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Inactivation of infectious hepatitis E virus present in commercial pig livers sold in local grocery stores in the United States

A. R. Feagins^a, T. Opriessnig^b, D. K. Guenette^a, P. G. Halbur^b, and X. J. Meng^{a,*}

^aCenter for Molecular Medicine and Infectious Diseases, Department of Biomedical Sciences and Pathobiology, College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA

^bDepartment of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA

Abstract

Hepatitis E virus (HEV) is a zoonotic pathogen and pigs are a known reservoir. Recently we showed that approximately 11% of commercial pig livers sold in local U.S. grocery stores for food consumptions are contaminated by infectious HEV. In this study, a swine bioassay was used to determine if the infectious HEV in contaminated commercial pig livers could be inactivated by traditional cooking methods. Group 1 pigs (n=5) were each inoculated intravenously (I.V.) with a HEV-negative liver homogenate as negative controls, group 2 pigs (n=5) were each inoculated I.V. with a pool of two HEV-positive pig liver homogenates as positive controls, groups 3, 4 and 5 pigs (n=5, each group) were each inoculated I.V. with a pool of homogenates of two HEV-positive livers incubated at 56°C for 1 hr, stir-fried at 191°C for 5 min or boiled in water for 5 min, respectively. As expected, the group 2 positive control pigs all became infected whereas the group 1 negative control pigs remained negative. Four of the five pigs inoculated with HEV-positive liver homogenates incubated at 56°C for 1 hr also became infected. However, pigs in groups 4 and 5 did not become infected. The results indicated that HEV in contaminated commercial pig livers can be effectively inactivated if cooked properly, although incubation at 56°C for 1 hr cannot inactivate the virus. Thus, to reduce the risk of food-borne HEV transmission, pig livers must be thoroughly cooked.

Keywords

Hepatitis E virus; HEV; commercial pig liver; inactivation; Food safety; Swine

INTRODUCTION

Hepatitis E, an enterically transmitted form of viral hepatitis, is a disease of major public health concern in many developing countries of Asia and Africa (Emerson and Purcell, 2003; Meng, 2003) where the sanitation conditions are usually substandard. However, sporadic cases of acute hepatitis E were also reported in individuals from the United States, Japan, and other industrialized countries (Hsieh et al., 1999; van der Poel et al., 2001; Clemente-Casares et al.,

*Corresponding Author: X.J. Meng, M.D., Ph.D., Professor of Molecular Virology, Center for Molecular Medicine and Infectious Diseases, College of Veterinary Medicine, Virginia Polytechnic Institute and State University, 1410 Price's Fork Road, Blacksburg, VA 24061-0342, Phone: 540-231-6912, Fax: 540-231-3426, E-mail: xjmeng@vt.edu.

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2003; Yazaki et al., 2003; Mizuo et al., 2005; Takahashi et al., 2003). The mortality rate in individuals infected with hepatitis E virus (HEV) is generally low (< 1%), but it can be as high as 25–30% in infected pregnant women (Hussaini et al., 1997). There are at least four major genotypes of HEV worldwide: genotype 1 (epidemic strains from Asia and Africa), genotype 2 (a single epidemic strain from Mexico), genotype 3 (human and swine strains from industrialized countries), and genotype 4 (human and swine strains from sporadic cases in Asia) (Tam et al., 1991; Huang et al., 1992; Schlauder et al., 1998; Nishizawa et al., 2003; Huang et al., 2007).

Human hepatitis E is not frequently reported in industrialized countries despite the relatively high level of HEV antibody prevalence in healthy individuals from these countries (Mast et al., 1997; Thomas et al., 1997; Meng et al., 2002). It has been hypothesized that hepatitis E is a zoonotic disease and that there are animal reservoir(s) for HEV (Meng et al., 1998; Meng, 2000). The discovery of animal strains of HEV, swine HEV from pigs (Meng et al., 1997) and avian HEV from chickens with hepatitis-splenomegaly syndrome (Haqshenas et al., 2001; Huang et al., 2004), as well as their demonstrated ability to infect across species (Meng et al., 1998; Halbur et al., 2001; Sun et al., 2004) provide strong support for this hypothesis.

