathogen-free pigs were divided into 7 groups of 2 pigs each (Table 1). Pigs were inoculated intravenously each with 3 ml respective inoculum from each of the 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 primers 6205 and 6636 (second round, Supplementary 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, Supplementary Table 1). An enzyme-linked immunosorbent assay (ELISA) was used to detect IgG anti-HEV in the weekly sera (Meng et al., 1997).

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. 3). Fecal virus shedding was detected in both pigs from 1–2 wpi, and viremia was detected in pig 361 at 2 wpi (Table 2). 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, Table 2). 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, Table 2). 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 (Tsarev et al., 1994; Kasorndorkbua et al., 2004).

Since genotype 1 HEV does not infect pigs whereas genotypes 3 and 4 HEV infected 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 (Fig. 1B). One (pig 368) of the two pigs inoculated with chimeric virus rAB4-1h had a low level seroconversion with the OD values slightly above the 0.30 cutoff at 8 (OD=0.343) and 9 (OD=0.332) wpi (Fig. 3). However, neither pig had detectable viremia or fecal virus shedding (Table 2). 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 (Fig. 3, 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.

Although chimeras rABC3-1h, rAB4-1h, and rABC4-1h were replication competent in Huh7 cells, only chimera rABC4-1h produced infectious virus particles in Huh7 cells capable of infecting naïve HepG2/C3A cells (Table 1). The inability of chimeras rABC3-1h and rAB4-1h to produce infectious virus particles suggests a hindrance in the virion assembly process and it remains to be determined which cellular or viral factors are involved. Translation of ORF3, located within the JR and overlapping ORF2 at its 3' end, from the same bicistronic subgenomic RNA as ORF2 (Graff et al., 2006) was not directly tested in this study. 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; Huang et al., 2007) but is essential for virion release from HEV infected cells (Emerson et al., 2010). The use of cell lysates for the HepG2/C3A infectivity assay instead of culture media removed any potential blocks in virion release from Huh7 cells.

Genotypes 3 and 4 swine HEV has been identified from pigs in essentially all major swine-producing countries worldwide (Meng et al., 2010). 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 suggest that genotypes 3 and 4 HEV are of swine origin (Meng et al., 2010). 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 polypeptide 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

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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG. 1. Schematic diagrams illustrating the HEV genomic organization and the strategies for the construction of five intergenotypic chimeric viruses

(A) The HEV genome consists of three open reading frames (ORF1, ORF2, and ORF3), short 5' and 3' noncoding regions (NCR), a junction region (JR), a 5' cap structure, and a 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) Three chimeric viruses were constructed by using the genotype 1 human HEV infectious clone pSK-HEV-2 as the genomic backbone: chimera rAB4-1h with the JR+ORF2 region of genotype 4 human HEV strain TW6196E replacing that of genotype 1 human HEV; chimera rABC4-1h with the JR +ORF2+3' NCR region of genotype 4 human HEV strain TW6196E 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. (C). Chimera rA4-3sw and chimera rABC4-3sw were constructed by using the genotype 3 swine HEV infectious cDNA clone pSHEV-3 as the genomic backbone: chimera rA4-3sw with the ORF2 gene of genotype 4 human HEV strain TW6196E replacing that of genotype 3 swine HEV; and chimera rABC4-3sw with the JR+ORF2+3' NCR of genotype 4 human HEV strain TW6196E replacing that of genotype 3 swine HEV.

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 intergenotypic 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 on the lack of 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).

Table 1

Replication competency in Huh7 liver cells, and infectivity in HepG2 liver cells and in pigs of intergenotypic chimeric hepatitis E viruses

Group (pig ID#)	Inocula	Replication competency in Huh7 cells	Infectivity in HepG2 cells	Infectivity in pigs
1 (360, 362)	DMEM media	–	–	–
2 (357, 361)	Wildtype pSHEV-3	+	+	+
3 (364, 366)	Chimera rA4-3sw	+	+	+
4 (358, 369)	Chimera rABC4-3sw	+	+	+
5 (356, 368)	Chimera rAB4-1h	+	–	– [†]
6 (359, 365)	Chimera rABC4-1h	+	+	–
7 (363, 367)	Chimera rABC3-1h	+	–	–

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

Table 2

Detection of HEV RNA in fecal/serum samples collected weekly from pigs inoculated with cell lysates of Huh7 cells transfected with capped RNA transcripts of wildtype genotype 3 and intergenotypic chimeric hepatitis E viruses

Inocula ^a	Pig ID	0	1	2	3	4	5	6	7	8	9
Positive (+) or negative (-) of HEV RNA detected in fecal/serum samples at indicated week post-inoculation											
DMEM media											
	360	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	362	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
Wildtype pSHEV-3											
	357	-/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	361	-/-	+/-	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-
Chimera rA4-3sw											
	364	-/-	-/-	-/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-
	366	-/-	-/+	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-
Chimera rABC4-3sw											
	358	-/-	+/+	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	369	-/-	+/+	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-

^a 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 negative results of viremia, and fecal virus shedding in these three groups of pigs were not shown here.