Detection and characterization of infectious *Hepatitis E virus* from commercial pig livers sold in local grocery stores in the USA


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*Hepatitis E virus* (HEV) is a zoonotic pathogen of which pigs are reservoirs. To determine the presence of HEV RNA in commercial pig livers sold in local grocery stores in the USA, 127 packages of commercial pig liver were purchased and tested by a universal RT-PCR assay capable of detecting all four known HEV genotypes. Among the 127 livers tested, 14 were positive for HEV RNA. Sequence and phylogenetic analyses revealed that the 14 isolates all belonged to genotype 3. An animal study was subsequently conducted in pigs to determine whether the PCR-positive pig livers still contained infectious virus. The results showed that pigs inoculated with two of the three PCR-positive pig-liver homogenates became infected, as evidenced by the detection of faecal virus shedding, viraemia and seroconversion. The data demonstrated that commercial pig livers sold in grocery stores are contaminated by HEV and that the contaminating virus remains infectious, thus raising a public-health concern for food-borne HEV infection.

The disease caused by HEV, hepatitis E, is a major public-health concern in developing countries (Arankalle et al., 1994; Purcell, 1996) where sanitation conditions are poor, but is also endemic in many industrialized countries, including the USA (Hamid et al., 1996; Purcell, 1996). Only sporadic cases of acute hepatitis E were reported in the USA and other industrialized countries; however, a significant proportion of healthy individuals in these countries were found to be positive for HEV antibodies (Mast et al., 1997; Thomas et al., 1997; Meng et al., 2002). It has been hypothesized that an animal reservoir(s) exists for HEV (Meng, 2000a, b).

The first animal strain of HEV, swine HEV, was identified from a pig in the USA and shown to be related antigenically and genetically to human HEV (Meng et al., 1997). More recently, another animal strain of HEV, avian HEV, was identified from chickens with hepatitis–splenomegaly syndrome in the USA (Haqshenas et al., 2001). Like swine HEV, avian HEV is also related genetically and antigenically to human HEV (Huang et al., 2004). Cross-species infections have been demonstrated: swine HEV infected non-human primates (Meng et al., 1998a), human HEV infected pigs (Meng et al., 1998a; Halbur et al., 2001) and avian HEV infected turkeys (Sun et al., 2004).

Recently, cluster cases of acute hepatitis E in Japan were linked epidemiologically and genetically to the consumption of undercooked pig livers and deer meat (Yazaki et al., 2003; Tei et al., 2003, 2004). Therefore, the objectives of this study were to detect and characterize HEV in commercial pig livers sold in local grocery stores in the USA and to...
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determine whether the contaminating pig livers still contained infectious virus.

In total, 127 packages of commercial pig livers were purchased weekly from three local grocery stores in Blacksburg, VA, USA (125 packages) and Ames, IA, USA (two packages) from September 2005 to March 2006. The packaging material of each pig liver consisted of a foam-insulated tray and plastic wrap. Each package of pig liver was purchased frozen from the fresh-meat section of the grocery store and stored immediately in a −80 °C freezer until use. A portion of each pig liver was homogenized in 10% (w/v) sterile PBS. The liver homogenates were used for the detection of swine HEV RNA by a universal RT-PCR assay capable of detecting all four known genotypes of HEV essentially as described previously (Huang et al., 2002; Cooper et al., 2005). The expected size of the final product of the universal nested RT-PCR was 348 bp.

