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## Short Communication

## First detection of Hepatitis E virus (*Rocahepevirus ratti*) in French urban wastewater: Potential implications for human contamination

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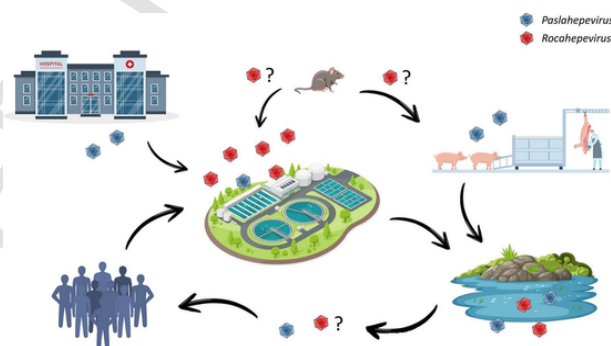
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## HIGHLIGHTS

- Detection of *Rocahepevirus* in urban wastewater and rivers waters
- High concentrations, compatible with a transmission to humans (as observed for *Paslahepevirus*)
- No detection in patients with suspicion of viral hepatitis (in the same geographical area)
- Need for optimized methods targeting emergent hepatitis E virus strains

## GRAPHICAL ABSTRACT



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## ABSTRACT

Hepatitis E virus (HEV) is considered as an emerging zoonotic pathogen circulating in a wide range of animals. In recent decades, the genus *Paslahepevirus* frequently isolated in pigs were the most involved in human clinical practice. In addition, the genus *Rocahepevirus* have been isolated in rodents, and transmission to humans is increasingly reported worldwide, although gaps remain regarding the exposure factors. In this study, the presence of HEV was investigated in urban wastewater, swine slaughterhouse wastewater and river waters, in a geographical area where its circulation had previously been reported. In addition to the expected detection of *Paslahepevirus* in almost all waters samples collected, *Rocahepevirus* strains were detected with the same frequencies in urban and river waters, at concentrations up to 40-fold higher. No *Rocahepeviruses* were detected in swine slaughterhouse wastewater. This is the first study demonstrating the presence of *Rocahepevirus* in French wastewater. Although no evidence of transmission was reported among patients followed for a suspected HEV infection in the same area between April 2019 and October 2023 (i.e. 135/3078 serological tests positive for anti-HEV IgM detection; 46/822 blood samples positive for *Paslahepevirus* genome detection but none for *Rocahepevirus*), the circulation of *Rocahepevirus* in waters in such concentrations raises the question of the possible zoonotic transmission to human. Indeed, the waterborne transmission of HEV is now well documented in industrialized countries,

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and the exploration of the growing number of human infections in Europe involving *Rocahepevirus* has not until now made it possible to clarify the transmission routes.

## 1. Introduction

Hepatitis E virus (HEV) is considered as the main cause of human hepatitis worldwide (Kamar et al., 2017). The infection is self-limited in the general population, including fever, asthenia, nausea and icterus associated to hepatic cytolysis. Severe acute and fulminant hepatitis are reported in patients with underlying liver disease while extrahepatic signs (i.e. renal and neuronal disorders) are also described (Pischke et al., 2017). In immunocompromised patients, chronic HEV infections may be observed (i.e. detection of HEV RNA up to 3–6 months in blood or stool) and are associated with a rapid progression to cirrhosis and potential carcinoma (Kamar et al., 2017).

The genus *Paslahepevirus* is the most important regarding human health. It includes at least 8 genotypes, infecting human through the fecal-oral route in developing countries for genotypes 1 and 2, or through a zoonotic way for genotypes 3 and 4 in industrialized countries, after consumption of contaminated food products (e.g. pork, wild boar, deer). Genotypes 5 and 6 are detected only in wild boar, while genotypes 7 and 8 typically circulate in camels, with currently a unique reported case of transmission to an immunocompromised individual (Lee et al., 2016).

