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Communication

First Report of Hepatitis E Virus in Shellfish in Southeast Italy

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Abstract: Hepatitis E virus (HEV) represents one of the principal causative agents of hepatitis globally. Among the five HEV genotypes affecting humans, genotypes 3 and 4 are zoonotic and are the main source of hepatitis E in developed countries. HEV has been detected in several foods. The present work investigated the presence of this virus in shellfish sold at retail in the Apulia region of Italy. The presence of HEV RNA was assessed by real-time RT-PCR in 225 shellfish samples collected during 2018. Overall, two (0.89%) of these samples tested positive for HEV RNA. To our knowledge, this is the first notification of the detection of HEV in mussels sold at retail in the Apulia region. These data highlight the potential role of shellfish as a vehicle for the transmission of viral pathogens.

Keywords: hepatitis E virus; shellfish; food-borne; risk assessment

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1. Introduction

Hepatitis E virus (HEV) represents one of the principal causative agents of hepatitis globally [1] and it is considered an emergent issue in industrialized regions, where transmission by contaminated food plays a key role [2]. HEV is a hepatotropic, single-stranded positive-sense RNA virus with a genome of approximately 7.2 kb, belonging to the *Hepeviridae* family, genus *Orthohepevirus*, *Orthohepevirus A* species [3,4]. Five of the eight genotypes of *Orthohepevirus A* (HEV-1, 2, 3, 4, and 7) infect humans [4]. HEV can exist in two forms: non-enveloped or "quasi-enveloped" [3]. All HEV genotypes belong to a single serotype [5]. The human viruses HEV-1 and HEV-2 genotypes are highly endemic in developing regions, including various regions of Africa, Asia, the Middle East, and Mexico, and are diffused through contaminated water. These viruses are accountable for a great number of outbreaks and cause severe hepatitis during pregnancy [6]. By contrast, genotypes 3 and 4 are revealed in both animals and humans and are the principal causes of hepatitis E in humans in several developed countries [7].

The World Health Organisation (WHO) has estimated that 20 million persons worldwide are newly infected with HEV annually, with 3.3 million developing acute hepatitis E and 70,000 dying of HEV-related causes [8]. Over the last decade, HEV infection has been reported to cause more than 21,000 cases of hepatitis E, with 28 deaths, in the European Union (EU), where food-borne transmission seems to be the major path of infection [9]. These data do not reflect the real number of infected people in the EU because HEV infection is not required to be reported to public health authorities in all member states of the EU, and the surveillance systems differ among countries.

In Italy, the number of individuals infected with HEV increased during the years 2007–2018, as well as doubling from 2018 to 2019, although not all regional laboratories are able to specifically differentiate HEV from other hepatitis viruses. Moreover, a peak of cases was

reached in 2019 when the number of cases doubled compared to the previous year [10]. In European countries, HEV genotype 3 is the most prevalent HEV genotype infecting humans, with relatively few patients being infected with genotype 4. These genotypes are thought to be transmitted through foods, especially the consumption of undercooked or raw pig, wild boar, and deer meat [9]. Domestic swine appear to represent the principal source of zoonotic HEV transmission in Europe and are considered the main viral reservoir [9,11]. The European Food Safety Authority has estimated that HEV can be transmitted to humans by the consumption of foods composed of meat from an infected animal and by foodstuffs fouled with HEV [9]. For the latter, virus shedding by feces (animal and human) represents an important public health risk. Indeed, environmental contamination, including contamination of organic fertilizers and water, can provide a source for HEV contamination of other foods, including shellfish, soft fruits, and leafy green vegetables [12]. In the feeding process, by filtering from their marine environment, shellfish can concentrate viral particles, becoming a potential source of human infection [13]. Although the consumption of raw shellfish, liver sausage, and wild boar meat was recognized as a hazard factor for locally acquired HEV infection in Italy [5], only a small number of surveys to date have evaluated the presence of HEV in shellfish for human consumption [13–17].

The Apulia region of southeast Italy, with an 860 km coastline [18], is a major source of shellfish for consumption throughout Italy. Shellfish are usually consumed in the undercooked or raw state in the Apulia region. To our knowledge, however, no survey to date has assessed the attendance of HEV in mussels sold at retail in the Apulia region. The present study, therefore, evaluated whether HEV could be detected in shellfish sold at retail in the Apulia region, and assessed the potential risk of HEV transmission associated with shellfish consumption.

