Title: Prevalence, genotyping and subtyping of hepatitis E virus in market weight pigs in the United States (NPB #18-021)

Investigator: X.J. Meng, M.D., Ph.D.

Institution: Virginia-Maryland College of Veterinary Medicine, Blacksburg, VA

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Industry Summary:
Pigs are animal reservoirs for hepatitis E virus (HEV), an important human pathogen. Although HEV does not cause overt clinical disease in infected pigs, the virus can transmit to humans through consumption of undercooked pork or direct contact with infected pigs and cause acute and chronic hepatitis E in humans. According to the World Health Organization, each year an estimated 20 million HEV infections occur in humans worldwide leading to an approximately 3.3 million symptomatic cases of hepatitis E, and 44,000 hepatitis E-related deaths. We previously demonstrated that commercial pork products such as liver (up to 11%) and chitterlings in grocery stores in the United States are contaminated by HEV and that medium-to-rare cooking conditions cannot inactivate the virus. This raises concerns over HEV-related pork safety. In fact, countless cluster and sporadic cases of hepatitis E have been definitively linked to the consumption of undercooked or raw pork products worldwide. To determine the prevalence and genotype of HEV in market weight pigs from slaughterhouses in the United States, in this project we tested a total of 5033 serum samples collected from market weight pigs from the 25 U.S. slaughterhouses in 10 different States for evidence of HEV infection. HEV RNA was tested by HEV-specific real-time quantitative PCR (RT-qPCR), and IgG anti-HEV antibody was determined by a commercial ELISA kit (Mikogen Inc). RT-qPCR positive samples were further tested by a nested RT-PCR assay, and the PCR products from positive samples were sequenced. The results show that approximately 6.32% (318/5033) of the serum samples from slaughterhouse pigs in the United States are positive for HEV RNA, and that approximately 39.88% of the samples are positive for IgG anti-HEV. Sequence and phylogenetic analyses of the HEV isolates revealed that the prevalent HEV genotype in slaughterhouse pigs in the United States is the zoonotic genotype 3 HEV, subgenotype Gt3 group 2 (i.e Gt3abchij). The finding from the project indicates a small proportion of the market weight pigs from slaughterhouses, the entry point to food chain, in the United States are still infected by HEV and viremic at the time of slaughter, and therefore the HEV-
contaminating blood during slaughter may subsequently contaminate pork products thus raising potential pork safety concerns since genotype 3 HEV is known to infect humans. The finding from this project will help swine producers, veterinarians and public health officials to devise more effective preventive strategies in the future to reduce the HEV-related pork safety concerns in the United States.

**Keywords (at least 5 words):**
Hepatitis E virus (HEV); genotype; subgenotype; IgG anti-HEV; pork safety; market weight pigs; slaughterhouse

**Scientific Abstract:**
Foodborne hepatitis E virus (HEV) infection is an emerging public health concern in industrialized countries including the United States. Swine is a major reservoir for genotypes 3 and 4 HEV which are known to infect humans. Since market weight pigs from slaughterhouses are the entry point into the food chain, knowledge of the prevalence and genotype of HEV in slaughtered pigs is of paramount importance since viremic pigs can potentially contaminate pork supply chains through HEV-containing blood at the time of slaughter. In this study, we tested serum samples collected from 5,033 market weight pigs from 25 slaughterhouses in different States for the prevalence of IgG anti-HEV and HEV RNA, using a commercial ELISA kit and RT-qPCR, respectively. RT-qPCR positive samples with high viral RNA copy number were further amplified for the viral capsid gene region by a nested RT-PCR assay, and the resulting PCR products are sequenced. The results show that approximately 6.32% (318/5033) of the sera are positive for HEV RNA, and approximately 39.88% of the sera are positive for IgG anti-HEV. Sequence and phylogenetic analyses reveal that the prevalent HEV genotype in slaughterhouse pigs in the United States is the zoonotic genotype 3, belonging to subgenotype Gt3 group 2 (i.e Gt3abchij). The finding from this comprehensive study indicates that a small proportion of the market weight pigs from slaughterhouses in the United States are still viremic at the time of slaughter and thus may contaminate pork supply chains causing potential pork safety concern.

