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Experimental infection of pigs with the newly identified swine hepatitis E virus (swine HEV), but not with human strains of HEV

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Abstract
A novel virus of pigs, swine hepatitis E virus (swine HEV), was recently identified and shown to be antigenically and genetically related to human HEV. In the present study, we attempted to infect specific-pathogen-free (SPF) pigs experimentally with swine HEV or with human strains of HEV. Serum samples collected from naturally infected pigs were used as the source of swine HEV. Pigs inoculated intravenously with serum samples containing swine HEV seroconverted to anti-HEV 4 to 8 weeks postinoculation, and the virus spread to an uninoculated pig. Swine HEV was detected in nasal and rectal swab materials as early as 2 weeks postinoculation and for 4 to 8 weeks thereafter. Viremia appeared 4 to 6 weeks postinoculation and lasted 1 to 3 weeks. The inoculated pigs appeared clinically normal and serum liver enzymes were not significantly elevated. In contrast, pigs were not infected when inoculated intravenously with about 105 monkey infectious doses of one of two human strains of HEV (Sar-55 or Mex-14).

Comments
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Brief Report

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Summary. A novel virus of pigs, swine hepatitis E virus (swine HEV), was recently identified and shown to be antigenically and genetically related to human HEV. In the present study, we attempted to infect specific-pathogen-free (SPF) pigs experimentally with swine HEV or with human strains of HEV. Serum samples collected from naturally infected pigs were used as the source of swine HEV. Pigs inoculated intravenously with serum samples containing swine HEV seroconverted to anti-HEV 4 to 8 weeks postinoculation, and the virus spread to an uninoculated pig. Swine HEV was detected in nasal and rectal swab materials as early as 2 weeks postinoculation and for 4 to 8 weeks thereafter. Viremia appeared 4 to 6 weeks postinoculation and lasted 1 to 3 weeks. The inoculated pigs appeared clinically normal and serum liver enzymes were not significantly elevated. In contrast, pigs were not infected when inoculated intravenously with about 10^5 monkey infectious doses of one of two human strains of HEV (Sar-55 or Mex-14).

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Hepatitis E virus (HEV), one of the causative agents of enterically transmitted non-A, non-B hepatitis, is currently classified in the family *Caliciviridae* [15, 24], although this classification has been questioned [18]. HEV is a single-stranded RNA virus without an envelope [24, 27, 28]. The positive sense viral genome of about 7.5 kb contains 3 open reading frames (ORFs). ORF1 probably encodes viral nonstructural proteins, ORF 2 the putative capsid protein and ORF3 a protein of unknown function [5, 22, 24, 27, 28].

The apparent primary source of infection for hepatitis E is human feces from infected individuals [1, 4, 24, 26, 36]. Antibodies to HEV (anti-HEV) have been found in many countries around the world, both in endemic and non-endemic areas, but hepatitis E has been largely confined to endemic areas comprising developing countries [1, 2, 6, 8–9, 10, 12, 16, 20, 23, 25, 26, 29, 30, 36, 38]. The disease is usually not fatal, although a high mortality rate, up to 20%, has been reported for infected pregnant women [11, 14, 24]. Hepatitis E has been successfully transmitted to various species of non-human primates [31, 32, 34, 35]. It has also been reported that domestic pigs, lambs and laboratory rats could be experimentally infected with a human strain of HEV [3, 19, 37]. Since naturally-acquired HEV antibodies have been detected in primates, rodents, and swine, it has been suggested that hepatitis E might be a zoonotic disease [7, 17, 19, 33].

Recently, we identified and characterized the first animal strain of HEV, swine HEV [21]. Swine HEV is a ubiquitous agent in pigs in the midwestern United States and antibodies to it cross-react with capsid antigen from strains of human HEV. The putative capsid gene (ORF2) of swine HEV shares about 79 to 80% sequence identity at the nucleotide level and 90 to 92% identity at the amino acid level with that of human HEV strains. The antigenic and genetic similarities between swine and human HEV suggested that the two viruses belong to the same family, and that swine HEV infection of pigs might serve as an alternative animal model for study of human HEV [21].

