RESEARCH REPORT
SWINE HEALTH

Title: **Attempt to transmit swine hepatitis E virus (HEV) by consumption of fresh and frozen pork loin or liver – NPB 04-087**

Investigator: Patrick G. Halbur DVM, MS, PhD

Institution: Iowa State University

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II. Abstract

Hepatitis E virus (HEV) was discovered in pigs in the U.S. in 1997 and swine HEV is now known to be widespread in the global swine population. Direct evidence has recently emerged that swine HEV is a food-borne zoonotic disease. The primary objectives of this study were to determine if swine HEV can be transmitted by oral consumption of uncooked pork contaminated with HEV-positive pig feces or consumption of raw livers from pigs infected with U.S. strains of HEV. Furthermore, we wanted to determine if conventional cooking or storage of HEV-contaminated pork loin or livers from HEV-infected pigs decreases the infectivity and risk of transmission of the virus.

The results demonstrated that HEV could be transmitted by oral consumption of pork meat contaminated with feces from HEV-infected pigs; however, based on the results of this study it appears that cooking does not destroy the infectivity of HEV in pork meat samples spiked with feces containing HEV. This was an unexpected finding. Similarly, we demonstrated that HEV could be transmitted through consumption of fresh liver from HEV-infected pigs and that cooking did not destroy the infectivity. We also demonstrated that HEV remains infectious in refrigerated or frozen meat samples.

These unexpected results could have substantial implications for food safety and pork trade if they can be extrapolated to human consumption of pork. Because of this, we are in the process of repeating parts of the experiment and conducting additional testing to further confirm these findings.

III. Introduction

Swine HEV is ubiquitous in the swine population (Bradley, 1992; Meng et al., 1997). Interspecies transmission of HEV has been demonstrated experimentally by infection of non-human primates with swine HEV and pigs with the US-2 strain of human HEV (Meng et al., 1998; Halbur et al., 2001). Pigs, whether infected with the U.S. human HEV or U.S. swine HEV, shed virus in feces for several weeks.

Pigs are now considered a potential reservoir and source of human HEV infection and it is now evident that HEV infection can be a food-borne zoonotic disease. Hepatitis E virus found in raw pig livers sold in grocery stores in Japan recently caused a clinical HEV infection outbreak in human patients who consumed the livers grilled or undercooked (Yazaki et al., 2003). Furthermore, another case of HEV-induced disease in two human patients was linked to the consumption of uncooked liver from a wild boar (Matsuda et al., 2003). It remains

unclear whether the HEV in these zoonotic cases came from consumption of tissues from HEV-infected pigs or tissues contaminated with feces from HEV-infected pigs.

There is also concern that individuals with occupational exposure to environmental sources of domestic animal waste may be at increased risk of HEV infection. Some researchers have proposed that pig manure and raw sewage from pig slaughterhouses could be a source of HEV contamination of environmental water.

III. Project Objectives

1. The first objective was to determine in the swine model if oral consumption of uncooked pork contaminated with HEV-positive pig feces is a possible means for transmission of swine HEV to humans.
2. The second objective was to determine in the swine model if consumption of raw livers is a possible means for transmission of HEV to humans.
3. The third objective was to determine if conventional cooking or storage of HEV-contaminated pork loin or livers from HEV-infected pigs decreases the infectivity and risk of transmission of the virus.

IV. Materials and Methods

Animals and Experimental design

Forty-two, two-week-old, segregated-early-weaned, crossbred pigs were purchased and brought to the research facility at Iowa State University. At the time of delivery, the pigs were randomly assigned to one of 14 groups with 3 pigs in each group. The experimental design is summarized in Table 1. The pigs were fed either fresh or frozen loin meat spiked with HEV-positive feces, or fresh or frozen liver from pigs in the early stages of HEV infection. Some of the groups received meat or liver that was cooked and others received raw meat or liver tissue taken directly from the refrigerator or freezer. All groups were housed in separate rooms. All pigs were confirmed to be free of swine HEV by rt-PCR on feces and by ELISA on serum prior to inoculation.

