RESEARCHREPORT
PUBLIC HEALTH/WORKER SAFETY

**Title:** Determination of the Presence and Infectivity of Swine Hepatitis E Virus (HEV) in Swine Manure Storage Facilities and Nearby Water Sources - **NPB# 02-123**

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**Abstract:** Hepatitis E virus (HEV) was discovered in pigs in the U.S. in 1997. Swine HEV has been shown experimentally to infect non-human primates. HEV does not cause clinical disease in pigs; however, there is concern that pigs are an important reservoir of HEV and that swine HEV may infect and cause disease in humans. Recent evidence of clinical outbreaks of HEV in Japan further supports this concern.

This study was conducted to get a better understanding of where HEV persists on a pig farms and if there is evidence of contamination of drinking water and nearby surface water with HEV from pigs. We visited 28 Iowa pig farms in the late summer and fall of 2002 and collected fresh feces from growing pigs, pit manure, lagoon manure, and water samples from the farm drinking water supply and the nearest upstream and downstream surface water (streams or rivers). We confirmed the presence of HEV in fresh feces (a pool of feces from 5 pigs per site) collected from the floors of pig barns on 7 of 28 farms. We demonstrated HEV in pit manure samples from 15 of 28 sites indicating that the virus nucleic acid persists in pit manure. Only eight of the sites we visited had outdoor lagoons and 3 of 8 lagoon samples were positive for HEV nucleic acid. Despite exhaustive testing using several accepted techniques, we were not able to detect the presence of HEV in the drinking water on the sites or in the upstream or downstream surface water sources.

Inoculation of HEV-free pigs with the pit and lagoon manure samples from 2 of the 28 farms with the highest titer of HEV was performed to determine if the HEV in the manure samples was infectious. HEV-free pigs were inoculated intravenously or orally with the inocula. Feces and blood was collected weekly from these pigs and tested by RT-PCR and serology for evidence of HEV transmission. Two of three pigs inoculated intravenously with pig manure from a pit sample were confirmed to shed the virus in feces by 3 weeks after inoculation. To date (5 weeks post inoculation), the pigs inoculated orally with pit manure and the pigs inoculated with lagoon manure (either intravenously or orally) have remained negative. The swine bioassay will be terminated at 8 weeks post inoculation (January 2, 2004).

This work suggests that HEV is commonly present in fresh pig feces and in manure stored in pits and lagoons on swine farms. HEV detected by RT-PCR in pig manure from storage pits is infectious. However, evidence that HEV from fresh pig feces, pit manure, or lagoon manure is contaminating drinking water supplies or surface water is lacking.

**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.

It is now clear 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).

Pigs are now considered a potential reservoir and source of human HEV infection. Individuals with occupational exposure to environmental sources of domestic animal wastes are at risk of HEV infection. Pig manure and raw sewage from pig slaughterhouses could be a source of contamination of environmental water with HEV. **Our hypothesis is that pig manure from deep pits and lagoons and surface water collected from or near pig farms contains infectious swine HEV.** The goal of this project was to collect pig manure samples from 25-30 swine farms, a sample of the drinking water supply (well or rural water) on each site, and an upstream and downstream surface water sample from the nearest surface watershed (river or stream) and determine if HEV was present in those samples. All samples were tested by RT-PCR for HEV. We further determined if the samples contained infectious viruses by inoculation of naïve pigs with manure samples containing HEV nucleic acid.

**Objectives:**

1. The first objective was to determine if, and if so how frequently, pig manure or water from or near pig farms contains swine HEV as measured by reverse transcriptase polymerase chain reaction (RT-PCR).
2. The second objective was to determine if the virus detected in manure or water samples is infectious as measured by a swine bioassay.

**Materials and Methods:**

**Feces, manure, and water sample collection**

Five fresh fecal samples were collected from each site. These samples were collected from five different piles of freshly dropped feces inside the finishing barns. Gloves were changed between each sample collection. All feces were stored in a whirl-pack, and placed in a designated cooler with ice packs for transportation back to the laboratory.