Zoonotic foodborne transmissions of HEV from undercooked pig liver and deer meat to humans have been reported in Japan (Tei et al., 2003; Yazaki et al., 2003; Tamada et al., 2004). It has been shown that commercial pig livers purchased from local grocery stores as food in Japan and the United States (Yazaki et al., 2003; Feagins et al., 2007) are contaminated by HEV and that some of the HEV-contaminated commercial pig livers still contain infectious virus (Feagins et al., 2007). The risk of HEV infection via the consumption of HEV-contaminated pig livers raises further public health concern since it is not clear whether cooking conditions will be effective in inactivating the virus present in the contaminated pig livers. The objective of this study was to determine if traditional cooking methods are effective in inactivating infectious HEV present in contaminated commercial pig livers.

MATERIALS AND METHODS

Source of commercial pig livers

Two packages of commercial pig livers (FL58 and FL91), previously found to contain genotype 3 infectious HEV (Feagins et al., 2007), and one package of commercial pig liver (FL1) negative for HEV (Feagins et al., 2007), were purchased from a local grocery store in Blacksburg, VA, and were used in this study. The livers were stored in a –80°C freezer until use.

Inactivation of HEV present in contaminated commercial pig livers by different cooking methods and preparation of inocula

A portion of pig livers FL58 and FL91 were separately homogenized in 10% (w/v) sterile phosphate-buffered saline (PBS). Liver homogenates were clarified by centrifugation at 3,000 rpm for 15 minutes at 4°C. Half of FL58 homogenate was pooled with half of FL91 homogenate, untreated and utilized as the positive control inoculum. The remaining half of FL58 and FL91 homogenates was pooled into a 50 ml conical tube and treated with a heat inactivation step by incubating in a 56°C water bath for 1 hour (the pooled homogenate in the tube was mixed every 10 minutes). A portion of HEV RNA-negative FL1 liver was homogenized in 10% PBS (w/v) and utilized as the negative control inoculum.

Consumers of pig livers typically cook them by either stir-frying or boiling. Therefore, equal amounts of FL58 and FL91 HEV-contaminated commercial pig livers were cut into 0.5 to 1.0 cm² cubes, pooled (a total of 24 g), and stir-fried at 191°C for 5 min in 15 ml of pure canola

cooking oil. The stir-fried pig livers were then homogenized in 10% (w/v) PBS and used as an inoculum. Similarly, equal amount of FL58 and FL91 HEV-contaminated commercial pig livers were cut into 0.5 to 1.0 cm² cubes, pooled (a total of 24 g), and boiled for 5 minutes in 1,420 ml of water. The boiled pig livers were then homogenized in 10% (w/v) PBS and used as an inoculum. A Rival 12" skillet with adjustable temperature control was utilized to stir-fry as well as boil the pig livers. A Taylor TruTemp instant read compact digital thermometer was used to ensure the internal temperature of the stir-fried and boiled pig livers reached at least 71°C.

Swine bioassay to assess the effect of different cooking and inactivation methods on virus infectivity

To determine the effects of different cooking and inactivation methods on virus infectivity, a swine bioassay (Feagins et al., 2007) was conducted since a reliable cell culture for HEV propagation is not available. The animal study was reviewed and approved by our Institutional Animal Care and Use Committee (IACUC). Briefly, twenty-five, four-week-old, specific-pathogen-free (SPF) pigs were purchased from a commercial source. Prior to inoculation, all pigs were confirmed to be negative for HEV antibodies by an enzyme-linked immunosorbent assay (ELISA) (Meng et al., 1998; Halbur et al., 2001). The pigs were divided into 5 groups of 5 pigs each. The 5 pigs in group 1 were each inoculated intravenously (I.V.) with 2 ml of liver homogenate from a PCR-negative commercial pig liver, FL1, as negative controls. The 5 pigs in group 2 were each inoculated I.V. with 2 ml of a pooled homogenate of two HEV-contaminated livers (FL58 and FL91) as positive controls. Pigs in groups 3, 4, and 5 were each inoculated I.V. with 2 ml of a pool of two HEV-contaminated liver homogenates (FL58 and FL91) incubated at 56°C for 1 hr, a pooled homogenate of two HEV-positive livers (FL58 and FL91) stir-fried at 191°C for 5 min or boiled in water for 5 min, respectively. The animals were monitored for a total of 8 weeks for evidence of HEV infection as previously described (Feagins et al., 2007).

