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# Molecular features of Hepatitis E Virus circulation in environmental and human samples

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ABSTRACT

*Background and objectives:* Hepatitis E virus (HEV) is emerging but its circulation between humans and the environment remains misunderstood. HEV ORF2 gene encodes the capsid playing a key role in viral interactions with surfaces, ORF3 products are involved in the viral cycle. Our aim was to study the molecular characteristics of ORF2 and ORF3 which could favor HEV fitness in patients and the environment.
*Study design:* Samples from 69 patients with hepatitis (blood/stools), 20 urban wastewaters, 20 effluents of a pig slaughterhouse, 22 farm pigs (stools), 20 wild boars (liver/stools) were collected in North-Eastern France. HEV strains were analyzed by direct sequencing within the ORF2 M region, of ORF2/ORF3, for phylogeny and physicochemical prediction and for ORF2 by ultra-deep sequencing.
*Results:* The results showed frequent HEV-positive samples: 9.1% of the patient bloods, 23.1% of their stools; 25.0% of wastewaters, 75.0% for the slaughterhouse, 10.0% of the boar livers, 5.3% of their stools. The strains were classified as HEV genotype 3. In ORF2, HEV highlighted one homogeneous major viral variant within quasispecies and a decrease in predicted antigenicity for two minor mutations (D442G, V402A). A cysteine signature at position 81 in ORF3 was observed in the boars.
*Conclusions:* HEV RNA genotype 3 was detected in patients and in animals, in a slaughterhouse effluent and in wastewater. Moreover, the low variability of amino acids in the ORF2 M region and molecular features in ORF2 and ORF3 suggested that HEV strains could be advantageous for key properties.

## 1. Introduction

Hepatitis E virus (HEV) is suspected to be a food- and water-transmitted virus with a zoonotic cycle especially for HEV genotype 3 (HEV3) [1]. HEV1 and HEV2 are restricted to humans and transmitted through contaminated water in developing countries. HEV3 and HEV4 genotypes can infect humans, pigs and other mammals [2,3]. HEV5 and HEV6 were detected in wild boars in Japan and HEV7 in camels [4,5]. However, HEV circulation between human beings, animals and the environment remains misunderstood and has been investigated for the present study in North Eastern France where quite a high HEV prevalence in humans was observed [6].

HEV is a non-enveloped RNA virus and a member of *Hepeviridae* family [7]. The HEV genome contains three open reading frames (ORFs): ORF1 encodes nonstructural proteins, ORF2 the capsid protein and ORF3 a small phosphoprotein. The HEV capsid plays a key role in the viral entry into hepatocytes, in the host-related immune response [8–10]. ORF3 has been reported to play a key role in viral cycle [11]. Genetic variation of ORF2 and ORF3 products may also, therefore, be an important factor for environmental transmission.

## 2. Objectives

Our aim was to explore a possible circulation of HEV between patients, animals and the environment and to investigate molecular features of HEV in the ORF2 and ORF3 genes, which could benefit the virus in both pathogenicity and transmission. We observed a HEV circulation in North-Eastern France, a tendency to molecular conservation of amino acid residues in ORF2 and an amino acid signature in ORF3, only found in HEV strains from wild boars.

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**Table 1**
Genome copies of HEV in stool, sera and liver from humans and animals, in slaughterhouse effluent and urban wastewater. [a] Concentrations are expressed in IU/g or IU/mL. [b] NA: Not Applicable.

| Sample Source | | Number of positive sample/Total number of samples | Concentration range[a] | Average |
|---|---|---|---|---|
| Patients | Blood | 8/88 | $1.4 \times 10^3$–$4.4 \times 10^7$ | $5.7 \times 10^6$ |
| | Stool | 3/13 | $2.1 \times 10^4$ – $7.7 \times 10^7$ | $3.0 \times 10^7$ |
| Urban | Wastewater | 5/20 | $2.7 \times 10^{-1}$ – $1.4 \times 10^1$ | 6.6 |
| Slaughterhouse | Effluent | 15/20 | $1.3 \times 10^{-1}$ – $6.4 \times 10^3$ | $5.3 \times 10^2$ |
| Wild boar | Liver | 2/20 | $7.5 \times 10^6$– $1.0 \times 10^7$ | $9.0 \times 10^6$ |
| | Stool | 1/19 | $2.0 \times 10^7$ | NA[b] |
| Farm pig | Stool | 0/22 | NA [b] | NA[b] |

## 3. Study design

The samplings were performed in North-Eastern France, in 2016 and 2017. Blood samples (n = 88) and stool samples (n = 13) were drawn from patients followed in the University Hospital of Nancy. The patients' non opposition was obtained, as approved by the "Comité de Protection des Personnes − CPP de Lorraine" (DC-2016-2790), in accordance with international guidelines (Helsinki). Urban wastewater was collected from an urban area of 260,000 inhabitants (n = 20) and effluents from of a pig slaughterhouse (n = 20). Samples were also collected from three farm pigs (n = 22 stool samples) and wild boars (n = 20 liver samples, 19 stool samples). The age of the animals ranged from two to 12 months old.