In the present study, we attempted to infect specific-pathogen-free (SPF) pigs with the swine HEV, and with two divergent strains of human HEV. Serum samples collected from naturally infected pigs during the acute phase of infection [21] were used as the source of swine HEV. The titer of viral genomes in the serum samples was determined by a nested-PCR with primers specific for swine HEV (see below). One genome equivalent (GE) is defined as the number of HEV genomes present in the highest serial dilution positive by RT-PCR. At least one serum sample from each of the four naturally-infected piglets contained detectable levels of swine HEV RNA (Table 1). The amount of virus in the inocula, however, was relatively low: 10^3 GE/ml of serum for piglets #4 and #14, $<10^2$ GE/ml for piglets #7 and #15. Therefore, we chose to infect pigs by the intravenous route because this route has required less virus to initiate an infection compared to the oral route in previous studies of human HEV [34].

Seven crossbred (Yorkshire, Hampshire, and Spotted Poland China) 4 week-old SPF pigs were randomly divided into 2 groups of 5 and 2 each (Groups A and B). Each group of pigs was housed in a separate room in a BL-2 facility and maintained under conditions that met all relevant requirements. Pig-to-pig contacts were allowed within the same room. Serum samples collected from four piglets in a prospective study [21] were used for inoculation of four SPF pigs in group A (Table 1). Two hundred μl each of 4 serial weekly samples of serum collected from one piglet were inoculated, in chronological order, via a pediatric catheter into the ear vein of an SPF pig (Table 1). The needle and catheter were flushed with PBS buffer between each injection. The fifth pig in group A was

Table 1. Experimental infection of specific-pathogen-free pigs with swine hepatitis E virus

	HEV markers in inocula (wk)[a]				Recipient	HEV markers in recipient pigs (wks PI[b])										
	3	2	1	0	Pig ID	0	1	2	3	4	5	6	7	8	9	10
	Serum				Group A											
	Piglet #7				A1											
Anti-HEV	−	−	−	+		−	−	−	−	−	−	−	−	+	+	+
Serum viral RNA	+	n/a	+	−		−	−	−	−	−	−	+	+	+	−	−
Fecal viral RNA						−	−	−	+	+	+	+	+	+	+	+
Nasal viral RNA						−	−	−	+	+	−	+	+	−	−	−
	Piglet #14				A2											
Anti-HEV	−	−	−	+		−	−	−	−	+	+	+	+	+	+	+
Serum viral RNA	−	+	+	+		−	−	−	−	+	−	−	−	−	−	−
Fecal viral RNA						−	−	−	+	+	−	+	+	−	−	−
Nasal viral RNA						−	−	−	−	+	−	+	+	+	−	−
	Piglet #15				A3											
Anti-HEV	−	−	−	±[c]		−	−	−	−	−	−	−	−	+	+	+
Serum viral RNA	−	−	+	+		−	−	−	−	−	−	+	+	−	−	−
Fecal viral RNA						−	−	+	−	+	+	+	+	+	−	−
Nasal viral RNA						−	−	−	+	+	−	+	+	+	−	−
	Piglet #4				A4											
Anti-HEV	−	−	−	+		−	−	−	−	−	−	−	−	+	+	+
Serum viral RNA	−	−	+	−		−	−	−	−	−	−	+	+	−	−	−
Fecal viral RNA						−	−	−	−	−	−	+	+	+	−	−
Nasal viral RNA						−	−	−	+	+	−	+	+	−	−	−
	uninoculated				A5											
Anti-HEV						−	−	−	−	−	−	−	−	+	+	+
Serum viral RNA						−	−	−	−	−	−	+	+	−	−	−
Fecal viral RNA						−	−	−	−	+	+	+	+	+	+	−
Nasal viral RNA						−	−	−	−	+	−	+	+	−	−	−
	PBS buffer				Group B											
Anti-HEV					B1	−	−	−	−	−	−	−	−	−	−	−
Anti-HEV					B2	−	−	−	−	−	−	−	−	−	−	−

[a] Group A: 200 μl each of weekly serum samples collected from naturally infected piglets (#4, 7, 14, 15) for 3 weeks (3, 2, 1) prior to and the week of (0) seroconversion or 2 days (0) before necropsy (piglet # 15) were inoculated, in chronological order, into the ear vein. n/a: not available. Group B: inoculated with PBS buffer via I.V. or oronasal route

[b] wks PI Weeks postinoculation

[c] The OD value was rising, but was still below the ELISA cutoff (±)

not inoculated. One SPF pig in group B was inoculated with 1 ml of PBS buffer intravenously and another with 4.5 ml of PBS buffer oronasally.