Ten samples were also collected from the concrete pit under the slats of each finishing facility. These samples were taken from ten randomly selected sites in each pit. Each sample was collected from a depth between one and two feet below the surface and directly below the pens. The samples were collected in 50 ml conical centrifuge tubes and labeled with: type of pit, finishing site identification number, and the date. All samples were then placed in a designated cooler with ice packs for transportation back to the laboratory.

Ten samples were collected from the lagoon on each site that had a lagoon. These samples were taken from ten random sites in each lagoon. Each sample was collected in sterile 50 ml conical centrifuge tubes using the tool designed to reach 12 feet into the lagoon (approximately 4-6 feet deep under the crust), and labeled with: type of lagoon, finishing site identification number, and the date. All samples were then placed in a designated cooler with ice packs for transportation back to the laboratory.

Water samples were taken from three different sources on each farm. These sources include: a sample from source water that the pigs drink (and in most cases people drink), a sample from the nearest upstream watershed and a sample from the nearest downstream watershed (river or stream). All samples were collected in a two gallon container. All containers were labeled with type of water sample (drinking, upstream, or downstream),

finishing site identification number, and the date. Each sample was then placed into a designated cooler with ice packs for transportation back to the laboratory.

**Manure sample concentration**

The protocol was modified from a published protocol by Pina et al., 1998. Equipment used included a refrigerated ultracentrifuge (Beckman L8-M with rotor 55.2 Ti, rotor 50 Ti, and Ultrabottle assemblies), a refrigerated superspeed centrifuge (Soverall RC-5B with rotor GSA and 250 polycarbonate bottles). The reagents used included 0.25M glycine buffer, pH 10.5 5 (18.7675 g of glycine in 1 liter of DEPC $H_2O$, adjusted to pH 10.5 by NaOH) and sterile DEPC-H2O-based phosphate buffered saline (PBS).

The concentration process required approximately 3 ½ hours. Manure samples were clarified at 3,000 rpm for 10 minutes at 4$^o$ C. The sediment was discarded and the supernatant was saved. Approximately 60 ml of the clarified manure sample supernatants were subjected to ultracentrifuge at 45,000 rpm for 1 hour at 4$^o$ C to pellet the viral particles. An equal volume of clarified manure samples was archived for a swine bioassay at -80$^o$ C. The pellets were then diluted with 10 ml of 0.25 M glycine on ice for 30 minutes and were centrifuged again to get rid of solid materials at 10,016 rpm for 15 minutes at 4$^o$ C. The supernatants were saved and ultracentrifuged at 50,000 rpm for 1 hour at 4$^o$ C to make a final virus concentrate. The final concentrate was resuspended with 400 µl of PBS. Two aliquots (200 µl each) were prepared for qualitative RT-PCR assay and for semi-quantitative RT-PCR assay.

**Water sample concentration**

The protocols used were modified from the protocol for detection of enteric viruses recommended by the Environmental Protection Agency (1995) and Abbaszadegan et al., 1999. Equipment used for the process included a filter cartridge (MD1 Virosorb®) and filter housing (Cuno, Inc). The reagents used included 0.25M glycine, buffer pH 10 and 1N HCl.

The filtering process lasted approximately one hour. This was started by attaching each two gallon water sample to a sterile plastic hose, which was also connected to the inlet of the absorbent filter housing. The water was then run through the hose and filter housing. All flow-thru water was then discarded. Once filtering was complete, the virus elution process was started. One liter of autoclaved 0.25M glycine buffer, pH 10.5 was poured into the filter housing inlet. After a fifteen minute wait the first virus eluate was collected in a one liter sterile container. This first eluate was then poured back into the filter housing inlet and allowed to sit for fifteen minutes. The second virus eluate was then collected in the same manner as the first. The second virus eluate pH was then immediately adjusted to a pH of 7-7.4 by adding 1N HCL, pH 1.5 and mixing vigorously. The virus eluate was then kept on ice before proceeding to the virus reconcentration.

**Virus reconcentration by organic flocculation in water samples**

The virus reconcentration by organic flocculation protocol used was modified from the Environmental Protection Agency's protocol for detection of enteric viruses (1995). The reagents used included 1N HCl, phosphate buffer saline (PBS) pH 7.4, and autoclaved beef extract powder (Sigma-Aldrich). Equipment used included a refrigerated ultracentrifuge (Beckman L8-M with rotor 55.2 Ti, rotor 50 Ti, and Ultrabottle assemblies), a refrigerated superspeed centrifuge (Soverall RC-5B with rotor GSA and 250 polycarbonate bottles), and sterile 50-ml polypropylene sample tubes.

