Localisation of swine hepatitis E virus in experimentally infected pigs

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Abstract

The distribution of intravenously inoculated swine hepatitis E virus (HEV) was assessed by in situ hybridisation for a period of 50 days. Evidence of apparent clinical disease was found in only one pig in the HEV infected group. The only gross lesion observed was mildly enlarged mesenteric lymph nodes at 50 days post infection (dpi). Histopathologically, mild lymphoplasmacytic infiltration and focal hepatocellular necrotic lesions were found in HEV-infected pigs. Swine HEV nucleic acids were detected by RT-PCR in the faeces at 3 dpi in 100% of the 18 pigs infected with the virus. Thereafter, the number of positives declined.

The most consistent and intense signal was found in the liver of infected animals using in situ hybridisation. The positive cells were hepatocytes, Kupffer cells, bile epithelial cells and interstitial lymphocytes. Swine HEV RNA was localised in the cytoplasm of the hepatocytes, with a slightly granular pattern of staining, but hybridisation signals were not observed in degenerative or vacuolated hepatocytes. HEV was much less frequently detected in extrahepatic tissues such as lymph nodes, tonsil, spleen and small and large intestine. It was concluded that swine HEV had replicated primarily in the hepatocytes and infection resulted in subclinical infection with minimal histopathological changes in the liver.

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Introduction

Hepatitis E virus (HEV) is an icosahedral, non-enveloped virus that has a positive sense, single-stranded RNA genome of approximately 7.5 kb and contains three overlapping open reading frames (ORFs) (Tam et al., 1991). HEV was classified originally in the Caliciviridae (Purcell, 1996), but its unique genomic organisation led to its reclassification in the genus Hepevirus (Emerson et al., 2004). Swine HEV, which is closely related to human HEV, was discovered in the United States in 1997 (Meng et al., 1997) and since then has been reported worldwide (Schlau- der and Mushahwar, 2001). Pigs are considered to be a good model for some aspects of HEV infection in humans (Meng et al., 1998b; Williams et al., 2001).

Although hepatocytes are the primary site for HEV detection (Williams et al., 2001; Meng et al., 1997, 1998b), little is known about viral detection and localisation in the liver and extrahepatic tissues (Choi and Chae, 2003; Ha and Chae, 2004; de Deus et al., 2007). HEV has been detected extrahepatically in rats experimentally infected with human HEV (Maneerat et al., 1996) and, using reverse transcription-polymerase chain reaction (RT-PCR), swine HEV has been reported in numerous extrahepatic tissues in pigs experimentally infected with either swine or human HEV (Williams et al., 2001). The purpose of the present study was to elucidate the pathogenesis of experimental infection with swine HEV by defining virus distribution and sites of virus detection in pigs using in situ hybridisation.

Materials and methods

Experimental design

All experimental methods were approved by the Seoul National University Institutional Animal Care and Use Committee.
The swine HEV strain SNUDVP3825 used was isolated from pigs in Kyounggi Province of Korea (Choi and Chae, 2003). Thirty 3-week old conventional pigs were confirmed as seronegative for HEV infection by ELISA (Genelabs Diagnostics), for porcine circovirus 2 (PCV2) by an indirect immunofluorescent test (Magar et al., 2000), and for porcine reproductive respiratory syndrome virus (PRRSV) by ELISA (IDEXX Laboratories). They were randomly allocated to an infected or a control group, and maintained in stainless steel isolators (two pigs per isolator).

An infectious pool of swine HEV was prepared as a 10% suspension of faeces collected from a colostrum-deprived pig experimentally infected with swine HEV. Each of the 18 pigs was then inoculated intravenously (IV) with 5 mL of this preparation, as previously described (Halbur et al., 2001). The 12 pigs in the control group were exposed in similar manner to uninfected PK-15 cell culture supernatants. Three pigs from the infected group and two pigs from the control group were humanely euthanased at 3, 7, 14, 21, 29, 50 days post-inoculation (dpi). Tissues were collected from each pig at necropsy.

