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Short communication

# Hepatitis E virus load in swine organs and tissues at slaughterhouse determined by real-time RT-PCR

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ABSTRACT

Although uncommon in North America, Hepatitis E virus (HEV) has been identified in some industrialized countries in patients without a history of travel to HEV-endemic countries. Its presence is ubiquitous worldwide in swine populations. Zoonotic transmission of swine HEV to non human primates has been achieved experimentally and transmission of HEV after ingestion of contaminated raw or undercooked meat is well documented. In Canada, so far, no HEV outbreak has been documented but HEV genotype 3 strains have been identified in sera and faecal samples of swine origin. The objective of the present study was to determine the viral load of HEV in liver, loin, bladder, hepatic lymph node, bile, tonsil, plasma and faeces samples of 43 pigs at slaughter. Feline calicivirus (FCV) was used as sample process control to validate the RNA extraction process, as a confirmation of the absence of sample inhibitors and as an amplification control. Using FCV/HEV multiplex TaqMan RT-qPCR system, HEV RNA was detected in 14 out of the 43 animals tested. HEV was detected in lymph nodes (11/43), bladder (10/43), liver (9/43), bile (8/43), faeces (6/43), tonsils (3/43), plasma (1/43) samples from infected animals. No HEV-positive loin samples were observed. Viral loads of $10^3$ to $10^7$ copies/g were estimated in positive liver and bile samples.



## 1. Introduction

Hepatitis E virus (HEV) is responsible for numerous epidemics in countries where the virus is endemic and sporadic cases in countries where the virus is non-endemic. HEV is transmitted mainly by ingestion of contaminated water (Purcell and Emerson, 2008) or food products (Tei et al., 2003) and is considered a public health concern.

Over the last decade, the number of reported hepatitis E (HE) cases in industrialized countries has increased (Teo, 2006). HE has long been associated with a history of travel in HEV-endemic areas. Since 1997, sporadic indigenous cases have been documented in countries such as the United States (Kwo et al., 1997), Italy (Zanetti et al., 1999), France (Mansuy et al., 2004), United Kingdom (Wang et al., 2001) and Germany (Wichmann et al., 2008).

HEV is ubiquitous in the swine population and natural infection is asymptomatic in domestic pigs. Swine and human sporadic cases share the same genotype of HEV (Meng et al., 1998; Emerson and Purcell, 2003). There is a high genetic homology between animal and human strains among the various subtypes of genotypes 3 and 4 suggesting the possibility of zoonotic transmission. Interspecies transmission has been experimentally demonstrated (Meng et al., 1998) and contamination of people by direct contact with infected animals through professional occupation has been reported. An HE case has been associated with exposure to pig blood during surgical training (Colson et al., 2007). A higher prevalence of swine anti-HEV antibody was observed in swine veterinarians compared to human blood donors (Meng et al., 2002). It is now believed that pigs act as a virus reservoir.

Presence of HEV RNA in faeces or plasma and high seroprevalence of anti-HEV antibodies in swine was reported in Japan (Takahashi et al., 2003), in China (Li et al., 2009), in Spain (Seminati et al., 2008), in Czechoslovakia (Vasickova et al., 2009), in Italy (Caprioli et al., 2007), in the UK (McCreary et al., 2008), in the Netherlands (Rutjes et al., 2007), in Canada (Leblanc et al., 2007) and in USA (Meng et al., 1997).

Recently reported cases of HE in Japan were linked to the consumption of raw or undercooked pig liver, boar or deer meat (Matsuda et al., 2003; Tei et al., 2003; Yazaki et al., 2003; Takahashi et al., 2004). The Risk Ranger tool, a system used to identify pathogen/product combinations that represent foodborne risk by analysing hazard assessment, classified HEV and raw pork products with high risk profile (Mataragas et al., 2008).

Canada is considered a non-endemic region for human HEV. Serological evidences indicate that 3% of the Canadian Inuit population have been exposed to HEV (Minuk et al., 2007). The source of infection remains unknown although it has been suggested that consumption of uncooked or insufficiently cooked caribou meat may be responsible. Previous works reported the presence of HEV in plasma and faeces of swine at time of slaughter (Leblanc et al., 2007) and in 34% of faeces samples from visited farms in Quebec (Ward et al., 2008).

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RT-qPCR has proven to be an effective tool to detect and quantify HEV. Ward et al. (2009) proposed a multiplex real-time detection system for HEV using feline calicivirus (FCV) as sample process control (SPC) to monitor the nucleic acid extraction and to identify the presence of possible amplification inhibitors in a sample in order to reduce and limit false negative results.