Preparation of inocula

Liver, loin muscle and feces were collected from 3 pigs experimentally-inoculated with swine HEV and necropsied at 7 days post infection (DPI). Pooled liver, pooled loin muscle and pooled fecal suspension were tested for HEV RNA by RT-PCR and stored at -80°C. Similar tissues were collected from pigs known to be HEV-negative.

Spiking of the loin muscles

The loin muscles from HEV-free pigs were injected (22 G hypodermic needle and 3 ml syringe) with 1 ml of swine HEV fecal suspensions containing $10^{4.5}$ PID^{50}.

Cooling

Livers or spiked loin muscles were stored at 4°C for a single day before inoculation into naïve pigs.

Freezing

Livers or spiked loin muscles were stored at -9°C for 10 days before inoculation into naïve pigs.

Cooking

An electric skillet was preheated at 375°F for 5 minutes. Liver pieces (1.7 cm thick; 30 grams) or loin muscle pieces (2.5 cm thick; 30 grams) were put into the lid-closed skillet and flipped every 5 minutes until the internal temperature of the livers and loin muscle pieces reached 160°F which took approximately 10 minutes for the liver pieces and 15 minutes for the loin.

Liver homogenate

Positive controls were inoculated intravenously with liver homogenate. Two grams of liver (from experimentally-infected pigs in the early stages of HEV infection) and 20 ml of PBS were placed in a stomacher

standard bag for 1 minute. The supernatant was clarified at 3,000 rpm for 10 minutes in a refrigerated centrifuge, filtered through a 5 nm filter and aliquoted into 1 ml tubes.

Inoculation

Each pig in groups 2-5 was fed 100 grams of HEV spiked loin, each pig in groups 6-10 received 100 grams of HEV positive liver, and each pig in group 11-14 received 10 grams pooled HEV positive feces in 100 ml PBS of fecal suspension on three consecutive days.

Each pig in group 1 received 100 grams of loin and liver free of HEV (determined by rt-PCR) on three consecutive days. This group served as the negative control group.

Each pig in groups 10, 13 and 14 received 5 ml of the HEV positive fecal suspension (groups 13 and 14) or the liver homogenate (group 10) intravenously for 3 consecutive days.

Nested HEV RT-PCR

The modified spin column method (QIAamp; Qiagen, Chatsworth, CA.) was used to extract total RNA from 140 µl of fecal suspensions. The RNA extract (11.5 µl) was then immediately used for the reverse transcription reaction for HEV cDNA synthesis.

The first round PCR primers comprised the forward primer F1 (5′-AGCTCCTGTACCTGATGTTGACTC-3′) and the reverse primer R1 (5′-CTACAGAGCGCCAGCCTTGATTGC-3′). The second-round PCR primers comprised the forward primer F2 (5′-GCTCACGTCATCTGTCGCTGCTGG-3′) and the reverse primer R2 (5′-GGGCTGAACCAAAATCCTGACATC-3′) (13). To synthesize HEV cDNA, portions (11.5 µl) of RNA extract was amplified in a reverse transcription reaction with R1 reverse primer and Superscript II reverse transcriptase (Invitrogen) at 42^{o} C for 1 h. The cDNA (10 µl) was used as template in a 100-µl PCR reaction. The first-round PCR reaction was initiated with the activation of ampli*Taq* Gold DNA polymerase (Applied Biosystems) at 95^{o} C for 9 min, followed by repeated 39 cycles of denaturation, annealing, and extension as follow; 94^{o} C for 1 min, 52^{o} C for 1 min, and 72^{o} C for 1.5 min, respectively, and finally, an incubation at 72^{o} C for 7 min. Similar PCR parameters were applied to the second round PCR reaction. The second-round amplified PCR product of 266 base pairs (bp) in length was visualized after 2% agarose gel electrophoresis.

Semi-quantitative nested RT-PCR for titration of HEV

To determine the virus titer in samples tested positive for HEV RNA, serial 10-fold dilutions in DEPC water-based PBS, ranging from 10^{-1} to 10^{-6} dilution were made and subjected to the universal RT-PCR. The virus titer was reported as genome equivalent (GE) at the highest dilution found positive by the RT-PCR.