**Introduction:**
Hepatitis E virus (HEV) causes an estimate of 20 million human infection worldwide annually leading to approximately 3 million cases of hepatitis E and more than 44,000 HEV-related deaths (1). In addition to humans, HEV has also been genetically identified from more than a dozen other animal species including pigs (2). The genome of HEV is a single-stranded positive-sense RNA virus (~ 7.2 kb size) belonging to the family Hepeviridae (3, 4) comprising two distinct genera (Orthohepevirus and Piscihepevirus). The genus Orthohepevirus consists of four species: Orthohepevirus A, B, C, and D. The species Orthohepevirus A, which contains viruses that infect humans, is further classified into at least eight distinct genotypes (5). Genotype 1 and 2 HEVs infect only humans. Genotypes 3, 4, 5 and 6 HEVs are known to infect pigs, and both genotypes 3 and 4 HEV also infect humans (2, 6). Genotypes 7 and 8 infect camels.

Foodborne hepatitis E due to zoonotic genotypes 3 and 4 HEV infection through the consumption of HEV-contaminated animal meats is increasingly becoming a public health concern in many industrialized countries especially in European countries and the United States (6, 7). Since 2000s, there has been a steady rise in reporting non-travel associated autochthonous zoonotic genotypes 3 and 4 HEV human cases in Europe (7-9). The zoonotic genotypes 3 and 4 HEVs can establish
chronic infection in humans especially in immunocompromised patients, and are also associated with numerous neurological sequela. The most common risk factor of foodborne genotypes 3 and 4 HEV infection is the consumption of raw or undercooked animal meat products (6, 8).

We previously demonstrated that genotype 3 HEV is prevalent in swine herds in the United States (10), and that swine veterinarians in the United States had a higher risk of HEV infection (11). We also previously showed that a small proportion of commercial pork products such as liver and chitterlings from grocery stores in the United States is contaminated with infectious HEV (12, 13). However, currently the HEV prevalence and infection status in market weight pigs at the time of slaughter, the entry point to food chain, remain unknown in the United States. Knowledge of the HEV prevalence, infectious status and genotype in pigs at the time of slaughter is of paramount importance, since viremic pigs can contaminate pork supply chains through HEV-containing blood at the time of slaughter and since genotypes 3 and 4 HEVs in pigs are known to infect humans.

Objectives:
The objective of the project is to determine the prevalence, genotype, and subgenotype of HEV in market weight pigs at the time of slaughter in the United States.

Materials & Methods:
Serum samples: Archived serum samples were obtained from a total of 22,940 market weight pigs from 25 slaughterhouses in 10 different States. This comprehensive set of serum samples was originally collected for an unrelated NPB-sponsored prevalence study for Toxoplasma and Trichinella in market swine. The serum samples were collected mainly from two types of slaughterhouses: market weight hogs of approximately 250 lb and 6 months old, and “sow only” plants of female pigs over 1 year old. The samples were collected on the kill floor at the slaughterhouses and shipped to the USDA-ARS Beltsville lab. Approximately 200 to 2000 samples were collected from each slaughterhouse plant depending upon daily volume.

Random selection of samples for the study: Stratified random selection of the 5,033 samples for this present study from a total of 22,940 available samples was performed by a biostatistician. Briefly, identification codes for the 22,940 available samples were entered into an excel worksheet along with state and zip code of origin. The combination of state and zip code of origin constituted the strata. Within each stratum, a number of samples that is proportional to the total number of samples were randomly selected (using simple random sampling) for processing. The number of samples selected from each stratum was constrained to sum to 5,033 as required by the study design. The sampling was performed using the SURVEYSELECT procedure of SAS version 9.4 (Cary, NC, USA).

RNA extraction and HEV real-time quantitative reverse transcriptase polymerase chain reaction (RT-qPCR): Total RNAs from 200µl of swine serum sample were extracted using Tri-reagent (Molecular Research Center, OH) as per manufacture’s protocol. The isolated RNA was resuspended in DNase/RNase-free water, and tested by HEV RT-qPCR for the presence and quantification of HEV RNA as per established protocol (14, 15). Briefly, the HEV RT-qPCR was carried out using SensiFast Probe One-step kit (Bioline, TN) in a 20µl volume comprising of 4µl extracted RNA and 16µl of master mix as per manufacture’s protocol. The concentration of primers and probe (Table 1) used per reaction were 800 nM and 200 nM, respectively. The One-
Step RT-PCR was performed under the following conditions: 45°C for 10 min, 95°C for 2 min, followed by 40 cycles of 95°C for 10 sec, and 60°C for 20 sec. A standard curve of HEV RNA ranging from $10^1$ to $10^8$ copy numbers was included during each run for verification of the assay sensitivity and efficiency.