Clinical samples (serum, nasal swab and rectal swab) were collected before inoculation and weekly thereafter for 10 weeks. Nasal and rectal swab samples were collected with the Cell*matics* viral transport system (DIFCO Laboratories,

Detroit, MI). For nasal swab collection, the external nares were wiped with a sterile towel prior to inserting the swab 1–2 inches into the nasal cavity. An ELISA for anti-HEV in swine was standardized as previously described [21]. An HPLC-purified 55 kD truncated form of the putative capsid protein, expressed from a recombinant baculovirus containing ORF2 of a human strain of HEV (Sar-55) was used as antigen for the ELISA [33]. Sera taken from the experimentally-inoculated pigs were tested in duplicate for IgG and IgM anti-HEV. Serum levels of the liver enzymes alkaline phosphatase (AP), alanine aminotransferase (ALT), gamma glutamyltransferase (GGT), and sorbitol dehydrogenase (SDH) were assayed weekly by standard methods at Iowa State University Veterinary Clinical Pathology Laboratory.

For detection of swine HEV by RT-PCR, total RNA was extracted with TriZol reagent (GIBCO-BRL, Gaithersburg, MD) from 100 μl of nasal or rectal swab material or serum. Total RNA was reverse transcribed with a swine HEV-specific primer and SuperScript II reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) at 42 °C for 1 h; cDNA was amplified by PCR using AmpliTaq Gold polymerase (Perkin Elmer, Norwalk, CT). A nested RT-PCR was used to detect low levels of virus and to confirm first round positives. The PCR reaction consisted of 39 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, and extension at 72 °C for 1.5 min, followed by a nested-PCR of 39 cycles using 10 μl of the first round PCR product. First round: forward primer, 5′-AGCTCCTGTACCTGATGTTGACTC-3′, reverse primer, 5′-CTACAGAGCGCCAGCCTTGATTGC-3′; second round: forward primer, 5′-GCTCACGTCATCTGTCGCTGCTGG-3′, reverse primer, 5′-GGGCTGAACCAAAATCCTGACATC-3′. Reagent preparation, RNA extraction, cDNA synthesis and PCR assembly were all performed in a laboratory separate from the one in which the PCR products were analyzed in order to avoid cross-contamination.

The swine HEV-infected pigs appeared clinically normal, and jaundice was not observed. Serum levels of liver enzymes remained normal, in contrast to what is often seen in primates infected with human strains of HEV [31, 32, 34]. Anti-HEV was detected in all four pigs inoculated with the serum samples as well as in the uninoculated control pig housed in the same room (group A, Table 1). The first pig seroconverted to IgG anti-HEV 4 weeks postinoculation and the four others seroconverted 4 weeks later (Table 1). IgM anti-HEV appeared earlier than IgG anti-HEV, but lasted only 1 to 2 weeks whereas the IgG anti-HEV was still present at the end of the 10 week experiment (Fig. 1). Pigs inoculated with PBS buffer (group B) remained seronegative throughout the experiment, and were not further tested (Table 1).

