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Detection of hepatitis E virus genome in pig livers in Antioquia, Colombia

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ABSTRACT. Hepatitis E is a form of endemic acute hepatitis found in humans in many countries worldwide and is caused by the hepatitis E Virus (HEV). Detection of HEV in pigs indicates that they may be carriers, possibly through zoonosis. The prevalence of HEV in pigs in Colombia is unknown. Studies in the US found that 11% of pig livers sold in grocery stores are contaminated with HEV. It is also known that HEV can be inactivated when cooked, as it is labile to high temperatures. The aim of this study was to determine HEV contamination in pig livers sold in Medellín, Antioquia. A total of 150 livers from 5 slaughterhouses and 100 livers in grocery stores from different social strata of the city of Medellin analyzed to detect a segment of the HEV open reading frame-1 using reverse transcription-polymerase chain reaction. The results showed that 41.3% of pig livers from slaughterhouses and 25% of livers from grocery stores tested positive for HEV. Thus, the HEV genome is present in pig livers sold in Antioquia, revealing the presence of this virus in pigs from Colombia and the need subject entrails to proper cooking processes before consumption. Further research is required to determine the role of this virus in public health and pork

production in Colombia.

Key words: Hepatitis E virus; Liver; Pigs; Zoonosis; Reverse transcription-polymerase chain reaction

INTRODUCTION

The hepatitis E virus (HEV) produces a form of acute hepatitis in humans that is endemic to many countries (Emerson and Purcell, 2006). The virus is non-enveloped and contains a positive-single strand RNA genome belonging to the genus *Hepevirus* from the Hepeviridae family (Emerson et al., 2004). HEV isolates that have been identified are divided into 4 genotypes in mammals, based on geographical distribution, range of hosts, and pattern of infection (Li et al., 2009). Genotype 1 includes strains from Asia and Africa; genotype 2 includes the Mexican strain and some variants from Africa; genotype 3 is uniformly distributed worldwide; and genotype 4 is most commonly found in China and Japan (Ahmad et al., 2011).

Several studies have been carried out throughout Latin America to determine the presence of HEV in pigs and humans. Villalba et al. (2010) found a seroprevalence of 10% (469 patients) in positive samples for anti-HEV IgG in healthy humans with no history of viral hepatitis in Cuba, while human HEV antibodies have been detected in healthy individuals in Brazil as well as molecular evidence for HEV (dos Santos et al., 2011). Similar studies have been conducted in Uruguay, where the prevalence of anti-HEV in human donors was 1.2% (Cruells et al., 1997) and sporadic cases of infection with genotype 3 HEV were also reported (Mirazo et al., 2011). In studies carried out by Munné et al. (2006), a strain of HEV was isolated from pigs in Argentina, showing a high degree of similarity in the nucleotide sequence to HEV strains previously identified in humans with sporadic cases of hepatitis E. In Bolivia, Dell'Amico et al. (2011) observed a positivity of 6.3% (236 patients) using an enzyme-linked immunosorbent assay (IgG anti-HEV), as well as 22.7 and 31.8% of positives by molecular testing for the HEV open reading frame (ORF)-2 in humans and pigs feces, respectively. The high levels of HEV detected in pigs from different countries (Clayson et al., 1995; de Deus et al., 2006; Baechlein et al., 2010) suggests that they could be carriers of HEV, and that this virus is a zoonosis transmitted to humans through water contaminated with pig feces or through contaminated viscera intended for consumption (La Rosa et al., 2010). However, swine HEV infection is asymptomatic; thus, it is difficult to determine when the infection peaks in order to optimize the sensitivity of tests for the virus (Di Bartolo et al., 2011).

Some studies have shown that HEV can cross the species barrier, suggesting that the infection is a major public health problem that can affect individuals who work or have contact with pigs, who are a higher risk of virus infection. Thus, HEV infection is considered to be a re-emerging zoonotic disease in industrialized countries as infections with genotype 3 HEV in humans have increased both in Western Europe and in North America (Pischke and Wedemeyer, 2010).

Additionally, some researchers have detected HEV in livers of pigs slaughtered for human consumption (Feagins et al., 2007, Wenzel et al., 2011), suggesting that some animals can be infected for periods exceeding 5-6 months of age, which is the average age of pig slaughter in Colombia. Pig liver is a common dietary component in many countries and is used as an ingredient in sausages or is grilled for consumption. In Germany, pig liver consumption was associated with HEV infection in a case study (Wichmann et al., 2008). Additionally, the consumption of raw sausages containing pig liver was identified as the probable source of an

HEV infection in France (Colson et al., 2010).