Knowing that ingestion of contaminated raw or undercooked pig meat has led to HE cases and may represent a health hazard, the objective of this work was to determine the presence and evaluate the viral load of HEV in different tissues, organs, plasma and faeces of pigs at time of slaughter using a multiplex RT-qPCR detection assay.

## 2. Materials and methods

### 2.1. Animals and samples

Liver, loin (*longissimus dorsi*), bladder, hepatic lymph node, bile, tonsil, blood and faeces samples were collected from 43 adult pigs, randomly selected from an experimental herd, at slaughter over a period of 7 weeks. To avoid cross contamination between samples, individually wrapped, sterile disposable material and new sterile scalpel blades were used for each specimen. Samples were stored at −80 °C until tested.

### 2.2. RNA extraction

RNA was extracted using Agencourt® RNAdvance Tissue kit (Beckman Coulter, MA, USA) according to the manufacturer's recommendations for soft and hard tissue. To minimize variation between RNA extractions, all samples from the same animal were treated simultaneously on the same plate. A total of $3.6\times10^3$ PFU of FCV particles were added to each tube. Homogenization of 10 mg of sample was achieved by a bead beating method using metal beads (5 mm) for 25 min with a Tissue lyser (Qiagen, ON, Canada). Plates were then incubated at 37 °C for 45 min. RNA was extracted from 400 µl of homogenized lysates and was eluted from in 50 µl of RNase-free water (Gibco, ON, Canada) containing 20 U of RNase inhibitor RNaseOUT (Gibco). Two extractions were carried out for each sample and final eluates were pooled for obtaining 20 mg of starting working material. For each set of animal samples, no-sample extraction controls were included. Because of the amount of organ sampled and the final elution volume tested, the lower limit of detection for HEV RNA was $1\times10^3$ copies per g of sample.

### 2.3. TaqMan RT-qPCR

HEV RNA was detected using a multiplex TaqMan RT-qPCR assay in a 25 µl final volume for the simultaneous detection of HEV and FCV according to Ward et al. (2009) using QTaq™ One-Step qRT-PCR kit (Clontech laboratories, CA, USA). Amplifications were performed with a Stratagene MX30005P® system combined with MxPro 4.01 software (Stratagene, CA, USA). Amplification profile was 20 min at 48 °C followed by 3 min at 95 °C and 45 cycles of 94 °C for 15 s and 60 °C for 1 min.

Standard curves for HEV were included on each plate allowing a relative quantification of all samples from the same animal assayed on the same plate. All runs included FCV and HEV-positive controls, a no-sample extraction control and no-template controls alongside tested samples to ensure that no cross contamination occurred during the assay preparation.

## 3. Results and discussion

### 3.1. Detection of HEV RNA in pigs

All samples obtained from pigs at slaughter were individually tested for the detection of HEV and FCV in a multiplex assay. HEV RNA was not detected in 29 of 43 animals (Table 1). Viral RNA was mostly detected in hepatic lymph node (11/43), followed by bladder (10/43), liver (9/43), bile (8/43), faeces (6/43), tonsils (3/43) and plasma (1/43) samples. HEV RNA was not detected in all loin samples tested. These results suggest that testing multiple sample sources is necessary to categorize an animal HEV free.

The presence of HEV RNA was reported in tissues, organs and excreta of experimentally infected pigs (Meng et al., 1998; Williams et al., 2001; Kasorndorkbua et al., 2004; Banks et al., 2004) and in samples from naturally infected pigs (de Deus et al., 2007; Vasickova et al., 2009). Bouwknegt et al. (2009) studied the course of infection in contact-infected pigs as a model for natural infection and compared it to intravenous (iv) injected pigs. The course of infection differed between inoculation routes. In contact-infected pigs, HEV RNA was present in faeces, serum, bile, liver, lymph nodes, spleen, kidneys, and ileum, jejunum and colon samples collected from pigs at 28 days post infection. Our results should be compared to data obtained from naturally infected pigs. They are in accordance with this previous work since, all sample types were positive except for muscle samples. Bouwknegt et al. (2009) reported the presence of HEV RNA in more than 50% of muscle samples examined and intended for human

**Table 1**
HEV RNA detection and viral load using a TaqMan RT-qPCR assay in swine tissues, organs and excreta from 43 animals.

