Evaluation of hepatitis E virus RNA persistence in experimentally contaminated cured pork liver sausages

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Abstract

Hepatitis E is a disease sustained by RNA viruses, which have four different genotypes, all of which are responsible for acute forms of hepatitis. Genotypes 1 and 2 infect only humans, causing epidemics mainly transmitted by contaminated water, while genotypes 3 and 4 are zoonotic, and the infection is linked to the consumption of raw or undercooked meat or meat products. Hepatitis E virus (HEV) genotypes 3 and 4 have been detected in domestic *Suidae*, considered the asymptomatic reservoir of HEV, and in wild animals such as wild boar and deer. Despite scientific studies that have highlighted the presence of HEV in cured meat products, such as pork liver sausages, the viral persistence in the different production steps of curing has not been evaluated. Therefore, this study aimed to evaluate the persistence of HEV genotype 3 during the different curing and storage times of experimentally contaminated pork liver sausages using biomolecular methods. The sausages tested positive at all curing and storage times. This study confirms the potential risk attributed to pork liver sausages in HEV transmission. However, to guarantee an efficient risk assessment, future studies will be performed to correlate the presence of HEV RNA with infectious viral particles.

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Introduction

Hepatitis E is an acute viral disease caused by the hepatitis E virus (HEV) that is classified in the family Hepeviridae. Strains affecting humans belong to the *Orthohepevirus A* species, recently re-named *Paslahepevirus balayani* from the *Paslahepevirus* genus, according to the new taxonomy classification by the International Committee on Taxonomy of Viruses (n.d.).

The characterization of the P. *balayani* genome showed eight different genotypes (HEV-1 to HEV-8) and only one serogroup (Smith *et al*., 2020). HEV-1 and HEV-2 are linked only to human infections, and they have been associated with significant outbreaks and epidemics, particularly in developing countries (Pallerla *et al.*, 2020). Instead, HEV-3 and HEV-4 are associated with zoonotic infections that cause small outbreaks (Said *et al*., 2009; Garbuglia *et al*., 2013; Hakim *et al*., 2017). These infections typically occur when consumers eat raw or undercooked food of animal origin, such as sausages, made with meat or liver from infected wild or domestic animals (boar, pigs, and deer) (Tei *et al*., 2003; Yazaki *et al*., 2003; Masuda *et al*., 2005; Deest *et al.*, 2007; Colson *et al*., 2010; Ricci *et al*., 2017). HEV-4 is spread in East Asia, while HEV-3 was detected worldwide and is recognized as an emerging pathogen in Western countries, including Europe, where, over the past decade, there have been more than 21,000 cases with 28 deaths (Ricci *et al*., 2017). In particular, there is an increasing trend of cases of HEV infection in Italy, mainly in central-northern Italy (Istituto Superiore di Sanità, n.d.). In Italy, the HEV seroprevalence detected in blood donors showed low to moderate rates (from 1.3% up to 27.20%). High levels in some regions and/or provinces were mainly attributable to eating habits (Spada *et al*., 2022). In Italy, the consumption of raw or undercooked pig/wild boar meat and liver sausages represents one of the main risk factors linked to human HEV infection (Garbuglia *et al*., 2021). In fact, the consumption of pork meat and its products increases every year, reaching 18.8 kg *pro capite* (Lenti, 2022).

The incubation periods (30-60 days) of this virus do not allow for a correlate of the infection with the food vehicle responsible for food-borne illness (Garbuglia *et al.*, 2021). Moreover, another critical point is that most of the suspected food was no longer available for the HEV investigation, at the onset of symptoms. So, to better understand the role of pork products in the transmission of HEV, this study aims to investigate the persistence of HEV RNA in artificially contaminated pork liver sausages cured and stored at different time intervals.

Materials and Methods

Virus stock preparation

The cell culture-adapted HEV genotype 3 strain 47832c (GenBank acc. no. KC618403), originally isolated from a serum sample of a chronically HEV-infected patient (Johne *et al.*, 2014), was used in all experiments. A cell line of A549 cells persistently infected with this virus strain (Johne *et al.*, 2014; Johne *et al.*, 2016) was used for the production of the virus. The persistently HEV-infected A549 cell line was grown in Dulbecco's modified eagle medium with 10% fetal bovine serum (10270106, Gibco, Waltham, MA, USA) and 100 µg/mL gentamicin (15750060, Gibco, Waltham, MA, USA) at 37°C and 5% CO_2 in a humidified incubator. After 7 days, some of the flasks were subjected to three cycles of freezing (-20°C) and thawing [room temperature (RT)] to release non-enveloped virus particles from inside the cells into the supernatant. The resulting supernatant was stored at -20°C. The viral load was evaluated with real-time polymerase chain reaction (PCR).

Sausage preparation

A total of 11 liver sausages were prepared in collaboration with a local sausage company. The liver sausages were prepared according to the following recipe: 60% pork, 40% pork liver, and 2.5% sea salt. All pork meat and liver were previously tested with biomolecular methods to assess the absence of HEV RNA. Fresh sausages were uniformly infected with a viral suspension containing 1000 GC/g by injection along the entire sausage. One sausage sample was used as the start point control (without curing and storing, T_0). Subsequently, all sausages were cured at a temperature between 20°C and 25°C. Six sausage samples (Tc 10, Tc 15, Tc 20, Tc 25, Tc 30, Tc 40) were analyzed after 10, 15, 20, 25, 30, and 40 curing days. Moreover, four sausage samples (Ts 20, Ts 30, Ts 40, Ts 45) cured for 40 days, were stored at 4 ºC for 20, 30, 40, and 45 days.

