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BRIEF COMMUNICATION

First Detection of Hepatitis E Virus in Shellfish and in Seawater from Production Areas in Southern Italy

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Abstract Shellfish samples ($n = 384$) from production areas, water samples from the same areas ($n = 39$) and from nearby sewage discharge points ($n = 29$) were analyzed for hepatitis E virus (HEV) by real-time and nested RT-PCR. Ten shellfish samples (2.6%) and five seawater samples (12.8%) tested positive for HEV; all characterized strains were G3 and showed high degree of sequence identity. An integrated surveillance in seafood and waters is relevant to reduce the risk of shellfish-associated illnesses.

Keywords Hepatitis E virus · HEV · Genotype 3 · Shellfish · Seawater · Molluscs

Introduction

Hepatitis E virus (HEV) is the sole member of the genus Hepevirus (family Hepeviridae). Four genotypes (G1 to G4) infect humans, with G1 and G2 restricted to human host, and G3 and G4 circulating also in different animals (swine, deer, wild boar, and rabbit) (Meng 2010; Pavio et al. 2010). Usually, the disease is self-limiting and resolves within 2–6 weeks, but a small number of cases experience more severe forms of the disease leading to hepatic failure. The major symptoms of hepatitis E are fever, nausea, abdominal pain, vomiting, and jaundice. Hepatitis E infection during pregnancy may result in fulminant hepatitis (Pérez-Gracia et al. 2017) and in patients with chronic liver disease, HEV may worsen rapidly to a syndrome called acute-on-chronic liver failure (Kumar and Saraswat 2013).

Foodborne transmission is a major pathway for HEV infection in Europe and is mainly associated to consumption of raw or undercooked meat and products derived from swine, wild boar, and deer (EFSA BIOHAZ Panel 2017). Shellfish can also be a potential transmission vehicle, since they can concentrate viral particles from the surrounding marine environment during their filter feeding process. Indeed, HEV RNA has been detected in shellfish worldwide (Li et al. 2007; Song et al. 2010; Crossan et al. 2012; Diez-Valcarce et al. 2012; Pol-Hofstad et al. 2013; Gao et al. 2015; Mesquita et al. 2016).

In Italy, few studies have assessed the occurrence of HEV in shellfish for human consumption and HEV has never been detected in shellfish from approved harvesting areas (La Rosa et al. 2012; Iaconelli et al. 2015; Fusco et al. 2017). Furthermore, no studies have been conducted to investigate the occurrence of HEV in seawater from shellfish production areas, even if there is evidence of HEV presence in other water environments, including sewage and river water (La Rosa et al. 2010; Iaconelli et al. 2017; La Rosa et al. 2017c).

In the present study, shellfish samples collected in growing areas in Southern Italy were analyzed for the presence of HEV, along with marine waters collected in production areas and nearby sewage discharge points.

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Materials and Methods

During 2015, 384 shellfish samples (298 mussels, 41 razor clams, 33 striped venus clams, and 12 wedge clams) were collected in 27 production areas of Campania Region, Southern Italy, distributed along 90 km of the coastal area. Sample preparation was performed according to ISO/TS 15216-1:2013 (ISO 2013). In the same period, 68 water samples (39 seawater samples from shellfish production areas and 29 water samples from discharge points) were collected in 10 selected production areas. Twenty liters of water samples was concentrated as previously described (La Rosa et al. 2017b). Viral nucleic acids from shellfish and water samples were extracted using NucliSense easyMAG (bioMérieux).

For shellfish samples, analysis of HEV was performed by real-time RT-(q)PCR assay according to Jothikumar et al. (2006) with slight modifications. PCR inhibition was ruled out by an external amplification control. A linearized plasmid containing the target sequence was used to generate the standard curve. Positive samples were subjected to genotyping through amplification of a portion of ORF1 using a broad range assay targeting the methyltransferase (MTase) gene of the ORF1 region of HEV (Fogeda et al. 2009). All positive PCR products were subjected to automated sequencing (Bio-Fab Research, Italy). Water samples were directly subjected to nested PCR as described for shellfish samples.