Reverse transcription-polymerase chain reaction (RT-PCR)

The primers from the genome of the swine HEV genotype III sequence (GenBank accession number, AF082843) used in this study were as previously described (Meng et al., 1998b) but with slight modification. For the outer RT-PCR, the forward and reverse primers were 5'-AGCTCCTGTACCTGATGTTGACTC-3' (nucleotides 5527–5550) and 5'-CTACAGAGC GCCAGCCTTGGATGC-3' (nucleotides 5954–5931), respectively. The primer set resulted in amplified fragments of 405 base pairs (bp). For the inner RT-PCR, the forward and reverse primers were 5'-GCTCACGTCATCTGTCGCTGCTGG-3' (nucleotides 5596–5619) and 5'-GGGGCTGTAACCCAAAATCTGACATC-3' (nucleotides 5884–5861), respectively. The primer set resulted in amplified fragments of 289 bp.

RNA was extracted from faeces and bile with Trizol LS Reagent (Gibco) according to the manufacturer's instructions. RNA extracts were treated with DNase I (Gibco BRL) to eliminate genomic DNA contamination. For the first-stranded cDNA synthesis, 1 μL of the swine HEV RNA (5 ng/μL) was supplemented in a total reaction volume of 20 μL with 1 × RT buffer (50 mM Tris-HCl, 8 mM MgCl₂, 30 mM KCl, 1 mM dithiothreitol [pH 8.3]), 0.5 mM (each) deoxynucleotide triphosphates (dNTPs), 2.5 μM random hexanucleotide mixture, 20 U of RNase inhibitor, and 50 U of Moloney murine leukaemia virus reverse transcriptase. After incubation for 45 min at 42 °C, the mixture was incubated for 5 min at 99 °C to denature the products. The mixture was then chilled on ice.

The composition of the PCR mixture (150 μL) was 30 μL of cDNA (5 ng/μL), 2 μL of each primer (250 nM), 15 μL of 10 × PCR buffer (10 mM Tris-HCl, 40 mM KCl, 1.5 mM MgCl₂ [pH 8.3]), 1.2 μL of each dNTP (0.2 mM), 29 μL of 2.5 unit of Taq polymerase, and 37.2 μL of distilled water. The PCR reaction for swine HEV was performed under the following conditions in a thermal cycler (Perkin-Elmer-Cetus): 1 cycle of 2 min at 94 °C; 35 cycles of denaturation at 94 °C for 45 s, annealing at 52 °C for 45 s, and elongation at 72 °C for 1 min.

In situ hybridisation

RT-PCR products prepared as previously described (Meng et al., 1998b) were purified using a 30 kD cut-off membrane ultrafiltration filter. The nucleotide sequences of the purified PCR products were determined before labelling by random priming with digoxigenin-dUTP (Boehringer Mannheim) according to the manufacturer's instructions. The cDNA probe for PRRSV was prepared as described by Cheon and Chae (1999) and used as negative probe.

In situ hybridisation was performed as previously described (Choi and Chae, 2003). The digoxigenin-labelled cDNA probe of PRRSV was used as negative probe (Cheon and Chae, 1999).

Morphometric analysis

Single sections from each formalin-fixed hepatic tissue were taken from the virus-infected pigs for morphometric analysis (Gomez-Villamandos et al., 2001). To obtain quantitative data, morphometric analysis of the in situ hybridisation slides was performed using the NIH Image J Program (National Institutes of Health). In each case, three fields were randomly selected, the number of positive cells per unit area (0.95 mm²) was counted, and the mean values calculated.

Statistical analysis

Statistical analysis was performed with the InStat package (GraphPad Software). The Wilcoxon matched pairs signed rank test was used for comparison of mean positive cells per unit area for swine HEV from experimentally infected pigs.