One-liter samples of the virus eluate from the water filtering process were reconcentrated by organic flocculation. This was done by immediately adjusting the pH to 3.5 with 1N HCL and adding 12g of autoclaved beef extract powder into the virus. The sample was then stirred vigorously for 15 minutes. The pH adjusted virus eluate was then centrifuged at 5,000 rpm for 10 minutes at 4$^o$ C. The supernatant was then discarded and the resulting pellet resuspended with 50 ml PBS. The sample was then made into two aliquots of 25 ml. One aliquot was subjected to the ultracentrifuge and the other was stored at -80$^o$ C.

The 25 ml aliquot of concentrated water samples was ultracentrifuged at 45,250 rpm for one hour at 4° C, in order to pellet all of the virus particles. The sediment was then kept and the supernatant again discarded. The sediment was then resuspended in 10 ml of .25M glycine buffer pH 9.5, on ice for 30 minutes. The resuspended material was then centrifuged at 10,016 rpm for 15 minutes. This time the supernatant was kept and the sediment was discarded. The supernatant was pelleted by ultracentrifugation at 45,250 rpm for one hour at 4° C, and the pellet resuspended with 400µl of DEPC-H2O-based PBS, 200 µl for qualitative RT-PCR testing and the other 200µl for archival purposes and to prepare a 10-fold dilution for semi-quantitative RT-PCR assay. When finished all concentrated water samples were stored at -80° C until RT-PCR testing.

After attempts to test the water concentrates yielded no HEV RNA by the ultracentrifugation method. We decided to re-test all the concentrated samples with an alternative concentration method by using Centricon© Centrifugal Filter Units. Each of the second aliquots (kept at -80° C) was applied to a Centricon© unit and was subjected to centrifugation at 3,000 rpm for 20 minutes at 4° C. The filtered concentrate was kept within the unit and the flow-through was disregarded. Then the filtered concentrate was retrieved by centrifuge the unit at 1,000 rpm for 3 minutes at 4° C. The retrieval process yielded approximately 200 µl of the sample concentrate. An aliquot of 140 µl was tested by qualitative RT-PCR assay and the other aliquot of 60 µl was used to prepare a 10-fold dilution for semi-quantitative RT-PCR.

**Swine Bioassay**

We have modified the swine bioassay from the original proposal. As none of the water samples had detectable HEV RNA, we decided not to use the water samples for swine bioassay and instead use both pit and lagoon samples with the highest virus titers to test the virus infectivity. We also decided to test the infectivity by both intravenous and oral inoculation routes. To determine if the virus nucleic acid detected by RT-PCR represents infectious virus, we selected the pit sample collected from Farm #12 ($10^3$ genome equivalent per 60 ml of pit sample) and the lagoon sample collected from Farm #19 (less than 10 genome equivalent per 60 ml of lagoon sample). Twenty-three HEV-seronegative, 7-week-old SPF pigs were randomly separated into seven groups of 3-4 pigs per group. The bioassay design is summarized in **Table 1**.

**Table 1. Swine bioassay design**

| Group | Pigs | Inocula | Source of Inocula | Route | RT-PCR Titer (total GE) |
|---|---|---|---|---|---|
| **1** | 3 | Non-inoculated control | - | - | - |
| **2** | 3 | sHEV-positive lagoon sample | Farm #19 | Oral | $< 10^1$ |
| **3** | 3 | sHEV-positive pit sample | Farm #12 | Oral | $\sim 10^3$ |
| **4** | 3 | sHEV-positive lagoon sample | Farm #19 | IV | $< 10^1$ |
| **5** | 3 | sHEV-positive Pit sample | Farm #12 | IV | $\sim 10^3$ |
| **6** | 4 | Positive fecal infectious pool | Swine HEV prototype strain | Oral | $10^3$ |
| **7** | 4 | Positive fecal infectious pool | Swine HEV prototype strain | IV | $10^6$ |

Feces and blood samples were/will be collected weekly for 8 weeks. Feces have been/will be tested for HEV RNA by RT-PCR assay and serum samples tested for anti-HEV antibodies by ELISA assay. We are currently at 5 weeks post inoculation. The bioassay experiment will be terminated at 8 weeks post inoculation on January 2, 2004.