Little is known concerning the circulation of HEV between human and the different environmental reservoirs. Recent data suggest that environmental water, shellfish or vegetables (e.g. green leaves, red fruits) may also contribute to spread HEV to human (Cook and van der Poel, 2015; Terio et al., 2017) and new animal reservoirs are also identified. Rabbit HEV, closed to genotype 3 strains has recently been described, and is able to infect humans (Abravanel et al., 2017; Baylis et al., 2022; Hartard et al., 2021). Although more genetically distinct, the other *Orthohepevirus* genera circulating in birds, rodents and bats, are also of concern. Among them, the genus *Rocahepevirus* was first identified in rats (*Rattus norvegicus*) in Germany in 2010. Now, its worldwide distribution is recognized (Ryll et al., 2017), especially among aged specimens (De Cock et al., 2023; Robinson et al., 2023) and transmissions to human have recently been documented, mainly in immunocompromised patients but also in immunocompetent or healthy individuals, including children (Andonov et al., 2019; Caballero-Gómez et al., 2024; Rivero-Juarez et al., 2022; Sridhar et al., 2021b). Currently, most cases have been described in Hong Kong, where the prevalence of the species *Rocahepevirus rattii* is as frequent as *Paslahepevirus* genotypes 1 and 3, and accounted for 8 % of all HEV infections (Sridhar et al., 2021b). Infection with *Rocahepevirus* leads to asymptomatic forms, as well as acute or chronic infections, associated or not with extra hepatic manifestations (Sridhar et al., 2021b).

The genus *Rocahepevirus* presents only 55 to 60 % of homologous sequence with HEV genotypes 1 to 4, leading to a new challenge in diagnosing HEV infection in patients. Indeed, the conventional RT-PCR assays usually used at the time of the diagnosis are designed to target *Paslahepevirus* genotypes and failed to amplify the other genera, while cross reactivity may be observed when using serological tests (Parraud et al., 2021; Sridhar et al., 2021a).

The prevalence of the genus *Rocahepevirus* is probably underestimated and the way of transmission to human remains unclear. Indeed, in addition to rats, *Rocahepevirus* may infect other animals. As an example, a recent study performed in Germany reported a seroprevalence up to 40 % in foxes (*Vulpes vulpes*) for the last 30 years (Eiden et al., 2020). Even if the direct transmission to human remains unlikely, foxes could contribute to spread *Rocahepevirus* in the environment and may ultimately constitute an important reservoir.

In this context, the objective of this study was to explore the circulation of *Rocahepevirus* in the North East of France, a geographic area

characterized by a heavy circulation of HEV (Fenaux et al., 2018; Mansuy et al., 2016), by screening patients addressed to the Nancy University Hospital, and by investigating the nearby potential environmental water reservoirs (i.e. urban wastewater, swine slaughterhouse wastewater and surface waters).

## 2. Materials and methods

### 2.1. Patients' samples

We tested 766 sera collected from patients followed at the University Hospital of Nancy, for whom conventional HEV RNA detection have been ordered as part of their medical care (meaning *Paslahepevirus*), and for which the RT-PCR result was negative. All sera were collected between April 2019 and October 2023. According to French Law (Loi Jardé), anonymous studies without additional sampling or data collection do not require the approval of an institutional review committee. Samples were stored at  $-80^{\circ}\text{C}$  until analysis.

### 2.2. Water samples

Water samples (urban wastewater treatment plant [WWTP,  $n = 11$ ], environmental surface waters [SW,  $n = 4$ ], and swine slaughterhouse [SH,  $n = 14$ ]) were collected in the Nancy area to explore the circulation of HEV in the patient's environment. The WWTP receives daily 70,000–110,000  $\text{m}^3$  of wastewater from an urban area with a population of approximately 400,000, including hospital effluent. Samples were collected at the WWTP's entry point using an automated sampler during a 24 h period. The WWTP's characteristics (capacity, treatment processes, and sampling) are detailed in Bichet et al. (2024), referred to as WWTP-1. Surface waters samples were collected 1 km upstream of the WWTP discharge, from the shoreline at the urban area's entrance. Lastly, the slaughterhouse processes around 25 pigs per day, and the sample collection was continuous throughout the slaughtering process, ensuring an average sample was obtained.

To concentrate environmental surface water, 500 mL of sample were filtered through Centricon Plus-70 centrifugal filters 100 kDa (Merck Millipore), as previously described (Bertrand et al., 2021). Briefly, 10 repeated passages of 50 mL were achieved. After each passage, the concentrates were recovered by inverting the system, applying centrifugation ( $1000 \times g$  for 2 min), and then pooled. After the last passage, the filters were washed with 5 mL of Lysis Buffer (bioMérieux) and added to the concentrate's pool. A final phenol-chloroform-isoamyl alcohol purification was performed as previously described (Bertrand et al., 2021), resulting in a 15-mL final volume.

