SUMMARY. Swine hepatitis E virus (HEV) antigen was detected immunohistochemically in formalin-fixed, paraffin-embedded hepatic tissue from 30 naturally infected pigs. Thirty pigs from 30 different herds were selected on the basis of positive results for reverse transcription-polymerase chain reaction. Positive cells typically exhibited a red reaction product in the cytoplasm without any observable background staining. Swine HEV antigen was consistently detected in liver from all 30 pigs tested. A strong immunohistochemical signal was seen within a variable number of hepatocytes in multifocal lobules. The signal involved the majority of hepatocytes diffusely or was confined to foci of liver cells. Positive immunohistochemical signals were also detected in small and large intestine, lymph node, tonsil, spleen, and kidney. The immunohistochemistry technique developed in this study proved useful for the detection of swine HEV in formalin-fixed, paraffin-embedded tissues taken from naturally infected pigs and may be a valuable tool in studying the pathogenesis of swine HEV infection.

Keywords: hepatitis E virus, immunohistochemistry, liver, swine hepatitis E virus.

INTRODUCTION

Hepatitis E virus (HEV), previously referred to as enterically transmitted non-A, non-B hepatitis, is responsible for sporadic infections as well as large epidemics of acute viral hepatitis in developing countries [1,2]. The disease generally affects young adults and reportedly has a mortality rate of up to 20% in infected pregnant women [3,4]. HEV was once considered to be a member of the family Caliciviridae, but the unique genomic organization of HEV has led to the removal of HEV from this family and it has been provisionally placed in an unassigned family of HEV-like viruses [5].

A new virus was discovered in swine from Illinois in the United States in 1997 and was designated swine HEV [6]. In the United States, two strains of human HEV isolated from patients with acute hepatitis were shown to have a striking genetic similarity to swine HEV [7,8]. In addition, strains of HEV from patients with acute hepatitis E were found to have a 92–97% nucleotide sequence identity with a strain of HEV of swine origin [9,10]. In addition, cross-species infection has already been demonstrated as swine HEV infects non-human primates, and there is also evidence of swine HEV infecting human beings [9, 11–13].

As a result of the lack of a suitable cell culture for HEV isolation [14], swine bioassay, positive and negative strand-specific reverse transcription-polymerase chain reaction (RT-PCR), in situ hybridization has been developed for the detection of swine HEV in clinical samples [9, 15–17]. A swine bioassay is time consuming, laborious, and expensive. Positive strand-specific RT-PCR has the potential to provide a sensitive, rapid, and specific method to detect swine HEV in clinical samples. Negative strand-specific RT-PCR can detect only the replicative, negative-strand HEV-RNA in infected tissues [16]. In situ hybridization detects positive strand HEV-RNA and provides details of cell localization and histological architecture [17]. However, the use of in situ hybridization is largely restricted to specialized laboratories because this technique requires greater technical complexity and expense compared with immunohistochemistry. Therefore, the objective of this study was to develop an immunohistochemical method for the detection of swine HEV from formalin-fixed, paraffin-embedded hepatic tissues.

Abbreviations: EIA, enzyme immunoassay; HEV, hepatitis E virus; ORF, open reading frame; PCV2, porcine circovirus 2; PRRSV, porcine reproductive and respiratory syndrome virus; RT-PCR, reverse transcription-polymerase chain reaction.

Correspondence: Dr C. Chae, Department of Veterinary Pathology, College of Veterinary Medicine and School of Agricultural Biotechnology, Seoul National University, San 56-1, Shillim-Dong, Kwanak-Gu 151-742, Seoul, Republic of Korea. E-mail: swine@plaza.snu.ac.kr

© 2004 Blackwell Publishing Ltd
MATERIALS AND METHODS

Animal samples
Thirty pigs (no. 1–30) from 30 different herds were selected on the basis of positive HEV-RNA results by RT-PCR. The age of these 30 pigs was between 60 and 114 days. All pigs were negative for porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus 2 (PCV2) as revealed by in situ hybridization [18,19]. Negative control sections were prepared from 1-day-old colostrum-deprived pigs that had not been exposed to any viral or bacterial pathogens. Liver sections from pig experimentally infected with PCV2 were also used as negative controls [20].

Tissue processing
Samples of liver, lymph node, tonsil, spleen, heart, lung, kidney, small and large intestine were collected from the animals, fixed in 10% (w/v) neutral-buffered formalin for 24–48 h, and then embedded in paraffin following standard laboratory procedures. Samples were cut into 4-μm thick sections, floated in a water bath containing diethylpyrocarbonate-treated (DEPC) water and mounted on positively charged slides (Superfrost/plus slides; Fisher Scientific, Pittsburgh, PA, USA).