Sample preparation and nucleic acid extraction

Virus extraction was performed following the method described by Di Pasquale *et al*. (2019) with appropriate modifications. Briefly, 1 g of liver sausage was shredded with a sterile blade and added to a tube containing 3.5 mL of TRIZOL Reagent (Life Technologies, Monza, Italy) and one sterile tungsten carbide bead (3 mm diameter). Samples were homogenized for 5 minutes at 25 hz/s in Tissue Lyser II (Qiagen, GmbH, Hilden, Germany). After mechanical disruption of the livers, samples were incubated at RT for 15 minutes and centrifugated at 8000× g for 20 minutes at 4°C. Then the supernatant was added to 0.7 mL of chloroform, vortexed for 15 seconds, and incubated for 15 minutes at RT. The aqueous phase obtained by centrifugation at 8000× g for 15 minutes at 4°C, was recovered and stored at -80°C until use.

Touchdown reverse transcription-polymerase chain reaction and touchdown hemi-nested polymerase chain reaction

The touchdown reverse transcription-PCR (TD/RT-PCR) and assay-touchdown hemi-nested PCR (TD/hn-PCR) were carried out as described in detail by Lorusso *et al*. (2022). Briefly, the RNA extract was retrotranscribed by TD/RT-PCR and subsequently amplified with TD/hn-PCR to increase sensitivity using primers reported by Drexler *et al.* (2012), amplifying a fragment of 346 bp belonging to RdRp of ORF1.

Detection of amplified products

TD/hn-PCR-amplified products were displayed by electrophoresis on 1.5% (w/v) agarose NA (Pharmacia, Uppsala, Sweden) gel in 1X tris-borate-EDTA buffer containing 0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.0 (USB, Cleveland, OH, USA), and stained with Green Gel Safe 10000X Nucleic Acid Stain (5 ll/100 ml) (Fisher Molecular Biology, USA). The Gene RulerTM 100 bp DNA Ladder molecular weight marker (MBI Fermentas, Vilnius, Lithuania) was used. Image acquisition was performed with Gel DocTM EZ imager Bio-rad (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Results

All sausage samples analyzed at different curing and storage times tested positive for HEV RNA, as described in Table 1.

Discussion

In our study, all pork liver sausages experimentally contaminated with HEV viral particles tested positive for HEV RNA at all curing and storage times (Table 1). Despite our study being focused on experimentally contaminated pork liver sausages, the results obtained have been enforced by previous studies on liver sausages marketed in Europe that reported a prevalence of HEV RNA from 4.3% to 70% (Giannini *et al*., 2018; Boxman *et al.*, 2019; Montone *et al*., 2019). In addition, these studies suggest that the curing process potentially preserves the HEV RNA (Di Bartolo *et al.*, 2015).

Table 1. Results of hepatitis E virus RNA analysis.

		Curing time						Storage time			
Day	T0	Tc10	Tc 15	Tc 20	Tc 25	Tc 30	Tc 40	Ts 20	Ts 30	Ts 40	Ts 45
HEV RNA test	+	+	+	+	+	+	+	+	+	+	+

HEV, hepatitis E virus.

Although curing is a technique useful in increasing the shelf life of food by reducing its pathogenic and altering microbial load, it has been demonstrated that it is not able to reduce the viral load (Sankaranarayanan *et al*., 2019; Colavita, 2023). Especially, the antimicrobial action of curing is mainly due to the reduction of activity in water and the lowering of pH (Colavita, 2023). Previous studies reported that food-borne viruses are characterized by high resistance to extreme pH, drying and thermal stress, and additives normally used in sausages since they are without envelope (Straube *et al*., 2011; Johne *et al*., 2016; Bosch *et al*., 2018; Wolff *et al*., 2020; Wolff *et al*., 2022). To date, it is well known that cooking contaminated pork or wild boar meat and their meat products, at 71°C for no less than 20 minutes, is useful in reducing the risk of HEV infection (Barnaud *et al*., 2012; Ricci *et al*., 2017).

Our study detected a high persistence of HEV RNA in pork liver sausages. It could be due to the presence of fat in their composition, which could act as protection against degrading enzymes of viral particles, such as proteases and RNAsi. This hypothesis, however, requires further investigations that allow us to understand the role of fat in the potential protection of HEV viral particles and how/if it could interact with them.

Moreover, we used reagents dissolving the fat portion to improve the extraction efficiency since it could prevent the extraction step of viral particles, producing false negative results (Sair *et al*., 2002; Martínez-Martínez *et al*., 2011; Schrader *et al*., 2012; Szabo *et al*., 2015).

In the present study, we evaluated only the occurrence of viral RNA without determining the presence of infectious viral particles in the liver pork sausages analyzed (Kamar *et al*., 2014). Nevertheless, when foods have a high GC/g of viral particles, we can assume that there are also infectious particles (Giannini *et al*., 2018). To date, the infectious dose of HEV is not known, but the severity of the disease depends on the presence of infectious viral particles and the host immunity (Tsarev *et al*., 1994; Kamar *et al*., 2014), particularly for fragile individuals such as immunocompromised persons, pregnant women, and persons with liver disease (Giannini *et al*., 2018). Finally, to guarantee an efficient risk assessment, a future study will be performed to correlate the presence of HEV RNA with infectious viral particles.

Conclusions

Our study shows that curing is not an effective method to eliminate the presence of HEV RNA in pork liver sausages. Therefore, to prevent potential epidemic outbreaks of HEV due to the intake of meat and/or liver products consumed raw or undercooked, several approaches should be followed: i) educating consumers through information campaigns regarding the consumption of pork and wild boar meat and sausages cooked; ii) providing systematic sampling to evaluate the presence of HEV RNA in meat products to ensure the consumers' health.

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