**Results:**

**Objective #1: Detection of HEV in feces, manure, and water samples.**

Twenty-eight finishing sites of various types in Iowa were sampled in the summer and fall of 2002. Table 2 contains all of the HEV detection results from feces, pit manure, lagoon manure, and water samples. Of 28 farms from which we collected feces samples, there were seven farms that we detected swine HEV in feces collected from pigs in the barns. Fifteen of 28 sites had swine HEV-positive samples from pit manure. There were three positive samples out of a total of 8 lagoon samples collected. None of the on-site drinking water samples nor the nearest surface watershed samples were positive for HEV by RT-PCR assay.

**Table 2. Detection of hepatitis E virus nucleic acid in pig feces, pit and lagoon manure samples, drinking water, and surface water on 28 Iowa farms.**

| Farm | Type of farm | Feces | Pit/ Virus titer | Lagoon/ Virus titer | Drinking water | Nearby Water* |
|---|---|---|---|---|---|---|
| **1** | Farrow to finish | Negative | **Pos/$10^3$** | NA | Negative | Neg/Neg |
| **2** | Farrow | Negative | Negative | Negative | Negative | Neg/Neg |
| **3** | Farrow to finish | Negative | Negative | Negative | Negative | Neg/Neg |
| **4** | Farrow to finish | Negative | Negative | NA | Negative | Neg/Neg |
| **5** | Grow/finish | Negative | **Pos/<$10^1$** | NA | Negative | Neg/Neg |
| **6** | Grown/finish | Negative | NA | Negative | Negative | Neg/Neg |
| **7** | Grow/finish | Negative | **Pos/<$10^1$** | NA | Negative | Neg/Neg |
| **8** | Grown/finish | Negative | Negative | NA | Negative | Neg/Neg |
| **9** | Grow/finish | **Positive** | **Pos/<$10^1$** | NA | Negative | Neg/Neg |
| **10** | Wean to finish | Negative | Negative | **Pos/<$10^1$** | Negative | Neg/Neg |
| **11** | Farrow to finish | Negative | **Pos/<$10^1$** | NA | Negative | Neg/Neg |
| **12** | Grow/finish | **Positive** | **Pos/$10^3$** | NA | Negative | Neg/Neg |
| **13** | Farrow to finish | Negative | **Pos/<$10^1$** | NA | Negative | Neg/Neg |
| **14** | Grow/finish | Negative | **Pos/<$10^1$** | NA | Negative | Neg/Neg |
| **15** | Grow/finish | Negative | **Pos/$10^2$** | NA | Negative | Neg/Neg |
| **16** | Grow/finish | Negative | **Pos/<$10^1$** | NA | Negative | Neg/Neg |
| **17** | Farrow to finish | Negative | NA | **Pos/<$10^1$** | Negative | NA |
| **18** | Wean to finish | **Positive** | **Pos/<$10^1$** | NA | Negative | Neg/Neg |
| **19** | Farrow to finish | **Positive** | NA | **Pos/<$10^1$** | Negative | Neg/Neg |
| **20** | Wean to finish | Negative | Negative | NA | Negative | Neg/Neg |
| **21** | Farrow to finish | **Positive** | **Pos/<$10^1$** | NA | Negative | Neg/Neg |
| **22** | Grow/finish | **Positive** | **Pos/<$10^1$** | NA | Negative | Neg/Neg |
| **23** | Farrow to finish | Negative | NA | NA | Negative | Neg/Neg |
| **24** | Grow/finish | Negative | NA | NA | Negative | Neg/Neg |
| **25** | Grow/finish | **Positive** | **Pos/$10^2$** | Negative | Negative | Neg/Neg |
| **26** | Grow/finish | Negative | **Pos/$10^2$** | NA | Negative | Neg/Neg |
| **27** | Grow/finish | Negative | Negative | NA | Negative | NA |
| **28** | Wean to finish | Negative | NA | Negative | Negative | Neg/Neg |
| **Total** | | 7/28 | 15/22 | 3/8 | 0/28 | 0/26 |

NA = Not Available at this site

*The nearest upstream/downstream surface water (stream or river) was sampled.