The amplified PCR products from each positive liver homogenate were separated in a 0.8% agarose gel. The expected band was excised from the gel, purified by the glass-milk procedure with a GENECLEAN kit (Bio 101 Inc.) and sequenced for both strands at the Virginia Bioinformatics Institute (Blacksburg, VA, USA). The nucleotide sequences of the HEV isolates from the 14 positive pig livers have been deposited in GenBank with accession numbers EF107626–EF107639. Sequence analyses were conducted by using the MacVector computer program (Oxford Molecular Inc.). The HEV sequences amplified from commercial pig livers were compared with selected known human, swine and avian HEV strains. Phylogenetic analysis was performed by using the maximum-parsimony method in the PAUP program (Sinauer Associates Inc.). GenBank accession numbers and the geographical origins of the nucleotide sequences of the HEV strains used in the phylogenetic and sequence analyses are as follows: Arkell (AY115488, Canada), JRA1 (AP003430, Japan), SwJ570 (AB073912, Japan), JRT-Kan (AB091394, Japan), IKN-Sap (AB074918, Japan), US1 (AF060668, USA), US2 (AF060669, USA), swine (AF082843, USA), B1 (M73218, Burma), HeBei (M94177, China), pSK-HEV-3 (AF444003, Pakistan), Ugh 179 (D11093, China), Hev037 (X98292, India), Abb-2B (AF185822, Pakistan), TK15/92 (AF051830, Nepal), M1 (M74506, Mexico), T1 (AJ272108, China), IND-SW-00-01 (AY723745, India), swCH31 (DQ450072, China), JYI-ChiSai01C (AB197674, China), CCC220 (AB108537, China), JSN-Sap-FH (AB091395, Japan), JKK-Sap (AB074917, Japan), JAK-Sai (AB074915, Japan) and avian HEV (AY535004, USA).

To determine whether the pig livers positive by RT-PCR for HEV RNA still contained infectious HEV, an animal-transmission study was conducted in specific-pathogen-free (SPF) pigs. Twenty-five 4-week-old SPF pigs were purchased from a commercial source. Prior to inoculation, all pigs were confirmed to be negative for HEV antibodies by an ELISA (Meng et al., 1998a, b) with the exception of two pigs (1225 and 1227). These two pigs had a very low but detectable level of maternal HEV antibodies (0.47 and 0.45 A_{405} values, respectively; cutoff value, 0.30) and were assigned to the negative-control group. The pigs were divided into five groups of five pigs each. The five pigs in group 1 were each inoculated intravenously (i.v.) with 2 ml liver homogenate from a PCR-negative commercial pig liver as negative controls. The five pigs in group 2 were each inoculated i.v. with 1 ml standard swine HEV infectious stock with an infectious titre of $5 \times 10^{4.5}$. 50 % pig infectious doses (Meng et al., 1998a) as positive controls. Pigs in groups 3, 4 and 5 were each inoculated i.v. with 2 ml RT-PCR-positive liver homogenates from each of the three selected commercial pig livers. The rationale for selecting the three positive commercial livers for the pig-transmission study was based upon the sequence data of the 14 HEV isolates. To avoid testing the infectivity of positive pig livers that may originate from the same farm, we selected the three isolates that were genetically distinct from each other and had greater sequence divergence than the other 11 isolates when compared with the prototype swine HEV. The animals were monitored for 8 weeks for evidence of HEV infection. Serum samples from each pig were collected prior to inoculation and weekly thereafter for the detection of HEV viraemia by RT-PCR as described above and for anti-HEV IgG by ELISA as described previously (Meng et al., 1997, 1998a, b). Faecal samples from each pig were collected prior to inoculation and weekly after inoculation for the detection of HEV RNA by RT-PCR.

Of the 127 packages of commercial pig livers purchased from local grocery stores, 14 (11.0%) tested positive for HEV RNA. Sequence analyses of the resulting 276 bp ORF2 sequence (excluding the PCR primer sequences) revealed that the 14 HEV isolates from pig livers in local grocery stores shared approximately 84–100 % nucleotide sequence identity with each other, 86–94 % identity with the prototype genotype 3 US swine HEV, 87–93 % identity with the two genotype 3 US strains of human HEV (US1 and US2) and 85–90 % identity with a Japanese strain of human HEV (JRA1) thought to be of swine origin. The 14 HEV isolates shared 74–81 % nucleotide sequence identity with genotype 1 HEV strains, 70–72 % identity with the genotype 2 HEV strain and 73–79 % identity with the genotype 4 HEV strains (data not shown). Phylogenetic analysis revealed that all of the 14 isolates identified from commercial pig livers in this study clustered in genotype 3, together with the US strains of human HEV and swine HEV (Fig. 1).

