

First detection and molecular characterization of hepatitis E virus in water from wastewater treatment plants in Portugal

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Abstract

Hepatitis E virus (HEV) genotype 3 is widespread in industrialized countries and widely recognized as a serious public health issue. HEV genotype 3 is excreted in both human and animal stools, having the potential to be introduced in aquatic environments through wastewater discharge. For this reason, waterborne transmission of the HEV genotype 3 is drawing increasing attention from the scientific community. Although studies in Portugal have shown that HEV circulates in both humans and swine, no study has yet focused on the presence of HEV in wastewater. Hence, a total of 60 water samples (influent and effluent) from 15 Wastewater Treatment Plants (WWTP) of different regions of Portugal were tested for the presence of HEV using a broad spectrum real-time RT-PCR with amplification within the ORF2 region of HEV genome. Positive samples were retested by nested RT-PCR with amplification within the ORF1 region, sequenced followed by phylogenetic analysis. HEV sequences were retrieved from two influent samples collected in December 2013 from WWTPs of North and Central of Portugal and classified in subgenotype 3i and 3f. This is the first study showing the presence of HEV in aquatic environments of Portugal.

Key words

wastewater, hepatitis E virus, genotype 3, zoonotic

INTRODUCTION

Hepatitis E virus (HEV) is a positive sense, single-stranded, non-enveloped hepatotropic virus belonging to the *Orthohepevirus* genus, *Hepeviridae* family [1]. Genotype 3 HEV is widespread in industrialized countries and infection in humans is mostly the result of foodborne zoonotic transmission from swine, considered the most important animal reservoir for human disease [2, 3]. Since HEV is excreted in stools, it has the potential to be introduced in aquatic environments through urban and agriculture runoff, sewage outfall and vessel wastewater discharge. The involvement of environmental transmission in HEV genotype 3 infection is uncertain, but the hypothesis of waterborne transmission has been drawing increasing attention from the scientific community [4]. Further concerns are apparent since, unlike bacteria, viruses are not so efficiently eliminated by wastewater treatment plants and, as a consequence, they can be released in aquatic environments at levels that can have significance for public health [5, 6, 7]. Several countries worldwide have reported the detection

of HEV in wastewater samples [8, 9, 10]. Since Portugal is considered an endemic country for HEV [11, 12] and past studies have confirmed the presence of HEV in swine [2, 13], it is expected that HEV could be released into the aquatic environment. To the best of our knowledge, no study on the detection of HEV or any viral enteric pathogen in wastewater has ever been carried out in Portugal. This study presents data from the first detection and molecular characterization of HEV isolates retrieved from Wastewater Treatment Plants (WWTP) in Portugal.

MATERIALS AND METHOD

A total of 60 influent and effluent wastewater samples from 15 WWTPs located in 5 Portuguese regions – North, Centre, Lisboa and Vale do Tejo, Alentejo and Algarve – were studied. From each WWTP, time proportional 24-h composite influent (N=1) and effluent (N=1) samples (250 ml) were collected in September and in December 2013, in high-density polyethylene containers. Samples were transported refrigerated ($\pm 4^{\circ}\text{C}$) to the laboratory and kept frozen (-20°C) until analysis. Viral concentration was performed according to a previously described method [14] with minor modifications.

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Briefly, suspended materials of each wastewater sample were removed by 3 successive filtrations, through a glass microfiber filter, a 0.45 μm and a 0.2 μm polyamide membrane filters, respectively. 75 millilitres of filtrate were then ultracentrifuged at 15,2743 g (Ultracentrifuge L-80, Beckman) for 90 min at 18°C. The resulting pellet was re-suspended in 500 μL of supernatant. An equal volume of chloroform was then added. After homogenization and centrifugation at 405 g (Sigma 3–15) for 10 min, the aqueous phase was collected and stored at -20°C until nucleic acid extraction. Nucleic acid was extracted from 140 μL of this phase using the QIAmp® Viral RNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Each extraction protocol was performed along with a negative and a positive control, consisting of sterile water and HEV RNA added to a known negative sample, respectively. For HEV detection, a broad real time RT-PCR (RT-qPCR) TaqMan probe assay targeting the open reading frame (ORF) 2 region of the HEV was performed [15]. Briefly, to 12.5 μL of reaction mix (SuperScript® III Platinum One-Step RT-qPCR Kit, Invitrogen), were added 0.8 μL SuperScript TM III RT/Platinum Taq enzyme, 10 pmol of each primer, 3 pmol of probe and 5 μL of extracted nucleic acid, made up to 25 μL with RNase-free water. Thermal profile consisted of incubation at 50°C for 30 min, denaturation at 95°C for 2 min and then 45 cycles of amplification with denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min acquiring on the FAM and ROX channels. Real-time measurements were taken at each cycle.

