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ORIGINAL PAPER

Occurrence of HEV-RNA in Italian Regional Pork and Wild Boar Food Products

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Abstract
Hepatitis E is an emerging threat in industrialized countries. The foodborne transmission linked to consumption of pork and game meat is considered the main source of autochthonous infection. In Europe, small outbreaks have been reported linked to the consumption of pork liver sausages and wild boar meat. Based on previous findings and on increasing evidence of pork and game meat as a vehicle for HEV infections, the present study investigated the occurrence of HEV in 99 pork and 63 wild boar sausages and salami sold in Southern Italy. The HEV genome was detected in four wild boar sausages. Sequencing from 2 wild boar sausages confirmed that the HEV strains detected belonged to HEV-3 genotype, not assigned to any defined subtype. Data obtained confirmed the possible occurrence of HEV in pork products and in game. Although the detection rate is low, these products are frequently consumed raw after curing, whose effect on virus viability is still unknown.

Keywords HEV · Genotype 3 · Pork products · Wild boar · Sausages

Introduction

Hepatitis E is an acute human disease, usually self-limiting with a low mortality rate, but it can become chronic in transplant patients and may lead to extrahepatic manifestations. The etiological agent is a small quasi-enveloped positive RNA virus, belonging to the family *Hepeviridae*. The five genotypes infecting humans belong to the *Orthohepevirus A* species. Genotypes HEV-1 and HEV-2 are exclusively responsible for human infections causing large waterborne outbreaks in low income countries (Aggarwal and Jameel 2011). Genotypes HEV-3 and HEV-4 not only infect humans but also many other mammal species (pigs, wild boar, deer) and are the main cause of cases reported in developed countries (Kamar et al. 2017). Recently, the genotype HEV-7 has been detected in camels and once in an immunocompromised patient affected by Hepatitis E who regularly consumed camel milk in the Middle East (Lee et al. 2016).

The genotypes HEV-3 and HEV-4 are zoonotic; pigs, wild boar, and less frequently other animal species (such as deer) act as reservoirs. In Europe, the majority of human cases are associated with HEV-3 which is widespread in the European pig population and has also been identified in wild boar. Foodborne zoonotic transmission of HEV-3 was frequently reported in sporadic and clustered human cases. The first evidence of foodborne transmission was described in Japan, when cases of Hepatitis E were associated with the consumption of raw or undercooked pork and venison and the same viral sequences were detected in patients and leftovers (Matsuda et al. 2003; Tei et al. 2003; Yazaki et al. 2003; Li et al. 2005). More recently, small outbreaks have occurred in Europe linked to the consumption of raw pork liver sausages (*'figatelli'*) and undercooked wild boar meat (Colson et al. 2010; Rivero-Juarez et al. 2017), and confirmed by detection of the same virus in patients and leftovers.

Products containing raw liver such as *'figatelli'* are more frequently found to be positive for HEV-RNA (Colson et al. 2010; Martin-Latil et al. 2014). In Italy, HEV RNA was

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detected in raw and dry pork liver sausages (Di Bartolo et al. 2015).

Furthermore, while detection of HEV in muscles from pigs has rarely been reported (Di Bartolo et al. 2012), linked most probably to cross-contamination, a recent study in Germany showed that 82.9% of the wild boar found to be HEV positive in liver were also HEV positive in muscles (Anheyer-Behmenburg et al. 2017). Consumption of pork and wild boar raw sausages or cured meat is frequent and the occurrence of HEV in those food needs further investigation. Furthermore, the effect of process technologies (such as curing, salting, pH) commonly used to produce sausages and/or salami on HEV viability is unknown.

To improve knowledge on the risk associated to HEV transmission and develop a risk assessment model, the Istituto Zooprofilattico Sperimentale del Mezzogiorno, with the support of the Italian Ministry of Health, carried out an integrated surveillance on viral hepatitis E in the Campania region, assessing the risk of transmission from environmental matrices (ground water, surface water, and wastewater), livestock breeding, edible leafy vegetables (first and fourth range products, berries and aromatic herbs), and food of animal origin (pork and wild boar sausages).