Swine HEV ELISA

Blood was collected prior to inoculation and at 7, 14, 21, 28. 35, 42 and 49 days post inoculation. Serum samples were tested by an enzyme-linked immunosorbent assay (ELISA) using a purified 55-kDa truncated recombinant putative capsid protein of human HEV Sar-55 strain as the antigen. Mean optical density values above 0.350 were regarded as positive samples. Preimmune and hyperimmune sera were used as negative and positive controls, respectively.

Necropsy

All pigs were necropsied at 56 days post inoculation.

V. Results

Table 1. Serology results from negative control pigs and pigs inoculated with tissues from HEV-infected pigs or tissues spiked with HEV.

Grp	Pig #	Tissue	HEV Status	Spiked with HEV+ feces	Storage	Cooking	Route of inoc.	Serology Results
1	3	Loin and liver	-	-	Refrigerate	-	Orally	0/3*
2	3	Loin	-	+	Refrigerate	Cooked	Orally	0/3
3	3	Loin	-	+	Freezing	Cooked	Orally	1/3
4	3	Loin	-	+	Refrigerate	-	Orally	3/3
5	3	Loin	-	+	Freezing	-	Orally	3/3
6	3	Liver	+	-	Refrigerate	Cooked	Orally	3/3
7	3	Liver	+	-	Freezing	Cooked	Orally	3/3
8	3	Liver	+	-	Refrigerate	-	Orally	3/3
9	3	Liver	+	-	Freezing	-	Orally	3/3
10	3	Liver homog.	+	-	-	-	IV	3/3
11	3	Fecal susp.	+	-	Cooling	-	Orally	3/3
12	3	Fecal susp.	+	-	Refrigerate	-	Orally	3/3
13	3	Fecal susp.	+	-	Cooling	-	IV	3/3
14	3	Fecal susp.	+	-	Refrigerate	-	IV	3/3

*number positive by serology/number in the group at 35 days post inoculation.

PCR for detection of HEV nucleic acids was conducted on selected samples. The pigs in group #4 were negative by PCR for HEV nucleic acids on 7, 14, and 21 days post inoculation. The pigs in group #8 were positive by PCR for HEV nucleic acids in feces at 7, 14, and 21 days post inoculation.

VI. Discussion

Our hypothesis was that HEV could be transmitted by oral consumption of pork meat contaminated with feces from HEV-infected pigs and in raw liver from HEV-infected pigs but proper cooking of that meat would inactivate the HEV and eliminate the risk of transmission. The results confirmed that HEV could be transmitted by oral consumption of pork meat contaminated with feces from HEV-infected pigs; however, based on the results of this study it appears that cooking does not destroy the infectivity of HEV in pork meat samples spiked with feces containing HEV. This is an unexpected finding. Similarly, we demonstrated that HEV could be transmitted through consumption of fresh liver from HEV-infected pigs and that cooking does not destroy the infectivity. We also demonstrated that HEV remains infectious in refrigerated or frozen meat samples.

Because of these unexpected results with potential food safety and pork trade implications; we are in the process of conducting additional testing to further confirm these findings. In particular, we are repeating some of the serology and doing additional PCR's at different days post inoculation and sequencing HEV isolates from pigs where the results indicate that transmission occurred after consumption of cooked pork. This will further confirm that the isolates the pigs became infected with were from the HEV-spiked pork meat or liver tissue and were not environmental contaminants.

VIII. Lay Interpretation

Hepatitis E virus (HEV) infects pigs but does not cause clinical disease in pigs. There is concern that hepatitis E virus from pigs may infect people and that pigs may be an important reservoir for HEV. Results from this experiment indicate that HEV can be transmitted by oral consumption of fresh or cooked liver from HEV-infected pigs or by consumption of fresh or frozen pork meat contaminated with feces from pigs shedding HEV. Surprisingly, cooking to an internal temperature of 160°F did not appear to inactivate the HEV. Because of these unexpected results with potential food safety and pork trade implications; we are in the process of conducting additional testing to further confirm these findings.

This research project was lead by Dr. Pat Halbur (**pghalbur@iastate.edu**) at the Iowa State University Veterinary Diagnostic Laboratory, 1600 S. 16th Street, Ames, IA 50011, Ph:515-294-1950, Fax:515-294-6961.

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