All positive samples were further analyzed using two different assays targeting the ORF2 region, to allow characterization at subtype level. Primers used in the present study are listed in Table 1.

The phylogenetic tree was constructed using the neighbor-joining method by MEGA software version 6.0 and the reliability of the clusters was assessed by bootstrapping using 1000 replicates.

Results and Discussion

The presence of HEV was detected by real-time PCR in ten (eight mussels, two razor clams) of the 384 shellfish samples (2.6%), with positive samples collected from seven different production areas of the 27 included in the study (26%). Concentrations of HEV were below the quantification limit of the method and ranged between 30 and 90 genome copies/g of digestive tissue. Conventional amplification and characterization by sequencing was achieved only for two shellfish samples, characterized as G3.

In water samples, HEV RNA was detected in five of 39 seawater samples (12.8%) distributed in three production areas, all characterized as belonging to G3 HEV. Water samples from discharge points were all negative for HEV RNA. In one production area, HEV detection was achieved both in shellfish and in seawater samples (samples 46502B and MI-NA12) and sequence analysis showed an identity of 99.2–100% among seawater and shellfish samples.

Table 1 Primers used in the present study

Region	Primer (5′–3′)	Length (bp)	Cycle	Primer position[a]	References
ORF1	ORF1F: CCAYCAGTTYATHAAGGCTCC	348	First round	36–56	Fogeda et al. (2009)
	ORF1R: TACCAVCGCTGRACRTC			383–367	
	ORF1FN: CTCCTGGCRTYACWACTGC	172	Nested	53–71	
	ORF1RN: GGRTGRTTCCAIARVACYTC			224–205	
ORF2	HE044: CAAGGHTGGCGYTCKGTTGAGAC	506	First round	5912–5934	Shrestha et al. (2003)
	HE040: CCCTTRTCCTGCTGAGCRTTCTC			6417–6395	
	HE110-2: GYTCKGTTGAGACCWCBGGBGT	457	Nested	5922–5943	
	HE041: TTMACWGTCRGCTCGCCATTGGC			6378–6356	
	3156N: AATTATGCYCAGTAYCGRGTTG	731	First round	5687–5708	Huang et al. (2002)
	3157N: CCCTTRTCYTGCTGMGCATTCTC			6417–6395	
	3158N: GTWATGCTYTGCATWCATGGCT	348	Nested	5972–5993	
	3159N: AGCCGACGAAATCAATTCTGTC			6319–6298	
ORF3[b]	JVHEVF: GGTGGTTTCTGGGGTGAC	–	One-step	5261–5278	Jothikumar et al. (2006), Garson et al. (2012)
	JVHEVR: AGGGGTTGGTTGGATGAA		Real-time	5284–5301	
	JVHEVP: FAM-TGATTCTCAGCCCTTCGC-MGB		RT-PCR	5313–5330	

FAM 6-carboxy fluorescein, *MGB* minor groove binder

[a]Primer positions are based on GenBank sequence accession no. M73218

[b]Real-time RT-(q)PCR assay was performed using the RNA UltraSense™ One-Step qRT-PCR System (Life Technologies) with the following primers/probe concentrations: JVHEVF 500 nM, JVHEVR 900 nM, JVHEVP-MGB 250 nM; the amplification conditions were 60 min at 50 °C, followed by 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

Unfortunately, no amplification was obtained using the additional assays targeting the ORF2 regions and therefore subtyping of the strains was not feasible.

Figure 1 shows the phylogenetic tree constructed in the ORF1 region, including the study sequences (in bold), along with G3 human and swine sequences detected previously in Italy, and G1, G2, G4 sequences retrieved from GenBank. Sequences obtained from shellfish and water samples (GenBank accession numbers: MF796385–MF796391) grouped with G3 human and swine sequences previously detected in Italy.