**Objective #2: Determination of the infectivity of HEV in manure and water samples.**

At the time of submission of this report (12/10/03), we are into the 5th week post inoculation (PI). We have collected feces and blood samples and have tested feces from the pigs at 1, 2, and 3 weeks post inoculation (PI). Based on previous HEV inoculation

experiments, we expect HEV to be shed in feces beginning at 1-2 weeks PI and continuing for three weeks. Anti-HEV seroconversion is typically detected between 3 and 8 weeks PI. The results of HEV RNA detection in feces by RT-PCR assay to date are summarized in Table 3.

**Table 3. Swine bioassay; results of RT-PCR assay for detection of HEV RNA in feces**

| Group | Inocula | Route | 1 week PI | 2 weeks PI | 3 weeks PI |
|---|---|---|---|---|---|
| **1** | Non-inoculated control | - | 0/3* | 0/3 | NT[#] |
| **2** | sHEV-positive lagoon sample | Oral | 0/3 | 0/3 | 0/3 |
| **3** | sHEV-positive pit sample | Oral | 0/3 | 0/3 | 0/3 |
| **4** | sHEV-positive lagoon sample | IV | 0/3 | 0/3 | 0/3 |
| **5** | sHEV-positive pit sample | IV | 0/3 | 1/3 | 2/3 |
| **6** | Positive fecal suspension | Oral | 0/4 | 0/4 | 0/4 |
| **7** | Positive fecal suspension | IV | 3/4 | 4/4 | 4/4 |

* Positive pigs/number tested

[#] Not tested

**Discussion:** We were able to confirm the presence of HEV in fresh feces (a pool of feces from 5 pigs per site) collected from the floors of pig barns in 7 of 28 farms. The pigs in these barns ranged from 6-25 weeks of age. The number of pigs in the barns ranged from 200-1,100 so the sample size was actually quite small and therefore the incidence of HEV infection in grow-finish pigs in Iowa is likely even higher than our results indicate. This is consistent with other reports that HEV is common in the U.S. swine population (Meng et al., 1997; Huang et al., 2002).

We expected that by sampling pits we would find more positive samples than by testing fresh feces from the barns and that was the case. Demonstration of HEV in pit manure samples from 15 of 28 sites indicates that the virus is commonly present in pit manure and that this may be a good sample to confirm the status of HEV infection of a population of pigs. The current PCR technique is not able to differentiate swine from human or rat HEV and thus the HEV in the pit samples could be from other than swine sources.

Only eight of the sites visited had outdoor lagoons. All were earthen lagoons. There were 3 of 8 lagoon samples from which HEV was detected. On 2 of the 3 positive lagoons, HEV was not detected in fresh fecal samples from the barn. This may indicate that the current pigs had not been infected with HEV or were not shedding HEV at the time of sampling. The HEV in the lagoon may have come from previous groups of pigs on the site, other buildings on the site that drain into the lagoon, or other animal (rats, deer, chickens, birds) or human sources.

Despite exhaustive testing using accepted techniques recommended by the US Environmental Protection Agency and published scientific research, we were not able to detect the presence of HEV in the drinking water on the sites. Likewise, tests for HEV in the nearest upstream or downstream surface water sources were negative. Evidence that HEV from fresh pig feces, pit manure, or lagoon manure is contaminating drinking water supplies or surface water is lacking. It is possible that the virus amount present in the water samples was below the detection levels of the diagnostic methods we used (less than $10^2$ genome equivalent per 10 liters of tested water samples).