2. Materials and Methods

A total of 225 samples of different species of shellfish commercially sold in the Apulia region were collected from January to December 2018. These samples included mussels (*Mytilus galloprovincialis*, n = 182), oysters (*Crassostrea gigas*, n = 32), and other species, including *Modiolus barbatus* (n = 5), *Tapes decussates* (n = 2), *Solen marginatus* (n = 2), *Aequipecten opercularis* (n = 1), *Callista chione* (n = 1). The geographic origins of these shellfish samples are shown in Table 1.

Table 1. Geographic origins of the tested shellfish samples.

Species	Origin					Total
	Italy	Spain	Greece	The Netherlands	France	
Mytilus galloprovincialis	157	16	9			182
Crassostrea gigas	12			14	6	32
Modiolus barbatus	5					5
Tapes decussates	2					2
Solen marginatus	2					2
Aequipecten opercularis	1					1
Callista chione	1					1
Total	180	16	9	14	6	225

All samples were collected by local health services, in accordance with national official monitoring activities for microbiological criteria of shellfish (e.g., *Salmonella* and *Escherichia coli*). All the collected samples were transported to the Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata (Foggia, Italy) and stored at 4 °C until analyzed. The presence of HEV was determined as described [13], using a method that follows the structure and quality criteria of the standardized ISO 15216-2:2019 method [19]. Briefly,

15–60 specimens of each sample were casually chosen, and their digestive tissue was cut up, cleaned, and shredded with a sterile blade. Following digestion, 2 mL proteinase K (0.1 mg/mL) and 10 µL of mengovirus clone MC_0 as process control (supplied by the Istituto Superiore di Sanità, Rome, Italy) were added to an aliquot of 2.0 g of each sample, and incubated at 37 °C for 60 min with shaking, and then at 60 °C for 15 min to deactivate the enzyme. After centrifugation at 3000× *g* for 5 min, the supernatants were recovered. Viral nucleic acids were extracted employing the Nuclisens extraction kit (BioMerieux, Paris, France) according to the manufacturer's protocol, and the eluted RNA (100 µL) was immediately analyzed or stocked at −80 °C until use.

Real-time RT-PCR for HEV was carried out on a 7500 Fast Real-Time PCR system thermocycler (Applied Biosystems, CA, USA) using an RNA UltraSense One-Step Quantitative RT-PCR System (Life Technologies, Carlsbad, CA, USA), and primers and probe targeting the ORF3 region of HEV [20,21].

The thermal cycling conditions were 50 °C for 60 min and 95 °C for 5 min, followed by 45 cycles of 95 °C for 15 s, 60 °C for 1 min, and 65 °C for 1 min. An external amplification control (in vitro synthesized RNA supplied by the Istituto Superiore di Sanità, Rome, Italy) was added to the samples to evaluate the presence of PCR inhibitors. Samples found to be HEV positive were retested (another aliquot of digestive tissue from the same pooled samples) at an independent laboratory as described [22], both for confirmation (i.e., to rule out false-positive results) and for quantification, according to Di Pasquale et al. 2019 [22], with the results defined as the number of HEV genome copies (gc) per gram of digestive tissue, and calculated according to the standardized ISO 15216-1:2017 method [23]. In detail, the quantification was done using a linearized plasmid containing the target sequence of HEV to generate the standard curve. Analyses were performed in duplicate and the average concentration of the two replicate reactions were used for quantification. All samples that tested close to or below the LOD of the real-time RT-(q) PCR assay were retested in quadruplicate reactions; only samples in which at least two wells that showed amplification were considered positive [22]. Positive samples were subjected to HEV genotyping by nested RT-PCR amplification of a portion of the ORF1 region [24].

3. Results and Discussion

Of the 225 shellfish samples tested, two (0.89%) were positive for HEV RNA by real-time RT-PCR. These two samples included one (0.5%) of the 182 samples of *M. galloprovincialis* tested and one (3.1%) of the 32 *C. gigas* samples tested. The levels of virus were slightly below the limit of quantification of the method (3.9×10^2 gc/g) with estimated concentrations of 2.5×10^2 in the mussel sample and 7.0×10^1 gc/g in the oyster sample (Table 2).

Because of the small sample size, HEV prevalence did not differ significantly among the different bivalve species (Fischer's exact test; $p > 0.05$). The two HEV positive samples originated from two distinct locations, with the mussel sample taken in the northern part of Apulia and the oyster sample taken in the southern part of Apulia. Interestingly, both positive samples were collected during spring 2018 (Table 2). However, multi-year sampling is needed to evaluate the significance of a supposed seasonal pattern.

Attempts to genotype the two positive samples were unsuccessful, as the conventional nested RT-PCR amplification yielded negative results. This may be related to the low concentration of HEV in the two samples (Table 2), roughly corresponding to <1 gc/µL in the tested RNA.