The present study highlights the presence of HEV in shellfish from commercial harvesting areas in Southern Italy at low prevalence (2.6%) and with concentrations always below 10^2 genome copies/g. Viral infections are frequently associated to shellfish consumption, often as a consequence of sewage contamination of harvesting areas and undercooking of the product (Bellou et al. 2013). Few episodes of hepatitis E following shellfish consumption have been described (Koizumi et al. 2004; Said et al. 2009) even if shellfish consumption has been reported as a risk factor for HEV infection in several sporadic cases (Chau et al. 2006; Sadler et al. 2006; Renou et al. 2008; La Rosa et al. 2011; Inagaki et al. 2015). Furthermore, studies conducted in European countries have detected HEV RNA in shellfish with prevalence values ranging from 3 to 14.8% (Diez-Valcarce et al. 2012; Pol-Hofstad et al. 2013; Mesquita et al. 2016), though several studies reported no detection (La Rosa et al. 2010; Fusco et al. 2013; Grodzki et al. 2014; Krog et al. 2014; Prpic et al. 2015). In Spain, concentrations ranging from 67 to 8.6×10^4 genome copies/g were found (Mesquita et al. 2016). In Italy, HEV has never been detected in commercial harvesting areas (La Rosa et al. 2012; Iaconelli et al. 2015; Fusco et al. 2017), and only one study documented HEV contamination in bivalves used for biomonitoring and collected in areas not approved for harvesting (Donia et al. 2012).

Seawater samples collected in production areas tested also positive for HEV in this study. To our knowledge, no evidence of HEV contamination of coastal waters has been reported in Italy before. However, a few studies have documented HEV's presence in sewage and river waters in Italy (La Fauci et al. 2010; La Rosa et al. 2010; Idolo et al. 2013; Iaconelli et al. 2015; Marcheggiani et al. 2015), demonstrating environmental circulation of this virus. Moreover, slurry samples from farms located in Northern Italy were found to be HEV-positive, pointing out the possibility of surface water contamination with animal strains, as a consequence