The weekly serum samples as well as the nasal and rectal swab samples from group A pigs were tested for viral RNA by RT-PCR with primers specific for swine HEV (Table 1). PCR fragments amplified from serum, nasal and rectal samples of two inoculated pigs were cut from 1% agarose gels and purified by the glassmilk procedure with a Geneclean Kit (Bio101, La Ja Jolla, CA). The purified PCR fragments were sequenced (both strands) with an Automated DNA Sequencer using swine HEV-specific primers and confirmed to represent swine

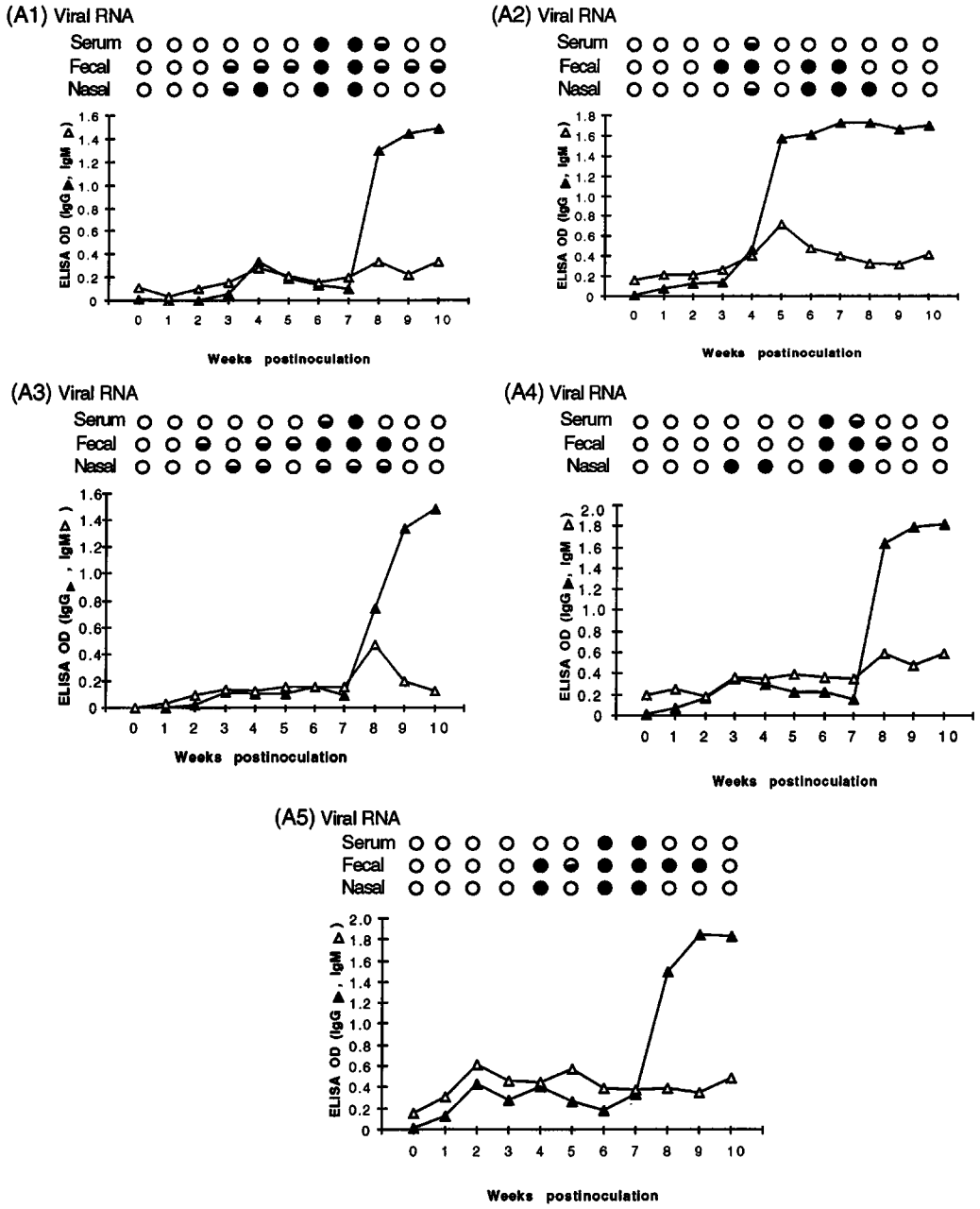

Fig. 1. Swine HEV infection in experimentally inoculated SPF pigs (A1, A2, A3 and A4) and in an uninoculated contact control SPF pig (A5). IgG and IgM HEV antibodies are plotted as ELISA OD values. Presence (● = detected by first round PCR; ◒ = detected only by a nested PCR) or absence (○) of swine HEV RNA in serum, fecal and nasal samples is indicated