Serum samples from each pig were collected prior to inoculation and weekly thereafter for the detection of HEV viremia by reverse transcription-polymerase chain reaction (RT-PCR) and for IgG anti-HEV by ELISA as described previously (Meng et al., 1997, 1998; Halbur et al., 2001). Fecal samples from each pig were collected prior to inoculation and weekly after inoculation for the detection of HEV RNA by RT-PCR.

RT-PCR to detect HEV RNA

To detect HEV RNA in fecal and serum samples of inoculated pigs, a universal RT-PCR assay was performed as previously described (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 10% fecal suspension or serum. 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 min with 1 µl of the reverse primer 3157N (5'- CCCTTA(G)TCC(T)TGCTGA(C)GCATTCTC-3'), 1 µl of Superscript II reverse transcriptase (Invitrogen), 1 µl of 0.1 M dithiothreitol, 4 µl of 5 × RT buffer, 0.5 µl of RNase inhibitor (Promega), and 1 µl of 10mM deoxynucleoside triphosphates. Ten microliters of the resulting cDNA was amplified in a 50 µl PCR reaction with *AmpliTaq* Gold DNA polymerase (Applied Biosystems).

The nested 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 min, followed by 39 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 42°C, extension for 1 min at 72°C, and a final extension at 72°C for 7 min. The expected final product of the nested RT-PCR was 348 bp.

Samples found to be positive using the universal RT-PCR assay were further confirmed by a RT-PCR assay specific for the FL58 and FL91 HEV strains. The degenerate primers for the specific RT-PCR assay were based upon the published sequences of FL58 and FL91 strains (Feagins et al., 2007): external primer set CL P1 (forward, 5'-CTGGA(T)GCATTGGGGCTT(C)CTTGA-3') and CL P2 (reverse, 5'-A(T)AGAGCA(T)ATACCT(G)CGG(A)CCCACC-3') (first round) and internal primer set CL P3 (forward, 5'-GACACCCGGGAACACTAACACC-3') and CL P4 (reverse, 5'-TGGTAAGCTCA(T)GCGGTCCCATC-3') (second round). Reverse transcription was performed at 57.5°C for 60 minutes with 1 µl of the reverse primer CL P2 (5'-A(T)AGAGCA(T)ATACCT(G)CGG(A)CCCACC-3'). PCR amplification was performed essentially as described above except that the annealing was 1 min at 57.5°C.

DNA sequencing and sequence analyses

The amplified PCR products from each positive sample were separated in a 0.8% agarose gel. The expected band was excised from the gel and purified by the glassmilk procedure with a GENECLAN kit (Bio 101 Inc., Carlsbad, CA). The purified PCR products were sequenced for both strands at the Virginia Bioinformatics Institute (Blacksburg, VA). Sequence analyses were conducted using the MacVector computer program (Oxford Molecular Inc.).

RESULTS AND DISCUSSIONS

As expected, all pigs in the group 1 inoculated with a HEV-negative liver homogenate remained seronegative throughout the study, and there was no detectable viremia, fecal virus shedding, or seroconversion in group 1 pigs (Fig. 1, Table 1). All pigs in the group 2 inoculated with a pooled homogenate of two HEV-positive commercial pig livers became infected by HEV, as evidenced by seroconversion to IgG anti-HEV as early as 3 weeks postinoculation (wpi) (Fig. 1), viremia, and fecal virus shedding in 4 of 5 group 2 pigs (Table 1). The infected pigs remain clinically normal, which is consistent with HEV infection in pigs. In general fecal virus shedding appears prior to viremia, generally at 1 to 2 weeks post inoculation followed by a transient or no viremia. The disappearance of viremia or fecal virus shedding is generally followed by seroconversion to HEV antibodies (Meng et al., 1998). The course of infection, viremia and fecal virus shedding pattern is consistent with acute HEV infection in pigs (Meng et al., 1998).