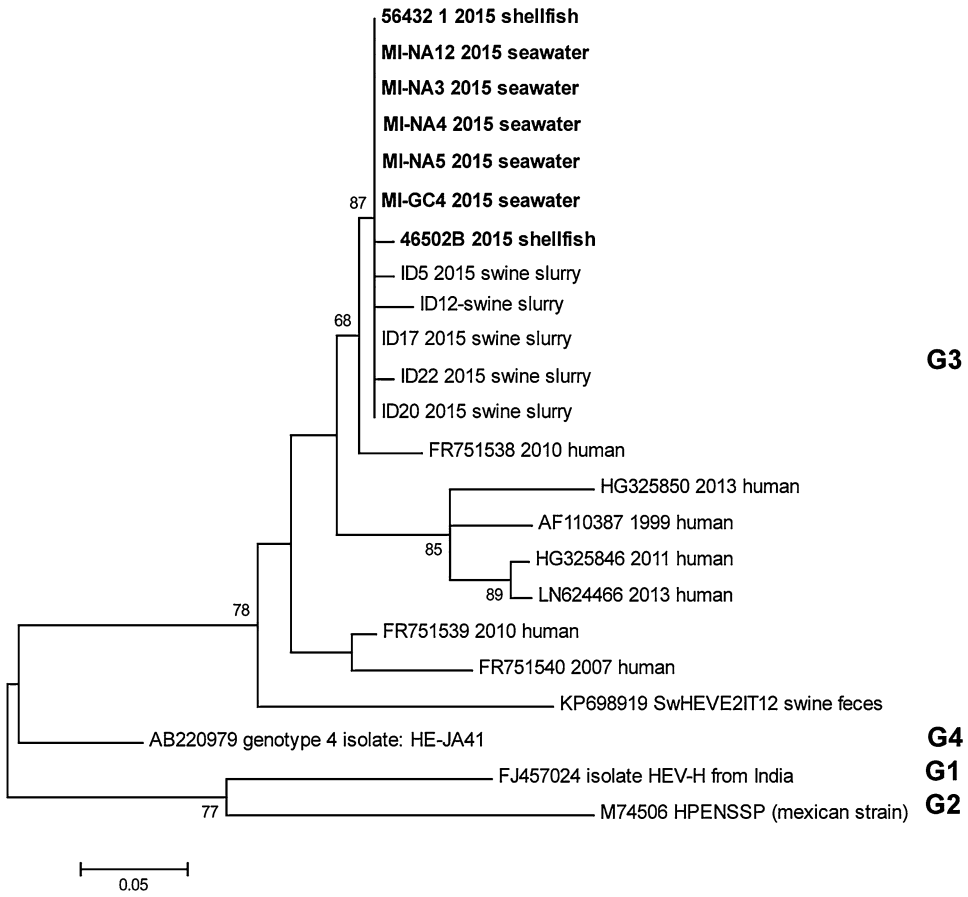

Fig. 1 Phylogenetic tree of hepatitis E virus. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 23 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + noncoding. There were a total of 132 positions in the final dataset. Evolutionary analyses were conducted in MEGA6

of run-off waters, percolation from pig farms or agronomic use of pig slurry (La Rosa et al. 2017a).

The present study is the first to demonstrate the occurrence of HEV both in shellfish from commercial harvesting areas and in marine waters collected in the same production areas in Italy, with strains showing a high degree of nucleotide identity. These data could help to refine risk assessment of foodborne transmission of HEV, and will support the establishing of an integrated surveillance to reduce the risk of shellfish-associated illnesses.

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References

Bellou, M., Kokkinos, P., & Vantarakis, A. (2013). Shellfish-borne viral outbreaks: A systematic review. *Food Environmental Virology, 5,* 13–23.

Chau, T. N., Lai, S. T., Tse, C., Ng, T. K., Leung, V. K., Lim, W., et al. (2006). Epidemiology and clinical features of sporadic hepatitis E as compared with hepatitis A. *American Journal of Gastroenterology, 101,* 292–296.

Crossan, C., Baker, P. J., Craft, J., Takeuchi, Y., Dalton, H. R., & Scobie, L. (2012). Hepatitis E virus genotype 3 in shellfish, United Kingdom. *Emerging Infectious Diseases, 18,* 2085–2087.

Diez-Valcarce, M., Kokkinos, P., Soderberg, K., Bouwknegt, M., Willems, K., de Roda-Husman, A. M., et al. (2012). Occurrence of human enteric viruses in commercial mussels at retail level in three European countries. *Food Environmental Virology, 4,* 73–80.

Donia, D., Dell'Amico, M. C., Petrinca, A. R., Martinucci, I., Mazzei, M., Tolari, F., et al. (2012). Presence of hepatitis E RNA in mussels used as bio-monitors of viral marine pollution. *Journal of Virological Methods, 186,* 198–202.

EFSA BIOHAZ Panel (EPoBH). (2017). Public health risks associated with hepatitis E virus (HEV) as a food-borne pathogen. *EFSA Journal, 15,* 7.

Fogeda, M., Avellón, A., Cilla, C. G., & Echevarría, J. M. (2009). Imported and autochthonous hepatitis E virus strains in Spain. *Journal of Medical Virology, 81,* 1743–1749.

Fusco, G., Aprea, G., Galiero, G., Guarino, A., & Viscardi, M. (2013). *Escherichia coli*, *Salmonella* spp., Hepatitis A virus and norovirus in bivalve molluscs in Southern Italy. *Veterinaria Italiana, 49,* 55–58.

Fusco, G., Di Bartolo, I., Cioffi, B., Ianiro, G., Palermo, P., Monini, M., et al. (2017). Prevalence of foodborne viruses in mussels in Southern Italy. *Food Environmental Virology*. doi:10.1007/s12560-016-9277-x.

Gao, S., Li, D., Zha, E., Zhou, T., Wang, S., & Yue, X. (2015). Surveillance of hepatitis E virus contamination in shellfish in China. *International Journal of Environmental Research and Public Health, 12,* 2026–2036.

Garson, J. A., Ferns, R. B., Grant, P. R., Ijaz, S., Nastouli, E., Szypulska, R., et al. (2012). Minor groove binder modification of widely used TaqMan probe for hepatitis E virus reduces risk of false negative real-time PCR results. *Journal of Virological Methods, 186,* 157–160.