Nested RT-PCR assay: The RT-qPCR positive serum samples were subsequently re-tested using a conventional nested RT-PCR assay that is known to broadly detect all 4 known major genotypes of HEV (10) (Table 1). Briefly, cDNA was synthesized using a Superscript-II Reverse Transcription Kit (Invitrogen-ThermoFisher, MA) as per manufacture’s protocol. The first round PCR was performed with primers 3156N and 3157N (Table 1, 600 nM concentration each) using the cDNA as template and the AmpliTaq Master Mix (ABI-ThermoFisher, MA). The second round PCR was performed with primers 3158N and 3159N (Table 1, 600 nM concentration each) using the first-round PCR product as the template. The PCR parameters were as follows: 95°C for 10 min, followed by 45 cycles of 95°C for 30 sec, 42°C for 30 sec, and 72°C for 1 min, and final elongation step at 72°C for 10 min.

Table 1: Oligo nucleotide primers used for HEV RT-qPCR, and nested RT-PCR

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>Sequence 5' - 3'</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>JHEV-FP</td>
<td>GGTGGTTTCTGGGGTGAC</td>
<td>HEV qRT-PCR</td>
</tr>
<tr>
<td>JHEV-RP</td>
<td>AGGGGTGTTGGATGAA</td>
<td>HEV qRT-PCR</td>
</tr>
<tr>
<td>JHEV-probe</td>
<td>5’FAM/TGATTCTCAGCCCTTCGC/3’BHQ</td>
<td>HEV qRT-PCR</td>
</tr>
<tr>
<td>HEV-ORF2-3156N-FP</td>
<td>AATTATGCYCATAYGCRGTGG</td>
<td>HEV-ORF2-1st round</td>
</tr>
<tr>
<td>HEV-ORF2-3157N-RP</td>
<td>CCCTTTCYTGCTGMCATTCCTC</td>
<td>HEV-ORF2-1st round</td>
</tr>
<tr>
<td>HEV-ORF2-3158N-FP</td>
<td>GTWATGCTYTGCATWCATGGCT</td>
<td>HEV-ORF2-2nd round</td>
</tr>
<tr>
<td>HEV-ORF2-3159N-RP</td>
<td>AGCCGACGAAATCAATTCTGTC</td>
<td>HEV-ORF2-2nd round</td>
</tr>
</tbody>
</table>

Sequencing, sequence and phylogenetic analyses: The expected RT-PCR product of 348 bp was visualized by gel electrophoresis on a 1% agarose gel, purified and sequenced using the 3159N primer (Table 1). The sequences were compared with reference strains of different HEV genotypes and with known human and swine HEV sequences retrieved from GenBank database (NCBI, MD) using software MEGA6 (16). The phylogenetic tree was constructed by using the Maximum Likelihood bootstrap method based on the Tamura-Nei model. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. Gaps/Missing data treatment was assumed to be partial deletion, with a 95% site coverage cutoff percentage. Codons included were 1st + 2nd + 3rd + Non-Coding.

ELISA: All swine serum samples were tested for IgG anti-HEV using a commercial HEV antibody assay, the PrioCHECK® HEV Ab porcine ELISA kit as per manufacture’s protocol (Mikogen Inc). Prior to purchasing the ELISA assay kits for this NPB project, we first compared the PrioCHECK® HEV Ab porcine ELISA kit with our in-house HEV ELISA assay by using a set of serum samples from pigs experimentally-infected with a genotype 3 HEV. The result showed that the PrioCHECK® HEV Ab porcine ELISA kit is comparable to and correlates well with our in-house HEV ELISA assay in detecting IgG anti-HEV (data not shown). Therefore, a decision was made to utilize the commercial PrioCHECK® HEV Ab porcine ELISA kit for the NPB project since the commercial assay contains standardized reagents that can generate reproducible results. Briefly, the serum samples were diluted 1:100 in sample dilution buffer. Approximately 100µl
diluted serum samples were then added on to the HEV antigen-coated ELISA plate, and incubated for 1hr at 37°C. The plates were then washed four times using 300µl washing buffer. Approximately 100µl of diluted anti-swine HRP antibody was then added onto the plate and incubated at 37°C for 30 min. The plate was washed for four times using 300µl washing buffer, and developed using the TMB (3,3′,5,5′-tetramethylbenzidine) substrate. The O.D. value was read at 450 nm. Positive control, cut-off control and negative control were all included in each test.