Samples positive by RT-qPCR were submitted to a nested broad-spectrum RT-PCR with amplification within the ORF1 region of HEV genome [16]. In short, the first round of RT-PCR was performed using the Qiagen One-Step RT-PCR Kit (QIAGEN, Hilden, Germany) with primers HEV-cs and HEV-cas that amplify a 472-bp fragment. Thermal profile consisted of 42°C for 60 min and 95°C for 15 min, followed by 40 cycles of 94°C for 1 min, 50°C for 1 min and 74°C for 1 min, with a final extension at 72°C for 10 min. For the second round PCR, HiFi Hotstart ReadyMix (KAPA Biosystems, MA, USA) was used, and the primers HEV-csn and HEV-casn that amplify a 334-bp fragment, using as template 5 μL of the first round products. The thermal profile consisted of 95°C for 5 min and 35 cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 1 min, with a final extension at 72°C for 10 min. Each set of amplification reactions in both RT-qPCR and conventional RT-PCR included a positive and a negative control consisting of sterile water added to the reaction mixture instead of nucleic acid extract. HEV genome containing plasmid was used as positive control for amplification reactions. Amplified products were separated by electrophoresis in a 1.5% agarose gel and appropriately-sized bands were excised and purified with the GRS PCR & Gel Band Purification Kit (GRISP, Porto, Portugal), and sequenced in both directions using the BigDye Terminator v1.1 Cycle Sequencing kit (PE Applied Biosystems, CA, USA). Sequence editing and multiple alignments were performed with the BioEdit software package, version 2.1 (Ibis Biosciences, CA, USA). Phylogenetic analysis was performed using MEGA version 6.06 software.

RESULTS AND DISCUSSION

Only 2 of the 60 tested wastewater samples were positive for HEV RNA by RT-qPCR. They were both influent wastewater samples collected in December, one (sample WW9) retrieved from a WWTP located in the North, and the other (sample WW19) located in the Centre of Portugal (Fig. 1). The WWTP located in the North is designed for 255,557 population equivalents, with a median average load of 15,000 m^3 per day. This WWTP treats domestic and industrial wastewaters, operating with tertiary treatments, having its discharge point in the Ave River. The WWTP located in the Centre of Portugal is designed for 213,000 population equivalents, with a median average load of 36,000 m^3 per day. This WWTP treats domestic and industrial wastewaters through secondary treatment processes, having discharge points in Mondego River. When these 2 samples were retested by nested RT-PCR with primers targeting the ORF 1 region, amplified products of the expected size (330 nt) were obtained. These 2 amplicons were sequenced and submitted to phylogenetic analysis. The nucleotide sequences determined in the presented study



Figure 1. Regions of Portugal showing the location of WWTPs sampled in the study. ○ – WWTPs where no HEV RNA was detected; ● – WWTPs with influents positive for HEV RNA

was to perform the molecular characterization of HEV isolates from the environment rather than to evaluate viral occurrence. Molecular diagnostic tools are known to be valuable for environmental surveillance, assisting in the assessment of the epidemiology of the circulating viral community in a given population [4,18]. In fact, reports have shown a correlation between viruses detected in wastewater and clinical cases, showing that this type of study is very useful for virus monitoring, and even for outbreak early warning systems [19, 20].

CONCLUSIONS

The presented study described the detection of HEV genotype 3 sequences in wastewater samples of WWTPs in the North and Centre of Portugal, and to the best knowledge of the authors is the first study to investigate the presence of HEV in aquatic environments of Portugal. Future studies should focus on the potential impact of waterborne HEV on public health.

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