Grodzki, M., Schaeffer, J., Piquet, J. C., Le Saux, J. C., Cheve, J., Ollivier, J., et al. (2014). Bioaccumulation efficiency, tissue distribution, and environmental occurrence of hepatitis E virus in bivalve shellfish from France. *Applied and Environment Microbiology, 80,* 4269–4276.

Huang, F. F., Haqshenas, G., Guenette, D. K., Halbur, P. G., Schommer, S. K., Pierson, F. W., et al. (2002). Detection by reverse transcription-PCR and genetic characterization of field isolates of swine hepatitis E virus from pigs in different geographic regions of the United States. *Journal of Clinical Microbiology, 40,* 1326–1332.

Iaconelli, M., Muscillo, M., Della Libera, S., Fratini, M., Meucci, L., De Ceglia, M., et al. (2017). One-year surveillance of human enteric viruses in raw and treated wastewaters, downstream river waters, and drinking waters. *Food Environmental Virology, 9,* 79–88.

Iaconelli, M., Purpari, G., Della Libera, S., Petricca, S., Guercio, A., Ciccaglione, A. R., et al. (2015). Hepatitis A and E viruses in wastewaters, in river waters, and in bivalve molluscs in Italy. *Food Environmental Virology, 7,* 316–324.

Idolo, A., Serio, F., Lugoli, F., Grassi, T., Bagordo, F., Guido, M., et al. (2013). Identification of HEV in symptom-free migrants and environmental samples in Italy. *Journal of Viral Hepatitis, 20,* 438–443.

Inagaki, Y., Oshiro, Y., Hasegawa, N., Fukuda, K., Abei, M., Nishi, M., et al. (2015). Clinical features of hepatitis E virus infection in Ibaraki, Japan: Autochthonous hepatitis E and acute-on-chronic liver failure. *Tohoku Journal of Experimental Medicine, 235,* 275–282.

ISO. (2013). *ISO/TS 15216-1 Microbiology of food and animal feed—Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR—Part 1: Method for quantification*. Geneva: ISO.

Jothikumar, N., Cromeans, T. L., Robertson, B. H., Meng, X. J., & Hill, V. R. (2006). A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. *Journal of Virological Methods, 131,* 65–71.

Koizumi, Y., Isoda, N., Sato, Y., Iwaki, T., Ono, K., Ido, K., et al. (2004). Infection of a Japanese patient by genotype 4 hepatitis e virus while traveling in Vietnam. *Journal of Clinical Microbiology, 42,* 3883–3885.

Krog, J. S., Larsen, L. E., & Schultz, A. C. (2014). Enteric porcine viruses in farmed shellfish in Denmark. *International Journal of Food Microbiology, 186,* 105–109.

Kumar, A., & Saraswat, V. A. (2013). Hepatitis E and acute-on-chronic liver failure. *Journal of Clinical and Experimental Hepatology, 3,* 225–230.

La Fauci, V., Sindoni, D., Grillo, O. C., Calimeri, S., Lo Giudice, D., & Squeri, R. (2010). Hepatitis E virus (HEV) in sewage from treatment plants of Messina University Hospital and of Messina City Council. *Journal of Preventive Medicine and Hygiene, 51,* 28–30.

La Rosa, G., Della Libera, S., Brambilla, M., Bisaglia, C., Pisani, G., Ciccaglione, A. R., et al. (2017a). Hepatitis E virus (genotype 3) in slurry samples from swine farming activities in Italy. *Food Environmental Virology, 9,* 219–229.

La Rosa, G., Della Libera, S., Iaconelli, M., Proroga, Y. T., De Medici, D., Martella, V., et al. (2017b). Detection of Norovirus GII.17 Kawasaki 2014 in shellfish, marine water and underwater sewage discharges in Italy. *Food Environmental Virology*. doi:10.1007/s12560-017-9290-8.

La Rosa, G., Fratini, M., Spuri Vennarucci, V., Guercio, A., Purpari, G., & Muscillo, M. (2012). GIV noroviruses and other enteric viruses in bivalves: A preliminary study. *New Microbiologica, 35,* 27–34.