Therefore, the aim of this study was to determine the presence of the HEV genome in pig livers both from slaughterhouses and commercial meat outlets in Antioquia.

MATERIAL AND METHODS

Ethical considerations

This project involved no damage to the environment or to human health. The protocols and procedures used in this study were approved by the Ethics Committee for Animal Experimentation from Universidad Nacional de Colombia, sede Medellín (Act No CEMED-226 of October 14, 2010).

Liver samples from processing plants in Antioquia

One hundred and fifty pig liver samples were acquired post-slaughter from 5 slaughterhouses in Antioquia. Locations were selected based on their high volume of slaughtered pigs. The animals sampled were aged 5-6 months and weighed 90-100 kg. These animals passed the relevant health inspection carried out by veterinarians from each slaughterhouse. For liver sampling, pigs were randomly selected at intervals of 10 animals, using systematic sampling techniques in the post-slaughter line, to include different sources from pig-producers municipalities of Antioquia. The pigs were slaughtered according to the guidelines of each slaughterhouse. Subsamples of approximately 3 g were taken from each liver and stored in 1.5-mL vials. To avoid cross-contamination between livers, samples were identified and transported to the Animal Biotechnology laboratory of the Universidad Nacional de Colombia, Medellín, in refrigerators at 4°C and stored individually at -80°C until processing.

Liver samples from grocery stores in Medellin

Ten commercial meat outlets from 5 different socioeconomic strata (stratum 1-5) were chosen in the city of Medellin. One hundred whole livers were acquired periodically (monthly) from December 2011 until April 2012 (10 livers from each grocery store) to ensure differences between samples. These samples were then transported to the laboratory and stored at -80°C until processing.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

To determine the presence of the HEV genome in each of the 250 liver samples, 250 mg were subjected to viral RNA extraction processes and then to RT-PCR. Each of the samples was subjected to RNA extraction using the QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer recommendations and the protocol described by Forgách et al. (2010). RNA quality and concentration was assessed by measuring the optical densities (OD_{260} and OD_{280}) in a Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA), and was verified by horizontal electrophoresis on a 0.8% agarose gel. RNA showing an OD close to 2 A_{260} was subjected to reverse transcription to obtain cDNA using 0.1 U/μL RevertAid Enzyme Mix (Fermentas, Vilnius, Lithuania), 4 μL 5X RT buffer, 2 μL 10 mM dNTPs,

1 µL 100 µM hexamer primers, 5 µL RNA 5, and deionized water to a final volume of 25 µL. The following temperature profile was used: initial period of 10 min at 25°C, 60 min at 43.5°C, and final extension for 5 min at 72°C.

The viral genome was detected from the cDNA using nested PCR using primers to amplify a region of the ORF-1 of HEV and reported by Fogeda et al. (2009) (Table 1). A final volume of 25 µL was used for the first round of PCR, which included: 2.5 µL 10X Buffer (Bioline, London, UK), 2 µL 50 mM $MgCl_2$, 1 µL dNTPs 10 µM of each dNTP, 2 µL of each primer (33.4 nm ORF-1 F, 31.5 nm ORF-1 R), 0.2 µL 5 U/µL *Taq* polymerase (Bioline), 3 µL 0.081 µg/µL cDNA, and deionized water to 25 µL. The temperature profile for the PCR consisted of initial denaturation at 94°C for 4 min followed by denaturation at 94°C for 38 s, alignment at 51°C for 45 s, and extension of 60 s at 72°C for each cycle, for 39 cycles. The first PCR amplified a fragment of 634 base pairs.

Table 1. Primers used to amplify part of the HEV ORF-1.

Primer	Direction	Sequence (5'-3')	Genome position
ORF1 F	Forward first round	CCAYCAGTTYATHAAGGCTCC	9-29
ORF1 R	Reverse first round	TACCAVCGCTGRACRTC	627-643
ORF1 FN	Forward nested	CTCCTGGCRTYACWACTGC	26-44
ORF1 RN	Reverse nested	GGRTGRTTCCAIARVACYTC	178-197

Fogeda et al. (2009).