In this study, we report the results on Hepatitis E virus detection and quantitative analysis on the 63 wild boar sausages and 99 pork products (salami and sausages) collected in Campania region during the surveillance period.

Materials and Methods

Sampling

Between June 2017 and March 2018, 99 pork products (2 raw bacon, 60 cold cuts, 4 salami with short-seasoned '*soppressata*,' and 33 raw cured meats) were purchased in small local markets in Campania, Southern Italy. Sixty-three wild boar sausages (home-made products) were sampled in the same region and period. Wild boars used for food production were hunted nearby protected areas as a part of the national program for wild boar control.

The samples were georeferenced (Fig. 1) with a GPS MAP 64st (Garmin, Milan, Italy) and the coordinates were calculated with geographical reference system wgs84/utm zone 33n (EPSG project; https://spatialreference.org). All matrices were accompanied by a sampling report containing

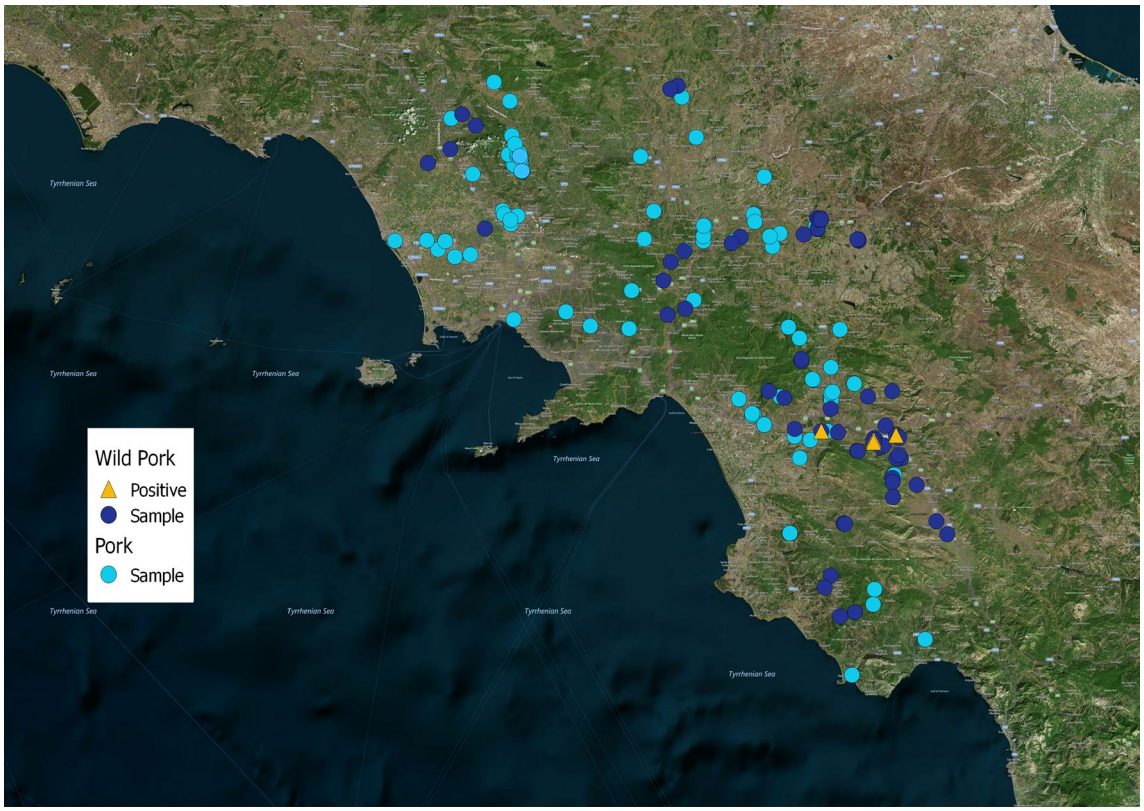

Fig. 1 Georeferentiation of collected samples. Pork and wild boar products collected and wild boar products positive for HEV obtained in the study are highlighted with different symbols and colors (see legend)

the descriptive record of the sample (code, sampling coordinates, type of meat, seasoning, etc.).