| HEV RNA positive | Liver | Muscle | Gallbladder | Bile | Hepatic lymph node | Tonsils | Plasma | Faeces |
|---|---|---|---|---|---|---|---|---|
| | 9 (20.9)[a] | 0 (0) | 10 (23.3) | 8 (18.6) | 11 (25.6) | 3 (7) | 1 (2.3) | 6 (13.9) |
| 1 | ++[b] | − | ++ | − | ++++ | + | − | + |
| 2 | ++++ | − | +++ | +++++ | ++ | − | − | ++++ |
| 3 | − | − | + | − | ++ | − | − | − |
| 4 | +++ | − | ++ | +++ | + | − | − | − |
| 5 | − | − | ++ | − | ++ | − | − | − |
| 6 | − | − | +++ | − | − | − | − | − |
| 7 | +++ | − | − | − | ++++ | − | − | − |
| 8 | +++++ | − | +++ | +++++ | ++++ | − | − | ++++ |
| 9 | + | − | + | +++ | + | + | − | + |
| 10 | − | − | − | − | +++ | − | − | − |
| 11 | +++++ | − | +++ | +++++ | +++ | − | − | +++ |
| 12 | − | − | − | +++ | − | − | − | − |
| 13 | +++++ | − | +++ | +++++ | ++++ | +++ | + | +++ |
| 14 | + | − | − | + | − | − | − | − |

[a] : Number of positive samples (results expressed in %).
[b] : + = values ranging from 1.0 to $9.9\times10^3$ copies/g; ++ = 1.0 to $9.9\times10^4$; +++ = 1.0 to $9.9\times10^5$; ++++ = 1.0 to $9.9\times10^6$; +++++ = 1.0 to $9.9\times10^7$, − = not detected.

consumption. Presence of HEV in muscle samples was observed up to 4 weeks after the onset of faecal shedding. These authors suggested that the time of infection has an impact on the presence of HEV in pork meat in retail stores. Late infection during fattening period may lead to HEV-positive muscle samples in retail stores. In this study, HEV RNA could not be detected in muscle (*longissimus dorsi*) samples tested. The loin samples assayed were obtained from the inner part of the muscle. If present in or on the muscle surface, it is possible that only a low quantity of HEV particles was present and fell under the detection limit of the detection system. Also, compared to Bouwknegt et al. (2009), half the amount of template (10% of final volume) was used for the same final RT-qPCR volume and that no data over a Ct of 40 were taken into account.

Numerous survey studies have estimated the prevalence of HEV RNA in marketed livers. HEV RNA was detected in 1.9% of 363 livers from supermarkets in Japan (Yazaki et al., 2003), in 6% of 62 packages in the Netherlands (Bouwknegt et al., 2007) and in 11% of 127 packages in the USA (Feagins et al., 2007). In United Kingdom, Banks et al. (2007) reported that all 80 packages from supermarkets were free of HEV. In Chinese abattoir, Li et al. (2009) found that 3.5% of liver samples tested were positive for HEV RNA. In the current study, 20.9% of liver samples obtained at the slaughterhouse tested positive for HEV RNA. However, substantial variability in viral loads of the infected animals was observed (Table 1). Quantification results estimated from $3.2\times10^3$ to $9.9\times10^7$ copies of HEV RNA per g of liver. Experimental data from non human primates indicate that the severity of infection with HEV is proportional to the infectious dose (Tsarev et al., 1994). Moreover, consumption of raw pig meat was associated with HE in the Netherlands (Melenhorst et al., 2007). In Hungary, the first case of human HE caused by a genotype 3 strain was reported and the suspected cause was the consumption of pork sausages prepared from house-slaughtered meat (Reuter et al., 2006). Our results indicate that no HEV RNA was detected in any of the muscle tested but high loads were observed in some liver samples and this may represent a risk if livers are consumed raw or undercooked.

A case of genotype 3 HE was reported in a slaughterhouse worker suggesting an occupationally acquired infection following handling of infected organs from pigs (Perez-Gracia et al., 2007). Our results also reveal that HEV is present in the slaughterhouse and may represent a risk for slaughterhouse staff.

This work reports for the first time the use of sample process control for the determination of HEV viral load of pig tissues and organs at slaughter. In a context of a dose dependent infection, these data add important information about the samples. The virulence of Canadian swine HEV genotype 3 strains remains to be evaluated. Swine bioassays revealed that HEV present in commercial pig livers can be efficiently inactivated if cooked properly (Feagins et al., 2008). Sanitary food preparation and sufficient heating of pork meat and liver before consumption should be observed.

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