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

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SUMMARY

Hepatitis E virus (HEV) is an important pathogen. The animal strain of HEV, swine HEV, is related to human HEV. The genotype 3 swine HEV infected humans and genotype 3 human HEV infected 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 3 groups of 5 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 indicated 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.

Keywords

Hepatitis E virus (HEV); genotypes; cross-species infection; swine 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; Takahashi et al., 2003; Yazaki et al., 2003; Clemente-Casares 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-

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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 (Thomas et al., 1997; Mast 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 previously (Purcell, R. H., unpublished data): $10^{5.3}$ 50% monkey infectious dose (MID₅₀) per ml for the US-2 strain (Meng et al., 1998b), and 2×10^3 MID₅₀ per ml for the TW6196E strain (Huang et al., 2002; Cooper et al., 2005).

Pigs

Fifteen, two-week-old, specific-pathogen-free (SPF) pigs were purchased from a commercial source (Geneticporc Inc, Nebraska). 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 (I.V.) with 1 ml of sterile phosphate buffered saline (PBS) as negative controls, group 2 pigs were each inoculated I.V. with 1 ml (10^3 MID₅₀) of the genotype 3 human HEV (strain US-2) as positive controls, and group 3 pigs were each inoculated I.V. with 1 ml (10^3 MID₅₀) of the genotype 4 human HEV (strain TW6196E) as the experimental group. The pigs were monitored for evidence of HEV infections 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 three 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 -80°C 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 minutes at 4°C (Eppendorf centrifuge 5810) and then used for the detection of HEV RNA by RT-PCR.

ELISA to detect anti-HEV antibodies

A purified, 55 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 11.0 μl of DNase-, RNase-, and proteinase-free water (Invitrogen). Reverse transcription was performed at 42°C for 60 minutes with 1 μl (10 μM) of the reverse primer 3157N [5'-CCCTTA(G)TCC(T)TGCTGA(C)GCATTCTC-3'], 1 μl (200 U/ μl) of Superscript II reverse transcriptase (Invitrogen), 1 μl of 0.1 M dithiothreitol, 4 μl of $5 \times$ RT buffer, 0.5 μl (40 U/ μl) of RNase inhibitor (Promega), and 1 μl of 10mM deoxynucleoside triphosphates. Ten microliters of the resulting cDNA was subsequently amplified in a 50 μl PCR reaction with *AmpliTaq* Gold DNA polymerase (Applied Biosystems).

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 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 minutes, followed by 39 cycles of denaturation for 1 minute at 94°C, annealing for 1 minute at 42°C, extension for 1 minute at 72°C, and a final extension at 72°C for 7 minutes. 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., 2005) 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) pCR®2.1 vector and the following primers: [forward, 5' – ATGCTGCCCGGCCACCG – 3'] and [reverse, 5' – AGGGTTGGTTGGATGAA – 3']. Plasmid DNA was purified using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich) and quantified using the Nanodrop ND-1000 according to the manufacturer's instructions. The mMACHINE® High Yield Capped RNA Transcription Kit (Ambion) 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) on an iCycler (Bio-Rad). The primers used for the real-time 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) and the probe (MWG Biotech) 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 –80°C until use. Reverse transcription was carried out at 50°C for 30 minutes, followed by denaturation at 95°C for 15 minutes. Real-time PCR amplification was performed with 50 cycles at 95°C for 10 seconds, 55°C for 20 seconds and 72°C for 15 seconds. 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 in 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 (Halbur et al., 2001; Meng et al., 1998). 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 days postinoculation (dpi). In group 2 pigs infected with genotype 3 human HEV, viral RNA levels in feces ranged from 4.49 to 10.10 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 to 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 5.21 to 10.26 log copies per mg of feces (Table 2). The fecal virus titers of most group 3 pigs peaked at approximately 9 to 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 HEV strains (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 (Meng et al., 1998b; Schlauder et al., 1998). Similarly, genotype 4 human HEV isolates obtained from hepatitis E patients in China and Taiwan (Hsieh et al., 1999; Wang et al., 2002; Wu et 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) has demonstrated, experimentally, 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 seroprevalence of IgG anti-HEV, 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) 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 three 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 two 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., 1999a) 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 (Banks et al., 2004; Cooper et al., 2005; Wang et al., 2002,