Results:
Prevalence of HEV RNA in serum samples of pigs from slaughterhouses in the United States: Of the 5033 sera from 25 different slaughterhouses in 10 States we tested (Table 2), 318 serum samples were positive for HEV RNA using an established HEV RT-qPCR assay. Therefore, the national average of serum HEV RNA positivity from slaughtered pigs in the United States is 6.32% (95% C.I. 6.32 ± 0.67%). The percentage of serum HEV RNA positivity varied from slaughterhouse to slaughterhouse ranging from 0% to 17.44%, as well as from State to State (Table 2). The highest serum HEV RNA positivity was found in three slaughterhouses from Iowa (17.44%, 9.5%, and 8.33%, respectively), 2 slaughterhouses from Illinois (8.54%, and 7.5%, respectively), one slaughterhouse from North Carolina (7.88%), and one slaughterhouse from Pennsylvania (7.5%).

Table 2: Detection of IgG anti-HEV and serum HEV RNA from market weight pigs from 25 different slaughterhouses in 10 different States

<table>
<thead>
<tr>
<th>Slaughterhouses from 10 States*</th>
<th># serum samples tested</th>
<th># serum samples positive for IgG anti-HEV by ELISA (%)</th>
<th># serum samples positive for HEV RNA by RT-qPCR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OK</td>
<td>455</td>
<td>269 (59.12%)</td>
<td>24 (5.27%)</td>
</tr>
<tr>
<td>TN</td>
<td>56</td>
<td>32 (57.14%)</td>
<td>1 (1.79%)</td>
</tr>
<tr>
<td>VA</td>
<td>213</td>
<td>89 (41.78%)</td>
<td>6 (2.82%)</td>
</tr>
<tr>
<td>IL-1</td>
<td>40</td>
<td>18 (45.00%)</td>
<td>3 (7.5%)</td>
</tr>
<tr>
<td>IL-2</td>
<td>55</td>
<td>22 (40.00%)</td>
<td>3 (5.45%)</td>
</tr>
<tr>
<td>IL-3</td>
<td>40</td>
<td>16 (40.00%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>IL-4</td>
<td>445</td>
<td>118 (26.52%)</td>
<td>38 (8.54%)</td>
</tr>
<tr>
<td>IL-5</td>
<td>259</td>
<td>60 (23.17%)</td>
<td>2 (0.77%)</td>
</tr>
<tr>
<td>WI</td>
<td>20</td>
<td>9 (45.00%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>IA-1</td>
<td>379</td>
<td>135 (35.62%)</td>
<td>36 (9.50%)</td>
</tr>
<tr>
<td>IA-2</td>
<td>455</td>
<td>304 (66.81%)</td>
<td>23 (5.05%)</td>
</tr>
<tr>
<td>IA-3</td>
<td>70</td>
<td>21 (30.00%)</td>
<td>1 (1.43%)</td>
</tr>
<tr>
<td>IA-4</td>
<td>105</td>
<td>39 (37.14%)</td>
<td>1 (0.95%)</td>
</tr>
<tr>
<td>IA-5</td>
<td>180</td>
<td>67 (37.22%)</td>
<td>15 (8.33%)</td>
</tr>
<tr>
<td>IA-6</td>
<td>37</td>
<td>17 (45.95%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>IA-7</td>
<td>453</td>
<td>153 (33.77%)</td>
<td>79 (17.44%)</td>
</tr>
<tr>
<td>IA-8</td>
<td>22</td>
<td>5 (22.73%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>MN</td>
<td>233</td>
<td>76 (32.62%)</td>
<td>10 (4.29%)</td>
</tr>
<tr>
<td>NC-1</td>
<td>245</td>
<td>61 (24.90%)</td>
<td>7 (2.86%)</td>
</tr>
<tr>
<td>NC-2</td>
<td>482</td>
<td>266 (55.19%)</td>
<td>38 (7.88%)</td>
</tr>
</tbody>
</table>
*State and the slaughterhouses from which the swine serum samples are collected. The total number of serum samples tested from each slaughterhouses in this project is listed in the column 2. The total number of serum samples tested positive for IgG anti-HEV and HEV RNA by ProCHECK HEV IgG ELISA and HEV RT-qPCR, respectively, is listed in columns 3 and 4, and the percentage (%) of positivity is indicated in parenthesis.