Reverse transcription-polymerase chain reaction
The primers used for swine HEV sequence amplification in this study were those previously described [9] and based on GenBank accession number, AF082843. For the outer RT-PCR, the forward and reverse primers were 5′-AG-CTCCTGTACCTGATGTTGACTC (nucleotides 5596–5619) and 5′-CTCACAGGCGCCAGCTTGTCG (nucleotides 5954–5931), respectively. The amplified fragment was 405 base pairs (bp) in length. For the inner RT-PCR, the forward and reverse primers were 5′-GCTCACGTCATCTGTCGCTGC (nucleotides 5884–5861), and 5′-CTCCTGTACCTGATGTTGACTC (nucleotides 5527–5550) respectively. The amplified fragment was 289 bp in length.

RNA was extracted from liver using Trizol LS Reagent (Gibco BRL, Grand Island, NY, USA) according to the manufacturer’s instructions. RNA extracts were then treated with DNase I (Gibco BRL) to eliminate genomic DNA contamination. For the first-strand cDNA synthesis, 1 μL of the swine HEV RNA (5 ng/μL) was made up in a total reaction volume of 20 μL with 1x RT buffer [50 mM Tris–HCl, 8 mM MgCl2, 30 mM KCl, 1 mM dithiothreitol (pH 8.3)], 0.5 mM of each deoxynucleotide triphosphate (dNTP), 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 and the mixture was then chilled on ice.

The composition of the polymerase chain reaction (PCR) mixture (150 μL) was 30 μL of cDNA (5 ng/μL), 2 μL of each primer (250 μm), 15 μL of 10x PCR buffer [10 mM Tris–HCl, 40 mM KCl, 1.5 mM MgCl2 (pH 8.3)], 1.2 μL of each dNTP (0.2 μM), 29 μL of 2.5 unit of Taq polymerase, and 72.8 μL of distilled water. The PCR reaction for swine HEV was performed under the following conditions in a thermal cycler (Perkin-Elmer-Cetus, Norwalk, CT, USA): 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.

Immunohistochemistry
Tissue samples from each pig were collected in 10% neutral-buffered formalin, and after 1 or 2 days’ fixation were dehydrated using graded alcohols and a xylene step and embedded in paraffin wax. Tissues were processed by routine methods, sectioned (4 μm), and mounted on positively charged slides (Superfrost/plus slides; Fisher Scientific, Pittsburgh, PA, USA). Liver sections from pig experimentally infected with PCV2 had not been exposed to any viral or bacterial pathogens. Negative control sections were flooded and incubated for 1 h at 36 °C with streptavidin–alkaline phosphatase conjugate (Roche Molecular Biochemicals, Mannheim, Germany). They were then incubated with antibody at 4 °C in a humid chamber overnight.

RESULTS
Reverse transcription-polymerase chain reaction
To detect swine HEV, RT-PCR analyses were performed using RNA extracted from liver tissues of pigs. Amplification of template cDNA with primers of swine HEV resulted in
amplified products corresponding to those of the predicted size, i.e. 289 bp. PCR products were sequenced, and their identity confirmed. Swine HEV was consistently detected in liver tissues of all 30 pigs examined. In contrast, swine HEV was not detected in negative control pigs.

**Immunohistochemistry**

A distinct immunohistochemical staining for swine HEV antigen was detected in all 30 pigs. A close cell–cell relation between adjacent serial sections from each of the 30 hepatic samples was confirmed by immunohistochemistry. Positive cells typically exhibited a red reaction product in the cytoplasm without any observable background staining. The signal intensity varied within and between anatomical structures in sections and between pigs.

Swine HEV antigen was consistently detected in liver from all 30 pigs tested. A strong immunohistochemical signal was seen within a variable number of hepatocytes in multifocal lobules. The immunohistochemical signal involved the majority of hepatocytes diffusely or was confined to foci of liver cells. At higher magnification, swine HEV antigen was localized to the cytoplasm of hepatocytes, with a slightly granular pattern of staining (Fig. 1). No immunohistochemical signal was observed in degenerative hepatocytes.

The intensity and extent of swine HEV antigen staining in other organs was less than in livers. Swine HEV-positive cells were detected in the mantle zone of the lymph nodes from eight naturally infected pigs. In the tonsil, swine HEV antigen was detected within cells in the centre of hyperplastic follicles from nine naturally infected pigs. In the spleen, swine HEV-positive cells, which were concentrated around periarteriolar lymphoid sheaths, was detected in 13 naturally infected pigs. Swine HEV antigen was scattered throughout cells located in the lamina propria of the small and large intestines from 23 naturally infected pigs (Fig. 2). Small numbers of swine HEV-positive cells were observed in the renal interstitium. Swine HEV-positive cells resembled lymphocytes with uniformly round nuclei and scant cytoplasm, and macrophages with large oval nuclei and abundant cytoplasm. The monoclonal antibody for PCV2 was consistently negative in all tissues tested. Sections from negative control pigs showed no immunohistochemical signal for swine HEV.