Nucleic Acid Extraction

Sample preparation was done according to Szabo et al. (2015) with slight modifications. Briefly, each sample was sliced and fat and other components such as casing, peppercorns, etc. were manually removed. The remaining meat tissue was then grounded and 5.0 ± 0.2 g were transferred to a stomacher bag and spiked with 10 µl of suspension of Mengovirus (strain MC_0, 1.6×10^5 $TDCI_{50}$/ml), which was used as a sample process control. Seven milliliters of TriReagent Solution (Life Technologies, Monza MB, Italy) was added and sample homogenized at maximum speed for 2 min. The liquid phase was clarified by centrifugation (10,000×*g*, 20 min, 4 °C), recovered, and 1.4 ml of chloroform was added. After 15 min at room temperature with gentle shaking, the supernatant was recovered after centrifugation (10,000×*g* for 15 min at 4 °C) and measured. One ml was subsequently used for RNA extraction using an automated extractor (NucliSENS miniMag; bioMerieux, Grassina FI, Italy) according to the manufacturer's protocol. One hundred µl of total RNA was eluted and used for testing.

For quantification of HEV in samples found positive, a second RNA extraction was performed, using the same protocol described above with some modifications: 100 mg of sample spiked with process control virus was homogenized with 50 mg zirconia beads using a mechanical disruptor (Tissue Lyser; Qiagen, Milan, Italy) for three runs for 2 min at 46 oscillations s^{-1}. After centrifugation at $10,000 \times g$ for 15 min at 4 °C, the recovered supernatant was subjected to RNA extraction as described above.

Extraction Efficiency

The RNA of Mengovirus process control was detected by real-time RT-qPCR as previously described (Costafreda et al. 2006). The extraction efficiency was estimated by comparative cycle threshold (Ct) method (Schmittgen and Livak 2008) between Mengovirus detected in spiked sample and Mengovirus used for spiking. RNA from samples negative for HEV and displaying < 1% of recovery were extracted again.

HEV Real-Time RT-qPCR

For each reaction, a 5 µl aliquot of sample RNA was analyzed using the RNA UltraSense One-Step qRT-PCR System (Life Technologies), 1.25 µl of RNA Ultrasense enzyme mix and the following concentrations for primers and probe: 500 nM for forward primer JVHEVF (5′-GGTGGTTTCTGG GGTGAC-3′), 900 nM for reverse primer JVHEVR (5′-AGGGGTTGGTTGGATGAA-3′), and 250 nM for probe JVHEVP-MGB (5′-FAM-TGATTCTCAGCCCTTCGC -MGB-3′) (Jothikumar et al. 2006; Garson et al. 2012). Reverse transcription was done at 50 °C for 60 min, followed by inactivation for 5 min at 95 °C and 45 cycles of 15 s at 95 °C, 1 min at 60 °C, and 1 min at 65 °C. A linearized plasmid containing the target sequence was used to generate the standard curve (dynamic range $1 \times 10^0 - 1 \times 10^4$ copies/µl); curves with a slope lying between − 3.1 and − 3.6 and a $R^2 \geq 0.98$ were used for quantification. Each sample was tested in duplicate reactions and the average concentration of the two replicate reactions was used for quantification. PCR inhibition was ruled out using an external amplification control (in vitro synthesized RNA) and amplifications were considered acceptable if inhibition was ≤ 50%.