**Figure 1:** A phylogenic tree based on the capsid gene region of reference HEV strains of genotype 1, 2, 3, 4 within species *Orthohepevirus* A, representative HEV strains from species *Orthohepeviruses* B, C, D, as well as Cutthroat trout virus in the genus *Piscihepevirus*. The phylogenetic tree was constructed with MEGA6 software by using the Maximum Likelihood bootstrap method based on the Tamura-Nei model. The bootstrap values of the nodes are represented in percentage. All the HEV sequences from slaughterhouse pigs in this study (N = 182 sequences obtained from market weight pig sera) belong to genotype 3 (designated under the collapsed clade Genotype 3 HEV).
Figure 2: A phylogenic tree of the capsid gene region of genotype 3 HEVs from market weight pigs in slaughterhouses in the United States along with reference strains of different HEV genotypes and representative known genotype 3 human and swine HEV sequences retrieved from GenBank database using MEGA6 software. The phylogenetic tree was constructed by using the Maximum Likelihood bootstrap method based on the Tamura-Nei model. The genotype 3-group 2 (Gt3-2) sequences are all highlighted in grey and marked as closed circles (●; N = 182 sequences from market weight pigs from slaughterhouses in this study; N = 19 reference sequences from GenBank database). The genotype 3-group 1 (Gt3-1) sequences are marked as open circles (○; N = 7 reference sequences from GenBank database). Reference HEV sequences from Genotypes 1/2/4 clade, and Orthohepevirus B/C/D clade are also shown as collapsed branches, and the Cutthroat trout virus in genus Piscihepevirus is shown as a separate clade.

Genotyping and subgenotyping of HEV in market weight pigs from 25 slaughterhouses in 10 different States: The RT-qPCR positive samples, originated from 25 different slaughterhouses in 10 different States, were further tested by a conventional broadly-reactive nested RT-PCR assay.
that is known to detect multiple HEV genotypes (10). The amplified 348 bp RT-PCR product was subsequently sequenced. Sequence analyses revealed that all HEV sequences identified in this study from market weight pigs in slaughterhouses in the United States belong to the zoonotic genotype 3 HEV (Figure 1). The HEV sequences from slaughterhouse pigs shared approximately 90-94% nucleotide sequence identities with the previously reported U.S. genotype 3 swine HEV (isolate US-C031008 and swHEV-20468D-USA13). Phylogenetic analysis based on the Maximum likelihood using bootstrap method and Tamura-Nei model revealed that the HEV sequences from slaughterhouse pigs in the United States all clustered in subgenotype Gt3-2 (i.e. Gt3abchij) (Figure 2). We did not detect any subgenotype Gt3-1 (i.e Gt3efg) or Gt4 HEV from the slaughterhouse pigs in this study.

Prevalence of IgG anti-HEV in market weight pigs from different slaughterhouses in the United States: The national average of HEV seropositivity from market weight pigs in slaughterhouses is approximately 39.88% (95% C.I. 39.88 ± 1.35%). It varies from slaughterhouse to slaughterhouse and from state to state (range 0 - 66.8%) (Table 2). One slaughterhouse from Pennsylvania tested seronegative (N = 50). The highest HEV seropositivity was obtained from a slaughterhouse in Iowa (68.81%), Oklahoma (59.12%), Tennessee (57.14%), and Carolina (55.19%) (Table 2). Iowa also had the highest HEV RNA positivity (17.44%) from one of the slaughterhouses (Table 2). Interestingly, the serum samples from Tennessee had a higher HEV seropositivity, but only 1.79% of these sera were tested positive for HEV RNA. This is largely due to the fact that HEV viremia is very transient in infected pigs, and thus there is a narrow window of detecting HEV RNA from sera (2, 17).