Yazaki et al., 2003;) 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 both genotypes 3 and 4 human HEV can infect pigs, thus 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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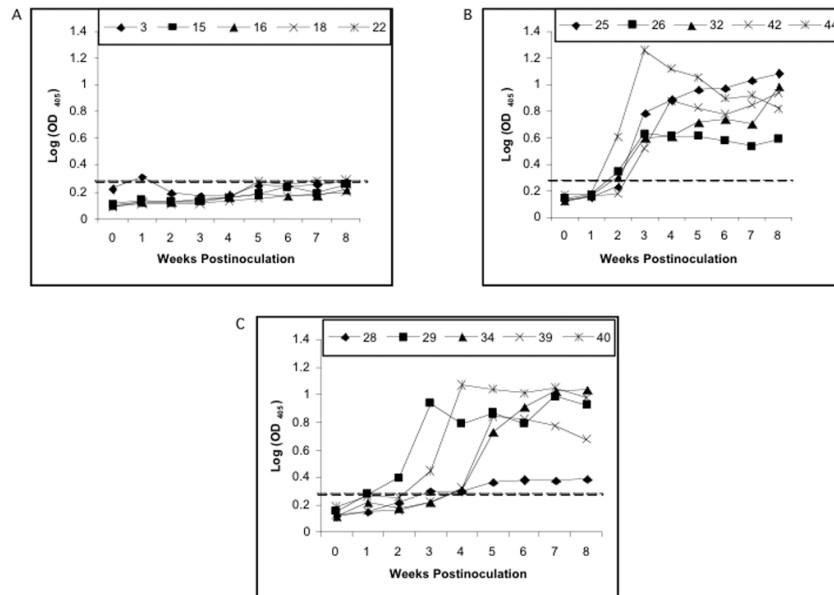


FIG. 1. Seroconversion to IgG anti-HEV in HEV-inoculated and control pigs. **Panel A**, pigs inoculated with sterile phosphate buffered saline (PBS) as negative controls (group 1); **Panel B**, pigs inoculated with a genotype 3 human HEV (strain US-2) as positive controls (group 2); **Panel C**, pigs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

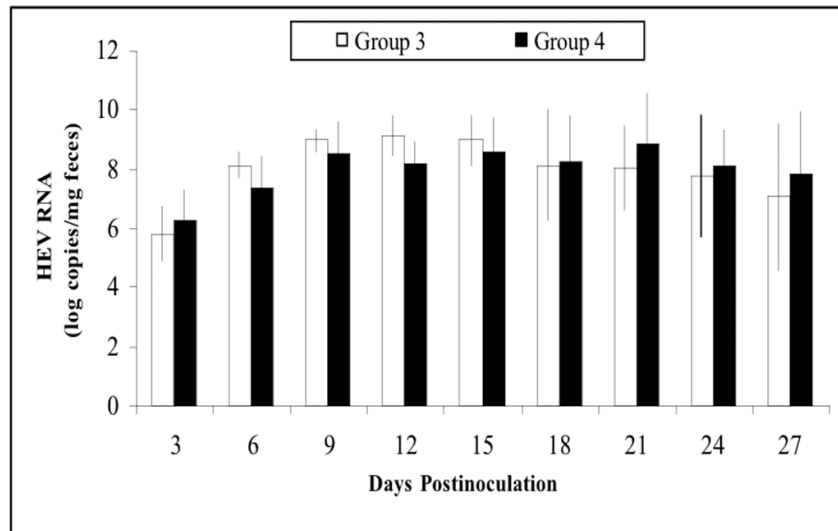


FIG. 2.

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 \pm standard deviation.

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	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
2 ^b	22	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	25	+/-	+/+	+/+	+/+	+/+	+/+	-/-	-/-
	26	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-
	32	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-
3 ^c	42	-/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-
	44	-/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-
	28	-/-	-/-	+/-	+/-	+/+	-/-	-/-	-/-
	29	+/-	-/+	+/+	+/+	-/-	+/+	+/+	+/+
	34	+/-	-/-	+/+	+/+	+/+	+/+	+/+	+/+
	39	-/-	-/-	+/+	+/+	-/-	+/+	+/+	-/-
	40	+/-	-/-	+/+	+/+	+/+	+/+	-/-	+/+

^ainoculated with sterile phosphate buffered saline, PBS (negative controls)

^binoculated with a genotype 3 human HEV strain US-2 (positive controls)

^cinoculated with a genotype 4 human HEV strain TW6196E (experimental group)

Table II

Quantification by Real-Time RT-PCR of HEV RNA in Fecal Samples Collected Every 3 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

Group 2—G3 US-2 Strain					
HEV RNA in fecal sample (log copies/mg)					
Days postinoculation	Fig 25	Fig 26	Fig 32	Fig 42	Fig 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	—

Group 3—G4 TW6196E Strain					
HEV RNA in fecal sample (log copies/mg)					
Days postinoculation	Fig 28	Fig 29	Fig 34	Fig 39	Fig 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

^aNot detected.