For the second round of nested PCR, a 171-base pair fragment was amplified using the ORF1 FN and ORF1 RN primers (Fogeda et al., 2009) (Table 1). The following mix was used: Product of the first round of PCR 3 µL, 10X buffer 2.5 µL (Bioline), 2 µL $MgCl_2$ 50 mM, 1 µL dNTPs 10 µM (from each dNTP), 2 µL of each primer (30.7 nm ORF-1 FN, 26.8 nm ORF-1 RN), 0.2 µL *Taq* polymerase (Bioline 5 U/µL), and deionized water to a final volume of 25 µL. The temperature profile for the second round of nested PCR was as follows: initial denaturation at 95°C for 3 min, and a denaturation at 94°C for 35 s, annealing at 48°C for 48 s, and extension of 60 s at 72°C for each cycle for 35 cycles. The amplified fragments were verified on a 2.5% agarose gel using EZ-Vision as an intercalating agent. Positive and negative (mixture free of cDNA) controls were used in all tests.

Sequencing

Some of the amplified samples were sequenced by MACROGEN (Rockville, MD, USA). Using these sequences, a BLASTn analysis of the HEV ORF-1 molecular marker for the liver samples was conducted.

Data analysis

Animal samples were taken at random using systematic sampling techniques. The sample size (number of livers evaluated), both in the slaughterhouses and grocery stores, was calculated with a confidence of 95%, an error percentage of 6%, and a prevalence of 50%, using the Epi-Info 2000 program. The information collected was analyzed statistically using the SAS® program (SAS Institute, Cary, NC, USA) and applying the Chi square test ($P < 0.05$). The descriptive analysis of the variables that were taken into account in the study was

expressed in percentages and categorized by slaughterhouse, region where the pigs originated from, and socioeconomic stratum.

Results

Detection of HEV OFR-1

After standardizing the RT-PCR technique to determine the presence of the HEV genome in the pig liver samples, the band of 171 base pairs was observed (Figure 1). This identified the positive liver samples for HEV ORF-1. Figure 1 show that the positive control (C+) and livers 1 and 2 tested positive for the viral genome, while livers 3, 4, and 5 tested negative.

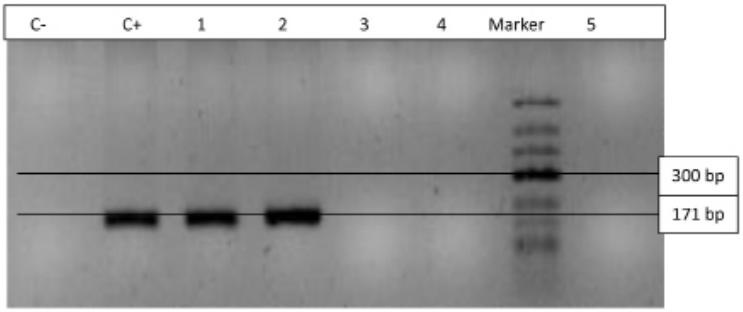

Figure 1. Amplicon of 171 base pairs from HEV ORF-1 in livers from slaughterhouses.

Livers in slaughterhouses from Antioquia

Table 2 shows the results of RT-PCR for 150 livers evaluated individually in the 5 slaughterhouses. The presence of the HEV genome in pig livers from slaughterhouses distributed for human consumption on average exhibited 41.3% positivity for the 5 slaughterhouses evaluated. There was a statistically significant difference ($P < 0.05$) observed, in which slaughterhouse 2 showed the lowest positivity (30%), while slaughterhouses 1 and 3 showed the highest (50%). For ethical considerations, the names of the slaughterhouses from which the samples were taken are not included.

Table 2. Percentage of pig livers testing positive and negative for HEV by RT-PCR, categorized by processing plant.

	Plant 1	Plant 2	Plant 3	Plant 4	Plant 5	Average
Total Samples	30	30	30	30	30	30
Positives	15	9	15	11	12	12.4
% Positives	50[a]	30[a]	50[a]	36.7[a]	40[a]	41.3[a]
% Negatives	50[a]	70[b]	50[a]	63.3[b]	60[b]	58.7[b]

[ab]Within just one column, means with a different superindex are statistically different ($P < 0.05$).

Positivity of HEV in pig livers in different subregions of Antioquia

Table 3 shows the distribution of pig livers obtained by slaughterhouse sampling in different subregions of Antioquia, as well as whether they were positive for the HEV genome.