The samples taken in 2017 (urban wastewater: n = 11, 1L; slaughterhouse: n = 9, 1L for eight and 500 mL for one) underwent a first concentration step through use of glass powder (20 g) (Grosseron, Couëron, France). The samples taken in 2016 (urban wastewater: n = 9, 150 mL; slaughterhouse: n = 11, 60 mL) and the products of the first concentration were concentrated in a Centricon® Plus-70 centrifugal filter device (Merck Millipore, Billerica, Massachusetts, United States). Viral genome was recovered using lysis buffer (5 mL) from NucliSens magnetic extraction reagents (bioMérieux, Marcy l'Etoile, France). For water samples, RNA was extracted using Nuclisens magnetic extraction reagents on Nuclisens MiniMAG. HEV extraction from stool, serum and liver samples was achieved with the QIAamp Viral Mini Kit from Qiagen (Valencia, Californie) with the Viral RNA Mini Spin protocol. We applied HEV RT-qPCR (ORF3; SuperScript™ III One-Step RT-PCR System with Platinum™ *Taq* DNA Polymerase, Invitrogen, Carlsbad, California) [12].

HEV RNA was amplified in a part of the ORF2 M region (capsid) by a nested PCR with combinations of primers [13,9] (Titan one tube RT-PCR kit −Sigma-Aldrich, Phusion master mix −Fisher Scientific, St Quentin Fallavier and Illkirch, France, respectively). This PCR system generates a 216-nucleotide long fragment, starting at nucleotide 1152 of ORF2 gene. For the ORF2/ORF3 overlap region, four primers were used [14] (Access RT-PCR, Promega France, Charbonnières-les-Bains, France) and the Phusion master mix, to amplify a 137 bp fragment beginning at nucleotide 174 of ORF3. Amplicons were submitted to direct sequencing using the BigDye Terminator v1.1 kit (Applied Biosystems, Foster City, CA) and an automated Sanger method DNA sequencer (ABI PRISM 3100, Applied Biosystems). For the investigation on viral quasispecies by ultra-deep sequencing (UDS), amplicons of the ORF2 nested PCR were used with MiSeq Reagent Nano kit V2 (Illumina, Paris France); each DNA fragment was sequenced onto MiSeq Equipment (Illumina). Sequence editing and analysis were performed with Geneious software (www.geneious.com) with a HEV reference sequence (AB369687_3f from GenBank).

HEV strains were studied by phylogeny with the MEGA6 software, in comparison with reference sequences [15]. The selection pressure was analyzed with MEGA6 software (Nei-Gojobori method with Jukes-Cantor correction), using Z-test [16]. The prediction of the antigenicity of aa sequences in the HEV capsid was performed with the AnTheProt software (ANTHEPROT 6.7.0; http://antheprot-pbil.ibcp.fr; IBMC Lyon) [17]; [18].

To evaluate the viral quasispecies features on the HEV ORF2 segment, the Mutation frequency (Mf) was obtained as the ratio of the number of observed mutations relative to the master sequence divided by the total number of nucleotides sequenced [19]. The genetic complexity which was determined by the Shannon entropy (Sn) of the nucleic acid sequences:

$$Sn = \frac{-\Sigma i(pi\ lnpi)}{ln(h)}$$

where pi is the frequency of the haplotype within the viral population. The result was normalized by the total number of haplotypes (h), the Sn varying from 0 (no complexity) to 1 (maximum complexity).

The statistical analyses were performed on the https://marne.u707.jussieu.fr/biostatgv/?module=tests/student site; the Student test was used in order to compare the viral loads between urban wastewaster samples and slaughterhouse effluent samples with $p < 0.05$ considered as significant.

## 4. Results

Among the 202 samples, for patients suffering from hepatitis, 9.1% blood samples and 23.1% stool samples were positive (Tables 1 and 2). For the HEV-infected patients, some developed an acute hepatitis (n = 4), while the information was not available for 3 patients (and the last one deceased shortly after the HEV diagnosis).

Moreover, 25.0% of urban wastewater and 75.0% of slaughterhouse effluents were also positive. No HEV-positive stool sample was found in pigs while 10.0% liver samples and 5.3% stool samples were positive in the wild boars.

The quantifications of HEV RNA were reported in Fig. 1 and Table 1. For the slaughterhouse samples, the concentrations were variable and could reach $6.4 \times 10^3$ IU/mL. In the urban wastewater samples, the concentrations reached $1.4 \times 10^1$ IU/mL. In the human and wild boar samples, the concentrations were comprised between $1.4 \times 10^3$ and $4.4 \times 10^7$ IU/mL (blood samples) and $2.1 \times 10^4$ and $7.7 \times 10^7$ IU/g (stool and liver samples). Statistical analyses have been conducted comparing the viral loads between urban wastewaster samples and slaughterhouse effluent samples: no significant result was observed.

The HEV ORF2 direct sequencing data were collected for five patients, with UDS for four of them and from one boar liver sample. The