**DISCUSSION**

This study demonstrated that swine HEV antigen could consistently be demonstrated by immunohistochemical methods in hepatocytes. Intense and consistent immunohistochemical signals of swine HEV antigen were demonstrated in normal hepatocytes but not degenerative hepatocytes. As the degenerative hepatocytes were negative for swine HEV antigen, they may represent reactive changes in the hepatocytes secondary to swine HEV infection. This explanation is supported by the observation that liver damage induced by HEV infection may be due to the immune response to the invading virus and may not be a direct cause of viral replication in hepatocytes [23].

Recent advances in xenotransplantation technology as a therapeutic approach have the potential to benefit human health. Pigs have been the major animal source considered for xenotransplantation because the physiology and the size of porcine organs are similar to those in human beings [24]. Human hepatic failure, such as liver cancer and decompensated liver cirrhosis, may someday be treated by xenograft implantation of porcine liver. Xenotransplantation of porcine organs to humans will allow micro-organisms
present in the donor organs to bypass the normal defense mechanisms of the human recipient. Swine HEV does not cause any clinical symptoms in the natural host but is a likely zoonotic agent that can infect human beings and cause hepatitis [25]. The use of immunohistochemistry as a monitoring and diagnostic tool for confirmation of the presence of swine HEV can be a tremendous asset for the management of swine HEV infection.

The great value of immunohistochemistry and in situ hybridization lies in the possible association of the signal with cells of particular types or with observable histological changes [14,17,18]. Although its major disadvantage is the greater technical complexity and expense compared with RT-PCR, immunohistochemistry and in situ hybridization are also able to detect swine HEV in formalin-fixed, paraffin-embedded tissues. However, the workload and time required for performance of in situ hybridization are prohibitive for many laboratories. In contrast, polyclonal HEV antibody is commercially available. However, the use of immunohistochemistry has possibilities for error caused by antigenic cross-reactivity or by the alteration of binding sites during tissue processing [26]. Formalin fixation can denature viral antigens, which can lead to false-negative results. Optimized antigen retrieval and a stringent procedure was devised to circumvent these problems in the present study.

The HEV genome is a single stranded, positive-sense RNA molecule approximately 7.2 kb in length and contains three overlapping open reading frames (ORFs) [27]. ORF1 probably encodes for nonstructural proteins responsible for viral genome replication and protein processing, and ORF2 encodes a capsid antigen. The peptide encoded by ORF3 may be a structural protein [28,29]. The polyclonal antibody used in this study is directed against the capsid protein derived from ORF2. In a serologic study, an ORF2-based enzyme immunoassay (EIA) performed better than an ORF-3 based one [29]. Moreover, the amino acid identities between HEV and swine HEV are 97.7% for ORF2 and 93.5% for ORF3. Thus, the capsid protein derived from ORF2 would have common epitopes and thus allow specific detection of swine HEV in formalin-fixed, paraffin-embedded hepatic tissues.

In all cases, the specificity of immunohistochemistry was confirmed by the rigorous observance of two controls, namely (i) immunohistochemistry performed on consecutive sections of swine HEV-infected hepatic tissues with PCV2 monoclonal antibody which showed no positive signal, and (ii) immunohistochemistry performed with swine HEV polyclonal antibody on tissues of negative control pigs which were consistently negative. In addition, the nucleotide sequences of 30 RT-PCR products of swine HEV were 97.3–100% identical to those of a US strain of swine HEV (data not shown) [6,9]. Therefore, these results confirmed that pigs tested in this study were indeed infected by swine HEV, and immunohistochemical signals detected in these pigs were specific for swine HEV. The development of an immunohistochemical technique is important as formalin fixation of tissues allows veterinary practitioners to readily ship tissue samples for swine HEV identification in a well preserved, noninfectious state to avoid accidental transmission of swine HEV to human beings during transportation.

It is difficult to interpret the results with naturally infected pigs especially as swine HEV does not cause any clinical disease in pigs [6] and the stage of infection in these naturally infected animals is not known. Swine HEV replicates in the liver for only a very limited period of time [16,30]. Therefore, further studies are needed to determine the viral distribution using tissues from experimentally infected pigs.

ACKNOWLEDGEMENTS

This research was supported by contract research funds of the Research Institute for Veterinary Science (RIVS) from the College of Veterinary Medicine, Seoul National University, and Brain Korea 21 Project, Republic of Korea.

REFERENCES