HEV sequences (data not shown). Viremia of 1 to 3 weeks' duration was detected in all four inoculated pigs and in the sham-inoculated control about 4 to 6 weeks postinoculation. The disappearance of viremia was temporally related to the appearance of anti-HEV (Table 1, Fig. 1). Swine HEV RNA was also found in rectal and nasal swab samples as early as 2 weeks postinoculation and persisted, even after viremia had disappeared, for up to 7 weeks (Table 1, Fig. 1). Fecal virus shedding appeared to increase for a 2 to 3 week period, usually at 5 to 7 weeks postinoculation (data not shown). In the sham-inoculated contact control pig (A5, Table 1), swine HEV was detected in the rectal and nasal swab samples about 2 weeks after the virus first appeared in the feces of an inoculated pig (A3, Table 1).

Viremia was detected variably from 4 to 6 weeks postinoculation in infected pigs. In contrast, viremia appeared as early as 9 days postinoculation in primates infected intravenously with human strains of HEV [31]. The difference may simply reflect a dose-dependent effect since the amount of swine HEV in the inocula was relatively low, and in primates infected with human strains of HEV [31, 32, 35], the time of virus appearance was related to dose. IgG anti-HEV was first detected up to one week after viremia ceased, and remained positive thereafter. Unlike human HEV infections in primates [31, 33, 35], elevation of serum liver enzymes, indicative of liver injury, was not associated with either viremia or seroconversion. The experimentally infected pigs remained clinically normal as had the naturally infected pigs in our prospective study [21]. However, dose dependence of clinical response to human HEV has also been documented [34]. Biochemical evidence of hepatitis was prominent in primates inoculated with higher concentrations of human HEV, but was absent in infected animals given a low dose of virus [34]. Therefore, further experiments are warranted to study whether swine HEV is pathogenic for pigs when a higher concentration of virus is administrated.

Previously, we found that swine HEV is highly contagious under natural conditions [21]. In the present study, the uninoculated control pig in group A quickly became infected, almost certainly through contact with the inoculated animals housed in the same room. The incubation period to fecal excretion of virus for the sham-inoculated control pig appeared to be 1 to 2 weeks. The rapid spread of the virus to the control pig means that only the first pig (A3) to be infected (Table 1) could be confirmed as an experimental inoculation. The other 3 inoculated pigs could have had a 3 week incubation period for the inoculated virus or a one week incubation period for a contact virus.

Human strains of HEV are normally transmitted by the fecal-oral route. Therefore, it was a surprise to detect swine HEV in nasal swab materials since that usually is indicative of viral transmission via a respiratory route. It is possible that the nasal swab samples were contaminated with a small amount of blood resulting from trauma to the nasal cavity during swabbing; however, swine HEV RNA appeared about 2 to 3 weeks earlier in the nasal swab samples than in the serum in 4 of the 5 pigs (Table 1), suggesting that this explanation was unlikely. More likely, the pigs' snouts may have been contaminated by fecal materials.

Clearly, the significance of swine HEV in the nasal swab materials needs to be further evaluated.

For inoculation of SPF pigs with human strains of HEV, stool suspensions containing human strains of HEV, Sar-55 [31] and Mex-14 [13], were used as the inocula. The infectivity titer of both strains was determined in primates ([34], Purcell et al., unpubl. data). The titer of viral genomes in inocula was also determined with a nested-PCR by using strain-specific primers as described below. The amount of virus in the inocula was about 10^5 monkey infectious doses (MID), which corresponded to 10^7 GE/ml for Mex-14, and 10^6 GE/ml for Sar-55. Three-week-old crossbred pigs from the same SPF herd used for the swine HEV inoculations were randomly divided into 3 groups of 3 pigs each. Each group was housed in a separate room in a BL-3 facility. One group was inoculated with HEV, strain Sar-55, and another group with HEV, strain Mex-14. Each pig received an intravenous injection of 0.5 ml of inoculum containing about 10^5 MID of HEV. The third group was inoculated with PBS buffer. The animals were monitored weekly for 14 weeks for evidence of hepatitis. Preinoculation values for anti-HEV and baseline serum levels of the liver enzymes were determined. A collection tray was used to gather feces.