Discussion:
Genotypes 3 and 4 HEVs are zoonotic in nature and infect both pigs and humans. There is a growing number of reported autochthonous hepatitis E cases due to consumption of raw or undercooked animal meats particularly pork. Genotype 3 HEV has been reported from swine herds in the United States (10), Asian countries and several European countries (7), whereas genotype 4 HEV is mainly endemic in China and Japan (2). In the United States, all known HEV isolates detected thus far belong to the genotype 3a, which is an Asian genotype. Pigs in Europe are reportedly infected by subgenotypes 3e and 3f, as well as 3c, 3h, and 3i. The HEV subgenotype 3efg are grouped as genotype 3 group 1 (Gt3-1) while subgenotype 3abchij are grouped as genotype 3 group 2 (Gt3-2). The Gt3-2 subgenotype in Europe has recently been incriminated in contaminated pork product in supermarket chain. Currently, there is no information regarding the HEV infection status or the genotype in market weight pigs at the time of slaughter in the United States. Therefore, determination of the prevalence, genotype and subgenotype of HEV from market weight pigs from slaughterhouses in the United States will fill in a critical knowledge gap and also help improve the competitive advantage of U.S. pork industry.

We showed that a national average 39.88% (95% C.I. 39.88 ± 1.35%; Range 0 - 66.81%) of the market-weight pigs from the 25 slaughterhouses in 10 States were seropositivity for IgG anti-HEV, and that approximately 6.32% (95% C.I. 6.32 ± 0.67%; range 0 - 17.44%) of them were still viremic and positive for HEV RNA. There was no apparent correlation between the IgG anti-HEV seropositivity and serum HEV RNA positivity: within IgG anti-HEV seropositive samples (N = 2007), only 145 sera were also positive for HEV RNA (i.e. 7.22%, 95% C.I. 7.22 ± 1.13 %). This is likely due to the fact that it generally takes 7-13 weeks to seroconvert to HEV antibodies during
the course of HEV infection in pigs (18). Sequence and phylogenetic analyses indicated that HEV genotype 3-group2 (Gt3-2) is the prevalent type in slaughtered pigs in the United States. The HEV sequences from market weight pigs in U.S. slaughterhouses share approximately 90-94% nucleotide sequence identity with known U.S. swine HEV isolates.

Globally, HEV seroprevalence in pigs varies from 30 - 98% (17). Higher levels of IgG anti-HEV seropositivity have been reported from farm pigs in Asia, Africa, and Europe. Approximately 54-82% of slaughterhouse pigs in Asian countries were also seropositive for IgG anti-HEV (19-21). High levels of HEV seropositivity have also been reported in farmed slaughter-aged pigs in Europe (59 - 100%) (18, 23-25) and in West Africa (~ 80%) (26). Unfortunately, the anti-HEV seroprevalence in slaughterhouse pigs in the United States remains unknown prior to this study. The results from this study indicated a national average of 39.88% (0-66.8%) of the slaughterhouse pigs in the United States were seropositive for IgG anti-HEV, which is consistent with our previous finding in farmed pigs (27, 28). The anti-HEV seroprevalence in the United States appears to be at the lower end of the global HEV seropositivity range. The Mirkogen HEV commercial kit reportedly has the highest detection limit for IgG anti-HEV in human samples (29). In this study, we first compared the Mirkogen PrioCHECK® HEV Ab porcine ELISA kit with our in-house HEV ELISA assay by using a set of serum samples from pigs experimentally-infected with a genotype 3 HEV. We found that the results obtained with the Mirkogen commercial kit was in concordance with our in-house HEV ELISA assay (data not shown). Taken together, the results from this study suggest that a significant proportion of the market weight pigs from slaughterhouses in the United States are seropositive for IgG anti-HEV, indicative of prior HEV exposure to pigs in the farms.

To determine whether active HEV infection is still present in market weight pigs from slaughterhouses in the United States, we first tested serum samples by a RT-qPCR, and the samples tested positive by RT-qPCR were further tested by a nested RT-PCR assay. The results showed that approximately 6.32% (318/5033 sera) of the slaughterhouse pigs are still viremic and positive for HEV RNA. The HEV RNA positivity rate in domestic farm pigs vary from region to region and among different age groups: 0.8-16.6% in South America (22, 30-32), 2.8-73% in Europe (7, 23, 33, 34), and 4.8%-30% in Asia (36-38). This large variation in HEV RNA positivity among farm pigs is mainly attributed to the age of the pigs, and to lesser extent, sample type and assay variability (7, 17). For example, swine liver and fecal samples have a higher HEV RNA positive rate, while plasma samples have lower positive rate (7, 24). Approximately 5.7% of slaughterhouse pig plasma samples in UK were tested positive for HEV RNA (24), while 44.4% HEV RNA positivity was reported from serum samples of slaughter-aged pigs samples in Scotland (39). The results from this study indicate that, although about 40% of the pigs from slaughterhouses are seropositive for IgG anti-HEV, only a small proportion of them (about 6%) are still viremic. Nevertheless, this does raise potential pork safety concern since blood containing infectious HEV during slaughter may contaminate pork supply chains.