Sequencing and Subtyping

The RNA from samples positive for HEV by real-time RT-PCR was analyzed by nested RT-PCR amplifying a 493-bp fragment in the ORF2 (Boxman et al. 2017). The DNA amplicons were sequenced by Eurofins Genomics (Germany) and submitted to NCBI (https://www.ncbi.nlm.nih.gov) under the accession numbers: Sal02NA17, MK801780; Sal04NA17, MK801781. The sequences were submitted to BLAST analysis and to the HEVnet Typing Tool (https://www.rivm.nl/mpf/typingtool/hev/) for genotyping (Mulder et al. 2019).

A Maximum Likelihood (ML) phylogenetic tree was constructed with the Tamura-Nei parameter model and Gamma distribution as suggested by the MEGA 7 software model test (https://www.megasoftware.net) based on 1000 bootstrap replications. The dataset used to determine the subtype includes the reference strains of HEV-3 suggested by Smith et al. (2016) and the HEV strain detected in Italy and Europe available on NCBI database.

Results

All samples were found positive for the process control (Mengovirus). However, the RNA extractions from 41 samples gave a recovery rate of less than 1% and were found negative for HEV. Subsequently, samples were retested. In Table 1 extraction efficiency results for each category of samples (type of food) are reported. Overall, the mean extraction efficiency was 3.97% (median 1.68%), with values ranging between 1.01 and 33.68%.

Table 1 Extraction efficiency for the different categories of samples

Sample category	Curing	No of samples	Extraction efficiency (%)			
			Median (%)	SD	Minimum	Maximum
Raw bacon	No	2	8.04	9.79	1.12	14.97
Pork cold cuts	Yes	60	4.45	7.35	1.03	33.68
Sausages	No	33	1.62	5.53	1	28.72
'Soppressata'	No	4	2.31	1.89	1	4.27
Wild boar salami	Yes	63	1.53	4.49	1.01	28.72

SD standard deviation

Overall, four wild boar sausages and one pork sausage resulted positive by RT-qPCR. Upon second RNA extraction, however, confirmation was achieved only for the four wild boar sausages, while a negative result was obtained for the pork sausage, possibly due to the low and non-homogeneous contamination within the sausage meat mix. To evaluate the amount of HEV in the four confirmed positive samples, a second extraction was performed using a smaller amount of the sample (100 mg). This second extraction gave an extraction efficiency ranging from 97 to 100% and the RNA was used to perform quantitative PCR that resulted in four samples below the LOQ (3.15 $\log_{10}$ genome equivalent per gram (GE/g)), with estimated viral loads of 2.04, 2.34, 2.73, and 3.03 $\log_{10}$ GE/g.

Two out of the four HEV-positive samples, collected from close sites, were further confirmed by conventional nested RT-PCR, amplifying a 493-bp fragment in the ORF2. Sequence analyses of Sal02NA17 (MK801780) and Sal04NA17 (MK801781) revealed that the two strains that shared 100% nucleotide identity (n.i.) belonged to HEV-3 and showed a high correlation (94% n.i., 100% aa. identity) with HEV strains detected from the liver of two wild boars hunted in the same geographical area (WB/HEV/NA21ITA15, MF959765; WB/HEV/NA18ITA15, KX54930) where the salamis had been purchased. The phylogenetic analysis on strains detected in this study showed clustering within the clade HEV-3abchij including the wild boar strains WB/HEV/NA21ITA15 and WB/HEV/NA18ITA15 (Fig. 2) and no correlation to any HEV reference sequences defined so far or with other sequences available in the HEVnet Typing Tool. The Italian strains showed the same p-distance (0.06) from WB/HEV/NA21ITA15 to WB/HEV/NA18ITA15, and < 0.11 with the other HEV sequences present in the NCBI database.

Discussion

There are many data to support the association between pork consumption and HEV-IgG seropositivity or the occurrence of hepatitis E (La Rosa et al. 2011). A direct link was proven by the detection of the same virus in patients and leftovers, most frequently raw pork sausages containing liver (Colson et al. 2010). To assess the occurrence of HEV in some of the typical Italian ready-to-eat food products, we investigated 162 samples containing pork or wild boar meat. The study was carried out as a part of a wider surveillance program in place in the Campania Region on HEV in environmental matrices, livestock breeding, and foods of animal and non-animal origin.