All negative-control group 1 pigs inoculated with a PCR-negative liver homogenate remained seronegative throughout the study and there was no detectable viraemia or faecal virus shedding in group 1 pigs (Fig. 2; Table 1). As expected, all positive-control pigs in group 2 inoculated with a standard infectious stock of swine HEV seroconverted to anti-HEV IgG by 5 weeks post-inoculation (p.i.) and were still seropositive at the end of the 8 week study (Fig. 2). Viraemia and faecal virus shedding were detected in all five positive-control group 2 pigs (Table 1).
Evidence of active HEV infection was detected in two of the three groups of pigs (groups 3 and 4) inoculated with one of the three PCR-positive commercial liver homogenates, respectively. In group 3 pigs, seroconversion started at 3 weeks p.i. By the end of the 8 week study, all group 3 pigs had seroconverted to anti-HEV IgG (Fig. 2). Viraemia and faecal virus shedding were detected variably in all five group 3 pigs (Table 1). Similarly, by 4 weeks p.i., all of the group 4 pigs had seroconverted to anti-HEV IgG (Fig. 2). Viraemia and faecal virus shedding were also detected in all of the group 4 pigs (Table 1). However, the pigs in group 5, inoculated with another PCR-positive liver homogenate, showed no evidence of infection as there was no seroconversion, viraemia or faecal virus shedding in group 5 pigs (Fig. 2; Table 1).

Viruses recovered from pigs 1250 (group 2), 1242 (group 3) and 1201 (group 4) were sequenced over the 276 bp ORF2 region. Sequence analyses confirmed that the viruses recovered from the infected pigs originated from the respective inocula.

HEV infection in pigs is ubiquitous worldwide (Meng, 2003). In some herds in the USA, approximately 60–100% of pigs are infected (Meng et al., 1997). Swine HEV isolates identified from pigs worldwide belong to either genotype 3 or 4 (Hsieh et al., 1999; Okamoto et al., 2001; van der Poel et al., 2001; Huang et al., 2002; Takahashi et al., 2003; Meng, 2005), although recently, a genotype 1 HEV strain was reportedly detected in a pig in Cambodia (Caron et al., 2006). Genotype 3 and 4 HEV strains are primarily responsible for sporadic cases of hepatitis E in humans, whereas genotype 1 and 2 strains are mainly responsible for hepatitis E epidemics (Emerson & Purcell, 2003; Meng, 2003, 2005). It has been demonstrated that a genotype 3 strain of human HEV (US2 strain), but not those of genotype 1 or 2, is readily transmissible to pigs (Meng et al., 1998a, b; Halbur et al., 2001). Conversely, genotype 3 swine HEV has been shown to infect non-human primates (Meng et al., 1998a). A genotype 4 swine HEV also infected non-human primates (V. A. Arankalle, personal communication; Meng & Halbur, 2005).

It is now known that hepatitis E is a zoonotic disease and that pigs are reservoirs of HEV (Meng, 2000a, b, 2003, 2005; Meng & Halbur, 2005). In a large, well-controlled, seroepidemiological study involving 465 swine veterinarians, Meng et al. (2002) found that US swine veterinarians were 1.51 times more likely (using genotype 3 swine HEV antigen, \( P = 0.03 \)) to be positive for HEV antibodies than age- and geography-matched normal US blood donors. Similarly, Drobeniuc et al. (2001) reported that about 51% of swine farmers from Moldova were positive for HEV antibodies, whereas only 25% of control subjects were seropositive. In North Carolina, swine workers were shown to have a 4.5-fold higher anti-HEV IgG prevalence than control subjects (Withers et al., 2002). Therefore, humans who consume contaminated pork products, such as pig livers, or come into contact with pigs or contaminated pig-waste materials are at potential risk of HEV infection (Matsuda et al., 2003; Tei et al., 2004).

Recently, swine HEV RNA was detected in approximately 2% of raw pig livers sold in Japanese grocery stores (Yazaki et al., 2003). Unfortunately, it is not known whether the contaminated commercial pig livers from grocery stores in Japan would still contain infectious virus, and no study has been conducted to assess the prevalence of HEV contamination in commercial pig livers in the USA. In the present study, we demonstrated that approximately 11% of
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commercial pig livers sold in local grocery stores in the USA are contaminated by HEV. The 14 HEV isolates recovered from contaminated pig livers all clustered in genotype 3, are contaminated by HEV. The 14 HEV isolates recovered from contaminated pig livers all clustered in genotype 3,

Table 1. Detection of HEV RNA in weekly samples (faecal/serum) collected from pigs inoculated with liver homogenates positive or negative for HEV RNA by RT-PCR

Entries denote faecal/serum samples that were positive (+) or negative (−) at the indicated week p.i.