Table 2. Sampling date, city, Cq values, and HEV concentrations (genome copies/g) of the samples positive for HEV RNA.

Sample ID	City	Sampling Date (Day/Month/Year)	Species	Cq Value	HEV Concentration (Genome Copies/g)
2882	Fasano	27/03/2018	Mussel *(Mytilus galloprovincialis)*	35.5	$\approx 2.5 \times 10^2$
5450	Cagnano Varano	24/05/2018	Oyster *(Crassostrea gigas)*	38	$\approx 7.0 \times 10^1$

Viral infections have been linked to shellfish consumption, often due to undercooking of the product and sewage contamination of harvesting areas [25]. Despite shellfish consumption being a risk factor for HEV infection [13], hepatitis E following shellfish consumption has been relatively rare [26]. Several studies in Europe have reported variations in the presence of HEV in mussels harvested from regulated waters, with rates of contamination of 2.9% [12], 14.8% [11], and 24.4% [27], whereas other studies have reported no evidence of HEV. In Italy, HEV has been observed in commercially harvested shellfish, with prevalence rates ranging from 0.9% in Sicily [17] to 2.6% in the Campania region [13]. The low prevalence ratio reported in this survey was comparable to that of shellfish from Sicily [17]. One study found that HEV was present in 8.1% of mussels from Italy, although that study included bivalves that were collected from areas not approved for harvesting [28]. HEV has been detected in the environment in Italy, including in sewage [29], river waters [15], and recently, in coastal waters [13].

The detected levels of HEV RNA shown in the present work were slightly below 10^2 genome copies/g tissue, in agreement with other European studies on shellfish. Two studies in northwestern Spain reported contamination levels varying from 6.7×10^1 to 8.6×10^4 gc/g [11] and from non-quantifiable to 1.1×10^5 gc/g [24]. In the latter study, 19.5% of positive samples presented viral genome copies below the limit of quantification, and one had a concentration of 4.0×10^2 gc/g. A study from Scotland also reported a very low virus concentration in commercial mussels with only one sample quantifiable at $<10^2$ IU/mL [12], and a study from Italy showed that concentrations of HEV in shellfish were always below 10^2 gc/g [13]. Although a low concentration was estimated in the present study (2.5×10^2 and 7.0×10^1 gc/g), this cannot be overlooked. For other foodborne viruses, several surveys on shellfish-related outbreaks showed as very low viral genome levels (10–100 gc/g) were correlated to the probability of infections with NoV or HAV [30,31]. Contrary, for HEV there are current data gaps regarding the relationship between genome copies numbers and infective dose [32].

The existence of HEV in shellfish may be associated with the pollution of the shellfish harvesting area by swine waste and urban sewage [11,12]. The increasing number of patients in Italy with hepatitis E over the last years [10] may support the recent reports of HEV in shellfish. Similar to other enteric viruses, variations in the prevalence of HEV may result from differences in contamination levels of shellfish harvesting areas. These differences in contamination may be caused by geographical (e.g., distances from the seacoast, rivers, and estuaries) and environmental (e.g., sewage discharge) factors [18].

Because of the small number of positive samples, differences in HEV prevalence among bivalve species could not be calculated. A similar result was obtained in a study conducted in Spain [27]. Further studies are needed to assess possible differences in contamination among bivalve species. In this study, no data on genotype were obtained from the two positive samples. The absence of amplification by nested RT-PCR confirms the lower sensitivity of conventional PCR compared with real-time RT-PCR assays, especially in food samples with low levels of contamination [9]. In particular, the assay utilized in this study [20] has been reported to be more sensitive than other RT-qPCR assays [9]. It is tricky to achieve a helpful sequence from positive real-time RT-PCR food specimens, due to several factors (e.g., failure of identification by the conventional primers, concomitant amplification of various strains, virus concentration below the limit of detection of

traditional reverse transcription PCR, not enough extraction of clean RNA suited for sequencing) [32]. Earlier studies carried out in Italy showed that HEV genotype 3 was present in shellfish [13,17,28]. Because this genotype is the most common in Italy, it was likely present in our positive samples. However, further investigations are needed to confirm this assumption.

4. Conclusions

The present survey is, to our knowledge, the first showing the presence of HEV in shellfish commercially available in the Apulia region of southeast Italy highlighting that these foods may play a potential part in the spreading of viral pathogens. Further investigations, especially of major shellfish harvesting areas in the Apulia region, may provide more information about the spread and genotypes of HEV in the environment of southeast Italy, as well as determining possible differences among bivalve species or seasonal patterns. Furthermore, continual surveillance, quantitative data, and evaluation of infectivity of detected viruses are necessary to more precisely estimate the risks associated with HEV in foods.

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