By grouping pig livers by departmental subregions, positive samples for the HEV virus were obtained from 6 of these subregions, indicating that the virus is circulating in pigs from different sectors in Antioquia. The positivity of the pigs livers by subregion of origin was 32.8% for the North and 83.3% ($P < 0.05\%$) for Northeast Antioquia. Notably, in municipalities in northern Antioquia, which produce the most swine in both the department and the country, the lowest levels of the HEV genome were found in livers. Additionally, the 4 regions with the highest levels of HEV in pig livers (Northeast, West, East, and Southeast) were those with a smaller sample size, which may have affected the results.

Table 3. Percentage of livers testing positive for HEV, categorized by subregion of Antioquia.

Subregion	Northeast	North	West	East	Aburrá Valley	Southeast	Total
Total Samples	6	67	10	6	48	13	150
Positives	5	22	7	3	19	6	62
% Positives	83.3%	32.8%	70%	50%	39.6%	46.1%	41.3%
% Negatives	16.7%	67.2%	30%	50%	60.4%	53.9%	58.7%

Positivity for HEV in livers at grocery stores in Medellín

Table 4 shows the results for detection of the HEV genome in pig livers sold in meat outlets in the city of Medellín. Based on grouping the 100 livers evaluated from meat outlets in different socioeconomic strata in Medellín (20 livers per strata), an average of 25% tested positive for HEV RNA; this difference was significant between the various social strata ($P < 0.01$), for which the percentage varied between 5% for stratum 2 and 50% for stratum 4.

Table 4. Percentage of livers testing positive and negative for HEV, categorized by socioeconomic stratum.

	Stratum 1	Stratum 2	Stratum 3	Stratum 4	Stratum 5	Average
Total Samples	20	20	20	20	20	20
Positives	3	1	6	10	5	5
% Positives	15[a]	5[a]	30[a]	50[a]	25[a]	25[a]
% Negatives	85[b]	95[b]	70[b]	50[a]	75[b]	75[b]

[ab]Within just one column, means with a different superindex are statistically different ($P < 0.01$).

Sequencing

Figure 2 shows the electropherogram for the nucleotide sequence of ORF-1 for HEV in swine liver found in this study compared with sequences from GenBank reported by Okamoto et al. (2001).

Figure 2. Sequence of nucleotides for ORF-1 for HEV in swine liver in Antioquia, compared with the results of Okamoto et al. (2001).

After sequence editing (MACROGEN), a reliable sequence of 131 bp was obtained. This was used to perform a BLASTn analysis of the HEV ORF-1 molecular marker in pig livers from Antioquia. We compared this sequence with that published by Okamoto et al. (2001) and found that it corresponded to nucleotides 70-200 of ORF-1 from HEV porcine genotype 3 detected in Japan and reported in the GenBank under the code AB073912; 95% similarity was observed. Similarly, the ORF-1 HEV sequence of pig livers from Antioquia was 95% similar to the HEV genotype 3 detected in the US (Dong et al., 2012).

DISCUSSION

The presence of the HEV genome in pig livers from slaughterhouses and grocery stores in Colombia has not been previously evaluated. Thus, we provided molecular evidence of the presence of HEV in pigs in this country.

Leblanc et al. (2010) detected HEV RNA in feces and samples from several organs (lymph node 25.5%, bile 18.6%, feces 13.9%, and liver 20.9%) of pigs killed in slaughterhouses in Canada. In China, Li et al. (2009) found that 3.5% of pig livers obtained from slaughterhouses tested positive for HEV RNA. The results of this study demonstrate that at the age of commercial slaughter of pigs in Colombia (between 5 and 6 months), a high percentage of pigs were positive (41.3%) for HEV RNA in their livers, with higher than those reported in other parts of the world.

This indicates that not only people who are in direct contact with the pigs in production plants and slaughterhouses (workers) are exposed to HEV, but also the end consumers of these types of foods. This viral agent is sensitive to the cooking process (Feagins et al., 2008), and thus pigs livers in Antioquia should be appropriately prepared to reduce the risk of infection.

Chang et al. (2009) found that at slaughter age, most pigs (> 70%) were immune (because of the presence of antibodies) to HEV, and did not have a history of clinical symptoms or abnormalities associated with viral infection. Studies in Brazil have demonstrated that this pattern is common in commercial pigs, as they contain high levels of anti-HEV antibodies at slaughter age; however, other studies showed that a significant percentage of animals exhibit viremia or have traces of HEV in their feces (dos Santos et al., 2011). The above the dynamic of viral excretion in pigs destined for human consumption should be further examined to determine whether these animals present a risk of active hepatic viruses at the moment of slaughter or if the presence of a viral genome exists only without infectious virions.