To determine the genotype and subgenotype of HEV in slaughtered pigs in the United States, we further tested the RT-qPCR positive samples by an established nested RT-PCR assay that is known to detect all 4 major HEV genotypes (10). The HEV capsid gene region (348 bp) was successfully amplified by RT-PCR and subsequently sequenced from 182 sera of the RT-qPCR positive samples. The inability to amplify all RT-qPCR positive samples by the nested RT-PCR assay in
this study is likely due to assay-to-assay variability and low levels of viral RNA in serum samples (17, 24, 40-42). Similar discrepancy has also been reported in other studies. For example, it was showed that ~5.7% of slaughterhouse pig plasma samples in UK were positive as tested by qPCR, however none of the samples were positive when tested by nested RT-PCR (24). Similarly, in Germany approximately 3.4-5.2% of blood samples obtained from wild-boar were tested positive by qPCR but partial sequences were obtained only from 12 of the qPCR positive samples (43). The target genomic regions by different PCR assays may influence the detection sensitivity as well: when comparing two PCR assays, in which one assay detected the ORF3-ORF2 overlap region (~70 bp), and the other detected ORF2 region (~140 bp), it was found that there is a greater chance of detecting the ORF3-ORF2 overlap products (44).

It has been reported that there is an epidemiological shift in circulating HEV strains in a given population. For example, genotype 4 HEV infections in China have recently overtaken genotype 1 HEV infections (45), while in United Kingdom emergence of a novel Gt3-2 strains has been observed (46) among the autochthonous human hepatitis E cases. Most of these genotypes 3 and 4 HEV cases in humans have been attributed to consumption of raw or undercooked animal meat products especially pork. Therefore, in this study we further analyzed the genotype and subtype of HEV strains in slaughtered pigs in the United States. Sequence and phylogenetic analyses of the HEV sequences obtained in this study reveal that the HEV isolates from the market weight pigs in U.S. slaughterhouses belong to genotype 3 that is clustered towards Gt3-2 subtype (i.e. Gt3abchij). The genetic diversity of the HEV isolates varies from region to region, and in some cases even within pigs from the same slaughterhouse, especially if the samples are from a slaughterhouse with a high volume sample set. The variation in genetic diversity within a slaughterhouse with high volume sample set could possibly be due to the fact that larger slaughterhouses process pigs from various geographic regions of the country. We did not detect HEV Gt4 or Gt3-1 (i.e. Gt3-efg subgenotype) in the 5033 samples tested in this study. HEV Gt4 is endemic in pigs from China (52), while the predominant European HEV strains in swine belong to Gt3-1 (i.e. Gt3e, and f) (9).

In summary, in this study we tested 5033 serum samples for evidence of HEV infection from pigs in 25 different slaughterhouses from 10 different States. We found that approximately 40% of the pigs tested are seropositive for IgG anti-HEV indicating prior HEV infection of the pigs in the farms. Despite the relative high seropositivity, however, only a small proportion (6%) of the pigs had detectable HEV viremia at the time of slaughter. Nevertheless the detection of HEV from blood of slaughtered pigs raise a pork safety concern as the blood-containing HEV may contaminate pork supply chains. Sequence and phylogenetic analyses indicated that the HEV sequences detected from pigs in U.S. slaughterhouses belong to the zoonotic genotype 3 of subgenotype Gt3-2 (i.e Gt3abchij), which is known to infect humans. Together, the results from this comprehensive study indicate that a small percentage of market weight pigs from slaughterhouses are still viremic at the time of slaughter and thus posing a potential concern of HEV contamination of pork.

Reference:
Consensus proposals for classification of the family Hepeviridae. The Journal of general virology 95:2223-2232.