Only 4 out of 63 (6.3%) wild boar salamis were found positive for HEV-RNA. None of the 39 not-cured pork products were found positive. In this work, we reported the HEV detection in home-made, cured wild boar sausages. The occurrence of HEV in wild boar sausages or salami was rarely investigated. Two studies reported either absence of HEV (5 samples analyzed) in Switzerland (Moor et al. 2018) or 1 HEV-RNA positive of the 10 investigated raw wild boar sausages not containing liver in Germany (Szabo et al. 2015). These findings support the concern that, despite wild boar being less frequently consumed, it could also play a role in the foodborne transmission of HEV-3.

Furthermore, in our study, the four wild boar salamis found positive for HEV-RNA only contained meat and no liver. In Germany, HEV-RNA was detected in 82.9% of muscle samples from animals whose liver was positive for HEV (Anheyer-Behmenburg et al. 2017). A previous study also reported detection of HEV-RNA in 1 out of 22 (4.5%) (Schielke et al. 2015) wild boar muscles, proving the occurrence of HEV in both liver, the main organ of HEV replication, and muscles. On the contrary, HEV-RNA was not found in the muscles of pigs which had been found positive for HEV-RNA in their liver (Feurer et al. 2018). Interestingly in our study, the two sequences obtained from wild boar salamis shared 94% identity with the sequence of one strain detected in the liver of a wild boar (Aprea et al. 2018) hunted two years before in the same area in which the salamis had been purchased. This finding supports the hypothesis of the persistence of the strictly related HEV-3 strains in animals over a two-year period. Furthermore, georeferentiation of samples collected highlighted clustering of positive samples in a restricted area of the region of Campania (Vallo di Diano/Alta Valle del Sele-Tanagro) where the use of locally hunted wild boar