Results

Evidence of apparent clinical disease was not found in pigs from any of the groups except one animal from the swine HEV infection group. This pig had mild diarrhoea at 3 dpi. The only gross lesions observed in the other pigs without diarrhoea were mildly enlarged mesenteric lymph nodes at 50 dpi.

Mild lymphoplasmacytic infiltration and focal hepatocellular necrotic lesions were seen histologically in experimentally HEV-infected pigs. Foci of hepatocellular vacuolation and foci of necrosis were occasionally observed at 7 dpi (2 pigs), 29 dpi (1 pig) and 50 dpi (1 pig). Multifocal lymphoplasmacytic infiltration was randomly distributed in liver at 14 dpi (1 pig), 29 dpi (1 pig) and 50 dpi (2 pigs). Histopathological lesions in other tissues were not seen in the HEV-infected pigs. No histopathological lesions were seen in pigs from the negative control group.

Results of RT-PCR examination of faeces and bile are summarised in Table 1. All pigs were negative for swine HEV RNA at 0 dpi, and negative control pigs remained negative throughout the experiment. Swine HEV nucleic acids were detected by RT-PCR in the faeces at 3 dpi in 100% of the 18 pigs experimentally infected with swine HEV. Thereafter, the number of positive results declined. Swine HEV nucleic acid was detected in faecal samples at 50 dpi. Swine HEV nucleic acids were also detected by RT-PCR in the bile from all experimentally infected pigs except for one pig at 29 dpi and one at 50 dpi. Sequence analysis confirmed that the virus detected in 18 faecal

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a Number of positive sample.
b Number of sample tested.
and three bile samples from the experimentally infected pigs at 3 dpi was the same as that in the inocula.

All pigs experimentally infected with HEV had distinct, positive hybridisation signals for swine HEV. The morphology of the host cells was preserved despite the relatively high temperature required during the incubation procedure. The labelling intensity varied within and between histological structures in any one section, and between pigs. Positive cells typically exhibited dark brown or black reaction products in the cytoplasm, but without background staining.

The liver was most consistently positive. The positive cells were hepatocytes (Fig. 1A), Kupffer cells (Fig. 1A), bile epithelial cells (Fig. 1B) and interstitial lymphocytes. Intense and specific staining was most often seen in hepatocytes at each sampling period. Swine HEV cDNA was located almost exclusively in the cytoplasm of hepatocytes, with a slightly granular pattern of staining (Fig. 1A). The hybridisation signal was diffusely found in the majority of hepatocytes or confined to the foci of the liver cells. When swine HEV cDNA was detected, it was not usually associated with lymphoplasmacytic inflammation. In addition, a hybridisation signal was not observed in degenerative or vacuolated hepatocytes. Kupffer cells hybridized for swine HEV cDNA for each pig from 14 to 29 dpi, but hybridisation signals were more intense and widespread at 14 and 21 dpi. Swine HEV was observed in bile epithelial cells at 3, 7, 14, 21 and 50 dpi. Occasionally, swine HEV nucleic acid was detected on the luminal surface of bile epithelial cells (Fig. 1B).

Swine HEV was much less frequently detected in extrahepatic tissues such as lymph nodes, tonsil, spleen, and small and large intestine (Fig. 1C). Comparison with haematoxylin and eosin-stained sections from the same block indicated that most of the positive cells were lymphocyte-like cells with uniformly round nuclei and scant cytoplasm (Fig. 1D). HEV-positive cells were detected in the mantle zone of the lymph nodes. In the tonsil, swine HEV nucleic acid was readily detected within cells in the centre of hyperplastic follicles and in the surrounding lymphoreticular tissues. HEV-positive cells were scattered throughout the peri-arteriolar lymphoid sheaths of the spleen. Positive cells were consistently detected in cells located in lamina propria of small and large intestines.