Regarding livers from grocery stores for human consumption, HEV RNA was detected in 1.9% (363 samples) of tested livers originating from supermarkets in Japan (Yazaki et al., 2003), 6% (62 samples) of packets of liver in Holland (Bouwknegt et al., 2007), and 11% (127 samples) of packets of liver in the USA (Faegins et al., 2007). Additionally, Wenzel et al. (2011) that in 200 liver samples in Germany, 4% were positive for HEV RNA. These reports found much lower HEV RNA-positive samples compared to grocery stores tested in this study, for which the average was 25%. This positivity average was 13-, 4.2-, 2.3-, and 6.3-fold greater than that found in Japan (Yazaki et al., 2003), Holland (Bouwknegt et al., 2007), USA (Faegins et al., 2007), and Germany (Wenzel et al., 2011), respectively.

Faegins et al. (2007) reported that livers from grocery stores in the US contained HEV RNA. Moreover, they found that all samples were in the genotype 3 group, which was the same as that found in humans in the US. This agrees with the results of our study, in which HEV genetic sequences from genotype 3 were found in pigs. This implies that HEV in pigs

used for human consumption circulate in Colombia. However, only a fragment of the viral genetic sequence was observed, and it is unknown whether other species can be infected.

Bio-studies carried out by Feagins et al. (2008) revealed that the HEV present in commercial pig livers can be rendered efficiently inactive if the food is prepared hygienically and the pork and liver are appropriately cooked before consumption. In Germany, the consumption of offal, such as pig liver and parts of wild animals (wild boar), was associated with native infections by HEV (Wichmann et al., 2008). Similar cases were reported from the consumption of venison with HEV in Japan (Tei et al., 2003). However, there have been no cases reported of hepatitis E related to the consumption of pork offal in Colombia. This is likely because of the conventional cooking processes routinely used in restaurants and homes in Colombia, which are in contrast to the traditions of Europe or Asia, where the consumption of offal in cured meats not subject to cooking processes is more common. The segment of the genetic sequence of HEV ORF-1 identified in this study is part of the genotype 3 group, in which there is a large quantity of sequences reported in both pigs and humans; supporting that this virus is zoonotic, and that it may become a public health issue if control and protection measures are not initiated.

The liver is considered to be the main infectious organ for transmitting the virus to humans. However, some extrahepatic areas of HEV replication have also been reported, such as in the small intestine and the colon, which may be involved in human exposure to the virus. Bouwknegt et al. (2009) reported the presence of HEV RNA in the viscera 28 days after infection in over 50% of animal samples infected experimentally. The meat samples evaluated in this study were often destined for commercial ends, primarily for the production of sausages or processed meats (Williams et al., 2001). HEV particles remain infectious and can resist external and internal temperatures of under or badly cooked meat (Emerson et al., 2005; Feagins et al., 2008). Additionally, after slaughter, animal parts are kept in cooling chambers that may favor HEV viability in the organs and tissues of animals that are infected during slaughter.

These findings support the results of our study. We found that the positivity of livers from slaughterhouses was much higher than in those from commercial meat outlets. This may be because of the seizure of livers which exhibit some type of macroscopic issue in the meat processing centers, and that the cold chain for the livers was interrupted at some stage during transport or storage. Subsequent to slaughtering the animals, some viral decay was observed under the environmental conditions and handling used at the slaughterhouses and cold chain. This leads to the breakdown of the HEV RNA, which is labile to environmental temperatures. In support of the hypothesis that the post-slaughter cold chain plays an important role in viral decay, we found that the percentage of samples testing positive for HEV was higher in meat outlets found in the higher strata (4 and 5) in the city, where cold chains were more accessible and strict than those found in lower strata. Thus, temperature may affect HEV.

HEV is labile to high temperatures, such as those reached during the full cooking process, highlighting the importance of consuming properly cooked foods to ensure that any virus present is inactivated (Feagins et al., 2008). Although we detected the presence of the HEV genome in pig liver samples, this does not indicate that the virus is infectious when detected, or that it can infect humans or other animals. Thus, additional studies focused on viral isolation and the detection of negative RNA for the virus may confirm or disprove their viability.

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