For detection of human strains of HEV, feces were resuspended in 10% (w/v) Mg^{++}/Ca^{++}-free PBS buffer, and clarified by low-speed centrifugation. RNAs from 100 μl of 10% fecal samples or 100 μl of sera were extracted with TriZol reagent. As a positive control, ten μl of stool suspension containing about 10 genome equivalents of HEV Sar-55 was mixed with 100 μl of 10% pig fecal suspension or 100 μl of serum. Superscript II reverse transcriptase was used as described by the manufacturer. The PCR reaction consisted of 40 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 1.5 min, followed by a nested-PCR of 40 cycles using 10 μl of the first round PCR product. The PCR primers were strain-specific and based on published sequences. PCR primers for the Sar-55 strain [31] were: first round, forward primer, position 4 184–4 205 and reverse primer, position 4 534–4 555; second round, forward primer, position 4 251–4 270 and reverse primer, position 4 521–4 540. PCR primers for the Mex-14 strain [13] were: first round, forward primer, position 4 437–4 460 and reverse primer, position 4 866–4 889; second round, forward primer, position 4 484–4 505 and reverse primer, position 4 812–4 833.

Clinically, all pigs inoculated with the two human strains of HEV remained normal throughout the entire experimental period, and jaundice was not observed in any of the inoculated swine. We did not observe significant elevation of liver enzymes tested (data not shown). We were unable to detect IgG anti-HEV by ELISA in any samples collected from these experimentally-inoculated swine. A nested RT-PCR was used in an attempt to amplify HEV RNA from the weekly serum and fecal samples of the inoculated swine. The RT-PCR assay was sensitive enough to amplify HEV product from a positive control containing about 10 viral genome equivalents. However, HEV RNA was not detected in either the serum or fecal samples of the inoculated swine.

Balayan et al. reported that Russian domestic swine could be experimentally infected with a Central Asian strain of HEV isolated from a naturally infected patient [3]. The experimentally-induced hepatitis appeared to be quite severe since the infected swine developed jaundice. In the present study, we examined the susceptibility of crossbred SPF swine to experimental infection with high doses of genetically divergent strains of human HEV from Asia (Sar-55) and from Mexico (Mex-14). However, we were unable to infect SPF pigs with either of these human strains although we inoculated 1000 to 10,000 times as much human virus as we had the swine virus.

A possible explanation for our failure to infect SPF pigs with human strains of HEV is that there is a difference in susceptibility of different swine breeds to HEV infection. Crossbred SPF pigs were used in our experiment. However, crossbred pigs have been widely used for virus transmission studies and have been shown to be highly susceptible to many viruses. Alternatively, the transmissibility of various human HEV strains may differ. Since the virus was not recovered from the pigs reportedly infected in the previous study [3], it is not known if it more closely resembled human HEV or swine HEV. It will now be very important to determine whether swine HEV can infect humans. As a first step, we are attempting to infect non-human primates with the swine virus.

In addition to swine, rodents and lambs are also reported to be susceptible to infection with HEV [17, 19, 37]. However, little is known regarding infection of these species with human strains of HEV, and the relative sensitivity to infection of these animals compared to nonhuman primates needs to be evaluated. These species should also be examined to determine if they have their own strains of HEV.

Our previous prospective study of naturally infected pigs revealed that swine HEV causes an asymptomatic infection in young pigs [21]. We have now experimentally reproduced this asymptomatic infection in SPF pigs, and recovered swine HEV genomic sequence from them. Although swine HEV infection in pigs was somewhat similar to human HEV infection in primates, differences in the course of infection and in clinical and pathological manifestations were noted. Nevertheless, swine HEV infection of pigs may be a useful alternative animal model to study the natural history of HEV as well as the ecological, biological and pathological differences between swine and human HEV. Studies of swine HEV infection of pigs may also prove useful in developing an effective vaccine for HEV infection in humans.

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