Fig. 1. Liver from a pig experimentally infected with the swine hepatitis E virus (HEV). (A) Swine HEV cDNA (dark brown) detected in the cytoplasm of hepatocytes and Kupffer cells (arrow) 400×. (B) Swine HEV nucleic acid (dark brown reaction) was detected in the luminal surface of bile epithelial cells 400×. (C) Swine HEV nucleic acid (dark brown reaction) was detected in the cells of the lamina propria in the large intestine 200×. (D) The positive cells were lymphocytes (arrow) with uniformly round nuclei and scant cytoplasm 400×. In situ hybridisation; cDNA probe; nitroblue tetrazolium/5-bromocresyl-3-indolylphosphate, methyl green counterstain.
positive cells were detected in the liver (\( P < 0.05 \)). Swine HEV-positive cells decreased from 29 to 50 dpi (\( P < 0.05 \)).

Swine HEV-positive cells were detected (\( P < 0.05 \)) in the liver from experimentally infected pigs. From 7 to 21 dpi, significantly less HEV-positive cells were detected in the liver (\( P < 0.05 \)). Swine HEV-positive cells decreased from 29 to 50 dpi (\( P < 0.05 \)).

The histopathological liver lesions observed in the swine HEV-infected pigs were mild. Lymphoplasmacytic inflammation and rare focal necrotic hepatocytes were observed as in previous studies (Meng et al., 1997; Halbur et al., 2001). In addition, swine HEV cDNA was detected in normal hepatocytes but not degenerative hepatocytes. These findings indicate that liver damage induced by HEV infection may be due to the immune response or via unknown pathways to the invading virus and may not be a direct cause of viral replication in hepatocytes.

The HEV genome has also been demonstrated in peripheral blood monocyte samples in experimentally infected rhesus macaque monkeys (Im et al., 2001). In the present study, swine HEV was detected in mononuclear cells in extrahepatic tissues. It would seem likely that swine HEV could replicate in circulating peripheral monocytes, at least to a limited extent, and that this contributes to virus distribution in extrahepatic tissues such as lymph nodes, tonsil, spleen, and small and large intestine.

Cytoplasmic localisation of HEV was different in duration of infection in rhesus monkeys. HEV RNA was located universally in the cytoplasm of hepatocytes in the acute period, in contrast to submembranous localisation seen in the prehepatic period in human HEV-infected rhesus monkeys (Kawai et al., 1999). However, this different cytoplasmic localisation of HEV was not observed in hepatocytes taken from swine HEV-infected pigs. It is not possible to explain the difference in distribution of HEV, but it could be due to different strains of HEV or to differences between the host species.

Serological studies have indicated that the increased prevalence of HEV infection among persons with occupational exposure to swine suggests animal-to-human transmission (Drobeniue et al., 2001; Meng et al., 2002). Viral excretion began approximately 3–7 dpi and persisted for nearly 7 weeks in the present study. In addition, HEV is transmitted by the faecal-oral route (Reyes, 1997). Therefore, long-term excretion of swine HEV in the faeces of infected pigs leads us to speculate that this may be a reservoir for transmission of swine HEV between pigs and pigs, or between pigs and possibly humans, and contaminated faeces may be the primary source of infection.

The major advantage of in situ hybridisation compared to RT-PCR is the ability to determine which cells (in a mixed population) or tissues are expressing the swine HEV in formalin-fixed, paraffin-embedded tissues. Furthermore, formalin fixation of tissues allows veterinary practitioners to ship swine liver tissues for swine HEV identification in a well-preserved, non-infectious state because cross-species infection between pigs and humans is likely, and swine may be a reservoir of human HEV (Meng et al., 1998b).
Conclusions

The present study showed that swine HEV induced very mild hepatic lesions but persisted in hepatocytes for 50 dpi. It is concluded that swine HEV replicated primarily in hepatocytes and infection resulted in subclinical infection with minimal liver histopathological changes.

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References