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<tr>
<th>Group/pig ID</th>
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*Inoculated with liver homogenate FL1, negative for HEV RNA by RT-PCR (negative controls).
†Inoculated with a swine HEV infectious stock (positive controls).
‡Inoculated with liver homogenate FL58, positive for HEV RNA by RT-PCR.
§Inoculated with liver homogenate FL91, positive for HEV RNA by RT-PCR.
‖Inoculated with liver homogenate FL114, positive for HEV RNA by RT-PCR.

Fig. 2. Seroconversion to anti-HEV IgG in inoculated and control pigs. (a) Pigs inoculated with a liver homogenate negative for HEV RNA by RT-PCR as negative controls (group 1); (b) pigs inoculated with an standard infectious stock of swine HEV as positive controls (group 2); (c–e) pigs inoculated with each of the three selected pig-liver homogenates positive for HEV RNA by RT-PCR (groups 3, 4 and 5, respectively). The ELISA cutoff A\textsubscript{405} value is 0.30.
together with the genotype 3 human HEV strains from patients in the USA, Japan and other countries. Our previous study found that the majority of pigs in the USA are infected at approximately 2–4 months of age (Meng et al., 1997). Therefore, viraemia and virus shedding are expected to be cleared in the majority of market pigs at slaughterhouses, which are approximately 6 months of age. The detection of infectious HEV in the livers of a proportion of slaughtered pigs suggested that some pigs may have protracted viraemia or may be infected beyond 4 months of age. In fact, HEV RNA has been detected in adult sows (Huang et al., 2002; Meng & Halbur, 2005).

The faecal–oral route of transmission indicates that HEV is resistant to inactivation by acidic and mild alkaline conditions in the intestinal tract. It has recently been shown that HEV is more heat-labile than Hepatitis A virus (HAV), another enterically transmitted hepatitis virus (Emerson et al., 2005). When faecal suspensions of the wild-type HM175 strain of HAV or the Sar-55 strain of human HEV were diluted in PBS buffer and compared in the same test by heating for 1 h at 45, 50, 56, 60, 66 or 70°C, HAV was only 50% inactivated at 60°C, but was inactivated almost totally at 66°C. In contrast, HEV was about 50% inactivated at 56°C and inactivated almost totally (96%) at 60°C. Therefore, the detection of HEV RNA by RT-PCR in commercial pig livers from grocery stores does not necessarily mean that the contaminating virus is still infectious.

In this study, we demonstrated that two of the three livers contaminated by HEV still harboured infectious virus, and the pigs inoculated with the PCR-positive liver homogenate developed an active HEV infection indistinguishable from that in pigs infected experimentally with a standard infectious stock of swine HEV. However, pigs inoculated with a third PCR-positive liver homogenate (group 5) did not become infected, suggesting that not all contaminated pig livers contain infectious virus. The storage conditions (4 or −20°C, or repeated freeze–thaw) in grocery stores probably inactivated the virus in the third PCR-positive pig liver. It is also possible that the infectious HEV titre in the third PCR-positive liver is too low to initiate an active HEV infection.

This is the first report demonstrating that commercial pig livers from grocery stores contain infectious HEV. The results from this study raise additional public-health concerns over pork safety and the risk of HEV infection via the consumption of undercooked pork products. It remains to be determined whether cooking is effective at inactivating the virus in contaminated pig livers and whether other pork products, such as pig intestines, sold in grocery stores also contain infectious HEV. It is possible that the relatively high HEV antibody prevalence in normal blood donors in the USA and other countries may be a result of individuals consuming HEV-contaminated pork products. Even if cooking can destroy the infectivity of HEV in the contaminated commercial pig livers completely, repeated exposure to foreign HEV proteins from contaminated pork products could elicit the production of a low level of HEV antibodies.

Acknowledgements
This project is supported by a grant from the National Pork Board (NPB#06-005) and in part by grants from the National Institutes of Health (AI065546 and AI050611). We thank Dr F. F. Huang for the development of the universal RT-PCR assay. We also thank Matt Boogerd and Paul Thomas for their assistance with the animal study.

References