La Rosa, G., Muscillo, M., Vennarucci, V. S., Garbuglia, A. R., La Scala, P., & Capobianchi, M. R. (2011). Hepatitis E virus in Italy:

Molecular analysis of travel-related and autochthonous cases. *Journal of General Virology, 92,* 1617–1626.

La Rosa, G., Pourshaban, M., Iaconelli, M., Vennarucci, V. S., & Muscillo, M. (2010). Molecular detection of hepatitis E virus in sewage samples. *Applied and Environment Microbiology, 76,* 5870–5873.

La Rosa, G., Sanseverino, I., Della Libera, S., Iaconelli, M., Ferrero, V. E. V., Barra Caracciolo, A., et al. (2017c). The impact of anthropogenic pressure on the virological quality of water from the Tiber River, Italy. *Letters in Applied Microbiology*. doi:10.1111/lam.12774.

Li, T. C., Miyamura, T., & Takeda, N. (2007). Detection of hepatitis E virus RNA from the bivalve Yamato-Shijimi (Corbicula japonica) in Japan. *American Journal of Tropical Medicine and Hygiene, 76,* 170–172.

Marcheggiani, S., D'Ugo, E., Puccinelli, C., Giuseppetti, R., D'Angelo, A. M., Gualerzi, C. O., et al. (2015). Detection of emerging and re-emerging pathogens in surface waters close to an urban area. *International Journal of Environmental Research and Public Health, 12,* 5505–5527.

Meng, X. J. (2010). Hepatitis E virus: Animal reservoirs and zoonotic risk. *Veterinary Microbiology, 140,* 256–265.

Mesquita, J. R., Oliveira, D., Rivadulla, E., Abreu-Silva, J., Varela, M. F., Romalde, J. L., et al. (2016). Hepatitis E virus genotype 3 in mussels (*Mytilus galloprovinciallis*), Spain. *Food Microbiology, 58,* 13–15.

Pavio, N., Meng, X. J., & Renou, C. (2010). Zoonotic hepatitis E: Animal reservoirs and emerging risks. *Veterinary Research, 41,* 46.

Pérez-Gracia, M. T., Suay-García, B., & Mateos-Lindemann, M. L. (2017). Hepatitis E and pregnancy: Current state. *Reviews in Medical Virology*. doi:10.1002/rmv.1929View/save.

Pol-Hofstad, I., Rutjes, S., Gerssen, A., Poelman, M., & van der Linden, A. (2013). Evaluation of the sanitary status of the Dutch shellfish production waters over a 7 year period. In *9th International Conference on Molluscan Shellfish Safety* Sidney, Australia.

Prpic, J., Cerni, S., Skoric, D., Keros, T., Brnic, D., Cvetnic, Z., et al. (2015). Distribution and molecular characterization of Hepatitis E virus in domestic animals and wildlife in Croatia. *Food Environmental Virology, 7,* 195–205.

Renou, C., Moreau, X., Pariente, A., Cadranel, J. F., Maringe, E., Morin, T., et al. (2008). A national survey of acute Hepatitis E in France. *Alimentary Pharmacology & Therapeutics, 27,* 1086–1093.

Sadler, G. J., Mells, G. F., Shah, N. H., Chesner, I. M., & Walt, R. P. (2006). UK acquired hepatitis E—An emerging problem? *Journal of Medical Virology, 78,* 473–475.

Said, B., Ijaz, S., Kafatos, G., Booth, L., Thomas, H. L., Walsh, A., et al. (2009). Hepatitis E outbreak on cruise ship. *Emerging Infectious Diseases, 15,* 1738–1744.

Shrestha, S. M., Shrestha, S., Tsuda, F., Nishizawa, T., Gotanda, Y., Takeda, N., et al. (2003). Molecular investigation of hepatitis E virus infection in patients with acute hepatitis in Kathmandu, Nepal. *Journal of Medical Virology, 69,* 207–214.

Song, Y. J., Jeong, H. J., Kim, Y. J., Lee, S. W., Lee, J. B., Park, S. Y., et al. (2010). Analysis of complete genome sequences of swine hepatitis E virus and possible risk factors for transmission of HEV to humans in Korea. *Journal of Medical Virology, 82,* 583–591.