At the time of submission of this report, the swine bioassay is at five weeks post inoculation. Two of three pigs inoculated intravenously with a pit sample containing the highest HEV titer (of all manure samples tested) were found to shed the virus in feces at 3 weeks PI. All four positive control pigs inoculated intravenously with a known positive fecal infectious pool shed the virus at 2 and 3 weeks PI (Table 3). We expect inoculated pigs to develop anti-HEV antibodies from 3 weeks PI onwards if the virus in the pit or lagoon samples (particularly the groups intravenously inoculated) is infectious. After completing the bioassay, we will perform a comparative sequential analysis of the genetic material of

HEV RNA amplified (by RT-PCR) from the pit sample (used as inocula) and from feces collected from the PCR-positive bioassay pigs to confirm that they are of identical origin.

**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. This study was conducted to get a better understanding of where HEV persists on a pig farm and if there is evidence of contamination of drinking water and nearby surface water with HEV from the pigs. We confirmed the presence of HEV in fresh feces (a pool of feces from 5 pigs per site) collected from the floors of pig barns in 7 of 28 farms and this is consistent with other reports that HEV is common in the swine population. As expected we found more positive farms by sampling concrete pits than by testing fresh feces from the floors in the barns. Demonstration of HEV in pit manure samples of 15 of 28 sites suggests that the virus does survive in pit manure. Only eight of the sites we visited had outdoor lagoons and 3 of these 8 lagoon samples were positive for HEV. Despite exhaustive testing, we were not able to detect the presence of HEV in the drinking water on the sites or in the nearest upstream or downstream surface water sources.

This work suggests that HEV is commonly present in fresh pig feces and in manure stored in pits and lagoons on swine farms. HEV detected by RT-PCR in pig manure from storage pits is infectious. However, evidence that HEV from fresh pig feces, pit manure, or lagoon manure is contaminating drinking water supplies or surface water is lacking.

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.

**References:**

Abbaszadegan, M., Stewart, P., LeChevallier, M. 1999. A strategy for detection of viruses in groundwater by PCR. Appl Environ Microbiol 65(2):444-9.

Bradley, D.W. 1992. Hepatitis E virus: epidemiology, aetiology and molecular biology. Reviews Med Virol 2:19-28.

Halbur, P.G., Kasorndorkbua, C., Gilbert, C., et al. 2001. Comparative pathogenesis of infection of pigs with hepatitis E viruses recovered from a pig and a human. J Clin Microbiol 39(3):918-923.

Huang, F.F., Haqshenas, G., Guenette, D., Halbur, et al. 2002. Detection by reverse transcription-PCR and genetic characterization of field isolates of swine hepatitis E virus from pigs in different geographic regions of the United States. J Clin Microbiol 40:1326-1332.

Kasorndorkbua, C., Halbur, P.G., Thomas, P.J., Guenette, D., Toth, T.E., Meng, X.-J. 2002. Use of a swine bioassay and a RT-PCR assay to assess the risk of transmission of swine hepatitis E virus in pigs. J Virol Methods 101:71-78.

Kasorndorkbua, C., Thacker, B.J., Halbur, P.G. et al. 2003. Experimental infection of pregnant gilts with swine hepatitis E virus. Can J Vet Res 67:303-306.

Matsuda, H., Okada, K., Takahashi, K., Mishiro, S. 2003. Severe hepatitis E virus infection after ingestion of uncooked liver from a wild boar. J Infect Disease 188:944.

Meng, X.-J., Purcell, R.H., Halbur, P.G., et al. 1997. A novel virus in swine s closely related to the human hepatitis E virus. Proc Natl Acad Sci USA. 94:9860-9865.

Meng, X.-J., Halbur, P.G., Shapiro, M.S., et al. 1998. Genetic and experimental evidence for cross species infection by swine hepatitis E virus. J Virol 72:9714-9721.

Pina, S., Jofre, J., Emerson, S.U., et al. 1998. Characterization of a strain of infectious hepatitis E virus isolated from sewage in an area where hepatitis E virus is not endemic. Appl Environ Microbiol. 64(11):4485-8.

U.S. Environmental Protection Agency. 1995. Detection of enteric viruses. Standard Methods. Section 9 Pp 89-102.

Yazaki, Y., Mizuo, H., Takahashi, M., et al. 2003. Sporadic acute or fulminant hepatitis E in Hokkaido, Japan may be food-borne, as suggested by the presence of hepatitis E virus in pig liver as food. J Gen Virol 84:2351-2357.