Interestingly, four of the five pigs in group 3 inoculated with a pool of two HEV-positive liver homogenates incubated at 56°C for 1 hr developed an active HEV infection. Seroconversion started at 2 wpi, by 4 wpi all but one pig seroconverted to IgG anti-HEV and remained seropositive at the end of the 8-week study (Fig. 1). Viremia and fecal virus shedding were detected variably in 4 of the 5 group 3 pigs (Table 1).

The pigs in group 4 inoculated with a pooled homogenate of two HEV-positive livers stir-fried at 191°C for 5 min had no evidence of infection as there was no seroconversion, viremia, or fecal virus shedding in any of the inoculated pigs. Similarly, the pigs in group 5 inoculated with a pooled homogenate of two HEV-positive livers boiled in water for 5 min also showed no evidence of HEV infection (Fig. 1, Table 1). All pigs from each group were necropsied at 8 wpi. Samples tested positive for HEV RNA by the specific RT-PCR assay were sequenced

for the ORF2 capsid gene region. Sequence analyses confirmed that the viruses recovered from the experimentally infected pigs originated from the respective inoculum (data not shown).

HEV transmission in industrialized countries is not well understood. It remains to be determined why only sporadic cases of hepatitis E occur in industrialized countries even though a high level of anti-HEV antibody prevalence has been detected in the population (Mast et al., 1997; Thomas et al., 1997; Meng et al., 2002). Because HEV antibodies have been detected in a number of animal species including chickens, swine, cattle, sheep, and goats (Meng, 2000), it has been suggested that HEV transmission is zoonotic. Recently, Yazaki et al. (2003) reported direct evidence of zoonotic HEV transmission via the consumption of grilled or undercooked commercial pig liver purchased from local grocery stores in Japan. The sequences of seven swine HEV isolates recovered from commercial pig livers from local grocery stores were closely related, or identical in a few cases, to the viruses recovered from the Japanese human hepatitis E patients. The majority of the patients in that study had a history of consuming undercooked pig livers prior to the onset of the disease, indicating that consumption of pig livers is a risk factor for hepatitis E.

In a previous study, we found that approximately 11% of pig livers purchased from local grocery stores in the United States are contaminated by HEV (Feagins et al., 2007). Most importantly, we demonstrated that two of the three contaminated pig livers still contain infectious virus that is transmissible to pigs, indicating that individuals who consume inadequately cooked pig livers may be at an increased risk of acquiring zoonotic HEV infection. Yazaki et al. (2003) suggested that the degree of cooking may affect the risk of acquiring an infection. Unfortunately, no such study has been done to assess whether the current pork safety cooking regulations are sufficient in inactivating HEV present in commercial pig livers. Only limited information is available regarding HEV resistance to inactivation by physical or chemical means. As a fecal-orally transmitted virus, HEV is likely resistant to inactivation by the acidic conditions of the stomach. The ability of HEV to survive harsh or extreme environments can be attributed to its non-enveloped viral structure. Emerson et al. (2005) recently demonstrated that HEV is more heat labile in comparison to hepatitis A virus, also an enterically transmitted hepatitis virus.

The United States Department of Agriculture (USDA) recommends a cooking method for fresh pork that will result in a minimum internal cooking temperature of 71°C (http://www.fsis.usda.gov/is_it_done_yet/, accessed on March 15, 2007). The United States National Pork Board (NPB) also recommends cooking pork to a minimum internal temperature of 71°C on an instant-read meat thermometer (<http://www.pork.org/NewsAndInformation/QuickFacts/default.html>, accessed on March 15, 2007). While internal cooking temperatures are used by industry for consistency when different cooking methods are used, many of the recipes do not specify a minimum cooking temperature. Instead, a time stipulation is suggested based on the level of heat. Stir-frying and boiling are the two most widely used and accepted methods for cooking pig livers by consumers. We therefore evaluated if stir-frying and boiling of HEV-contaminated pig livers can effectively inactivate the virus by using a swine bioassay to determine the virus infectivity. Pig livers were stir-fried at 191°C since, under the condition we used in the study, this was the cooking temperature that produced an internal cooking temperature closer to the recommended 71°C without burning the tissue. We demonstrated that pigs inoculated with the homogenates of HEV-positive livers that were stir-fried at 191°C for 5 min or boiled for 5 min (with a minimal internal temperature of 71°C) did not develop HEV infection. Therefore, these two cooking methods were effective in inactivating infectious HEV in contaminated pig livers purchased from local grocery stores. However, it is important to note that the time required to inactivate HEV in pig liver pieces larger than the 0.5 to 1.0 cm² cubes used in this study may need to be extended.