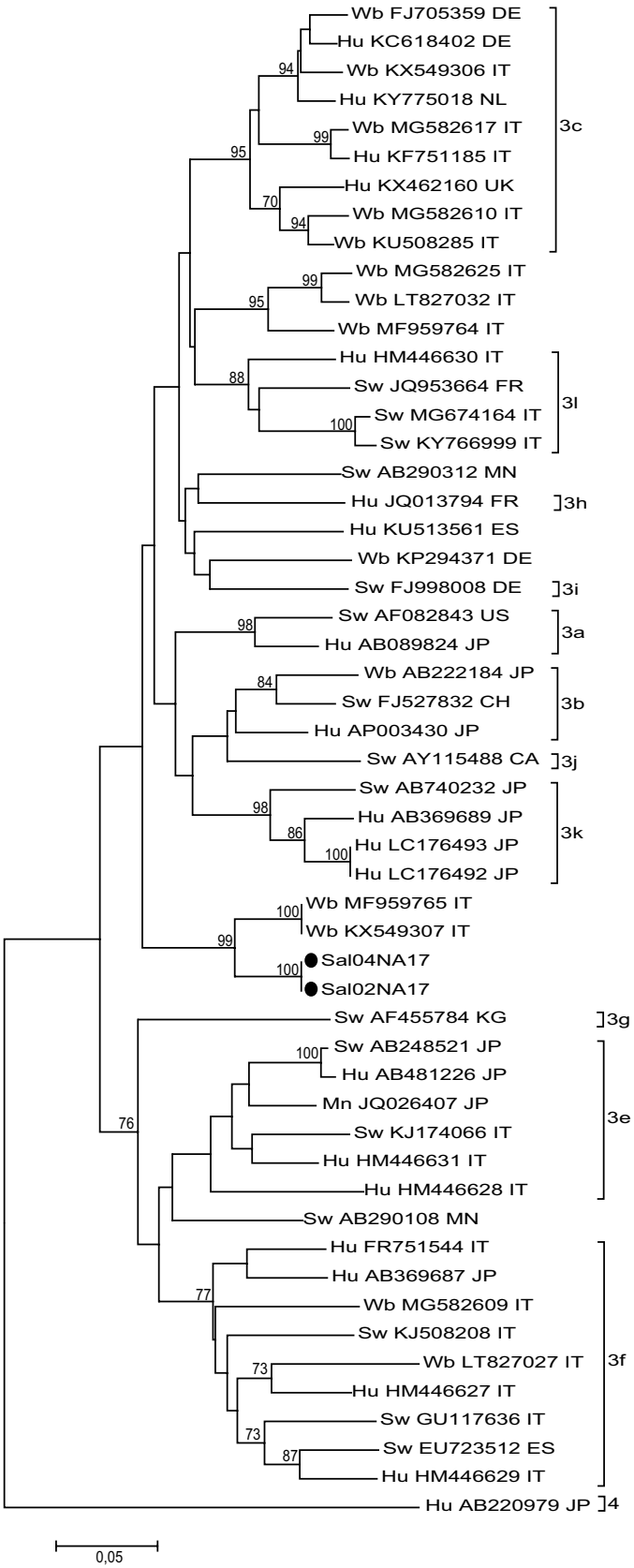

Fig. 2 Maximum likelihood phylogenetic tree built with GTR + G + I substitution model, by 1000 resampling, on 52 HEV-3 genotype partial capsid sequences including the Italian strains Sal02NA17 (MK801780) and Sal04NA17 (MK801781) indicated by black circles. HEV-4 strain as outgroup and bootstrap replicates > 70% were reported. Each entry includes host (Hu: human, Sw: swine, Wb: wild boar, Mn: monkey), accession number, and countries origin of strains

for preparation of salami and sausages is frequent and may result in an increase of exposure. To further assess the risk linked to consumption and professional exposure to wild boar (hunting and slaughtering), a study on seroprevalence in butchers and hunters operating in the area where the HEV-positive food were collected is ongoing. The role of wild animals, and in particular wild boar, as a source of HEV-3 in foodborne transmission is increasingly recognized, as proven by a recent outbreak in Spain associated with the consumption of undercooked wild boar meat (Rivero-Juarez et al. 2017). As a point of fact, wild boar is consumed less than pork but the dynamic of infection in the two reservoirs may be different. Wild boar is still found highly positive at the age of hunting (≥ 6 months) (Martelli et al. 2008), while in pigs the age of slaughtering (6–9 months) corresponds to the age at which the occurrence of HEV infection decreases (Capai et al. 2019). We have also reported detection of HEV (Ct > 40) in one pork salami, but we could not further confirm this result because, despite attempting a second extraction, the result was not confirmed.

In Italy, the notification of hepatitis E is mandatory thus providing data on acute cases of the disease, and until now none of the cases notified have been directly linked to foodborne transmission. However, epidemiological and indirect evidence such as the detection of closely related virus sequences from an Italian patient and French *'figatelli'* provides evidence for potential foodborne transmission (Garbuglia et al. 2015). Furthermore, two studies conducted in Italy identified the consumption of pork liver products and the hunting of wild boar as possible risk factors for HEV infection (La Rosa et al. 2011; Garbuglia et al. 2015). The main limit of this study is that the detection of HEV-RNA does not necessarily correspond to a viable virus and we do not know what food processes are used to produce home-made ready-to-eat products. In the absence of these data, it is not possible to fully estimate the risk associated to consumption of these foods.

In conclusion, our results provide data for the evaluation of risk of HEV transmission associated to consumption of pork and game products.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed Consent Informed consent was not required, as no human participants or individuals were included in this study.

Research Involving Human Participants and/or Animals This article does not contain any studies with human participants or animals, performed by any of the authors.

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