By using an *in vitro* system, Emerson et al. (2005) reported that HEV is approximately 50% inactivated when heated at 56°C for 1 hr, therefore a third experimental group was added to determine whether the heating of HEV-contaminated liver homogenate at 56°C for 1 hr would be sufficient to inactivate the virus by using the swine bioassay. We demonstrated that incubation of homogenates of the contaminated pig livers in a 56°C water bath for 1 hr did not inactivate the virus, as four of the five inoculated pigs developed an active HEV infection, thus supporting the *in vitro* results of Emerson et al (2005).

This is the first report demonstrating that adequate cooking of HEV-contaminated commercial pig livers will inactivate HEV in the tissue. This is also the first report that partially inactivated HEV (heat at 56°C for 1 hr) is still able to initiate an active infection *in vivo*. The results from this study indicate that commercial pig livers purchased from grocery stores for food consumptions should be thoroughly cooked to ensure proper inactivation of infectious HEV present in the livers, and to decrease the risk of potential food-borne HEV transmission.

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FIG. 1.

Seroconversion to IgG anti-HEV in inoculated and control pigs. **Panel A**, pigs inoculated with a liver homogenate negative for HEV as negative controls (group 1); **Panel B**, pigs inoculated with a pool of two HEV-positive pig liver homogenate as positive controls (group 2); **Panels C, D and E**, each with a pool of two HEV-positive liver homogenates incubated at 56°C for 1 hr, stir-fried at 191°C for 5 min or boiled in water for 5 min (groups 3, 4 and 5, respectively). The ELISA cutoff OD value is 0.30.

Table 1
Detection of HEV RNA in samples (fecal/serum) collected weekly from pigs inoculated with commercial pig liver homogenates positive or negative for HEV

Group	Pig ID	positive (+) or negative (-) in fecal / serum samples at indicated wpi							
		0	1	2	3	4	5	6	7 8
1 ^a	1905	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	1910	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	1920	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	1923	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	1943	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
2 ^b	1909	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	1912	-/-	+/+	+/+	-/-	-/-	-/-	-/-	-/-
	1924	-/-	+/+	+/+	-/-	-/-	-/-	-/-	-/-
	1931	-/-	+/+	-/+	-/-	-/-	-/-	-/-	-/-
	1944	-/-	+/+	-/-	-/-	-/-	+/+	-/-	-/-
3 ^c	1908	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	1914	-/-	+/+	+/+	-/-	-/-	-/-	-/-	-/-
	1929	-/-	+/+	-/+	-/-	-/-	-/-	-/-	-/-
	1946	-/-	+/+	+/+	-/-	-/-	-/-	-/-	-/-
	1948	-/-	+/+	-/-	-/-	-/-	-/-	-/-	-/-
4 ^d	1919	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	1921	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	1927	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	1928	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	1945	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
5 ^e	1911	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	1917	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	1922	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	1930	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	1951	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-

^a inoculated with a liver homogenate FL1 negative for HEV (negative controls)
^b inoculated with a pool of two HEV-positive pig liver homogenates (positive controls)
^c inoculated with a pool of two HEV-positive liver homogenates heat-treated by incubating at 56°C for 1 hr
^d inoculated with a pool of homogenates of two HEV-positive livers stir-fried at 191°C for 5 min
^e inoculated with a pool of homogenates of two HEV-positive livers boiled in water for 5 min