Water samples from the swine slaughterhouse were concentrate from 200 mL, using the Amicon® Stirred Cell 400 mL system (Merck Millipore) and Ultracel® membrane discs of regenerated cellulose with a cut-off of 10 kD, as previously described (Chaqrour et al., 2024).

For urban wastewater, 5 mL of sample were directly added with 10 mL of Lysis Buffer (bioMérieux) upstream the RNA extraction.

When a concentration method was applied (i.e., for environmental surface water and swine slaughterhouse samples), recovery rates were estimated as previously described (Chaqrour et al., 2024), by measuring and comparing the concentration of F-specific RNA phages subgroup II, used as surrogates, in the unconcentrated sample and after the concentration step.

### 2.3. RNA extraction and HEV detection

RNA was extracted using NucliSens easyMAG® or miniMAG® (bio-Mérieux) following the manufacturer recommendations. Extraction was performed from 200 µL of patient serum, or from 15 mL of lysed water sample concentrate. The purified RNA was recovered in a 100 µL volume of elution buffer.

To detect *Rocahepevirus* RNA in biological samples, reverse transcription and polymerase chain reaction (RT-qPCR) were performed from 15 µL of RNA extract as previously described (Parraud et al., 2021), using the qScript XLT 1-Step RT-qPCR ToughMix (Quantabio). The specificity of the *Rocahepevirus*-RT-PCR was confirmed using the Basic Local Alignment Search Tool (BLAST) program available on the NCBI network server, as well against a database including the entire HEV whole genome sequences isolated in Europe available in GenBank® (from humans [ $n = 301$ ], rodents [ $n = 45$ ] and other animals [ $n = 80$ ; i.e. *Sus scrofa*, *Oryctolagus cuniculus*, *Lepus europaeuscanis*, *Canis lupus*]). A plasmid containing the complete sequence of the rat HEV strain R63/ DEU/2009 (Li et al., 2015) was used as positive control (kindly provided by Prof Reimar Johnne). For water analysis, HEV quantification was performed using the QIAcuity Digital PCR System and the QIAcuity OneStep Advanced EG Kit (Qiagen) on an 8,5 k nanoplate. The same RT-PCR system as for biological samples was used to quantify *Rocahepevirus* (Parraud et al., 2021). *Paslahepevirus* were also targeted, using the primers/probe proposed by Abravanel et al. (2012).

For each sample, a negative control made of sterile phosphate buffered saline was included at the beginning of each nucleic acid extraction procedure.

### 2.4. Wastewater sample sequencing

To characterize the HEV strains circulating in wastewater, an approach using metagenomics was applied. Nucleic acid extract were treated using TURBO DNase (AM1907, ThermoFisher) and cDNA were synthesized using the ProtoScript® II First Strand cDNA Synthesis Kit (E6560S, New England Biolabs) and the NEBNext® Ultra™ II Non-Directional RNA Second Strand Synthesis Module (E6111S, New England Biolabs), following the manufacturer recommendations. Library preparation was performed using the Twist Enzymatic Fragmentation Library Prep Kit 2.0 (106,454, Twist Bioscience) and viral genome capture was then done using the Twist Comprehensive Viral Research Panel (103,549, Twist Bioscience). Sequencing was performed using the MiSeq Reagent Kit v2–300 cycles (Illumina). Analyses were achieved on the cloud-based metagenomics platform CZ-ID (czid.org).

## 3. Results

### 3.1. HEV in patients

Between April 2019 and October 2023, 3078 serology anti-HEV assays were performed at the Nancy University Hospital, and anti-HEV IgM were detected in 135 patients (4.4 %) (VIDAS® Anti-HEV IgM, bio-Mérieux). During the same period, 812 RT-qPCR were performed to detect conventional HEV RNA, meaning *Paslahepevirus* (RealStar® HEV RT-PCR Kit 2.0 – Altona Diagnostics), including patients positive for anti-HEV IgM detection, as well as patients for which physicians recommended HEV RNA exploration despite the absence of anti-HEV IgM based on clinical setting. Among them, 46 samples were positive (5.7 %, corresponding to 19 patients – collection DC 2016–2790 and 2018-A00117–48). According to the sequencing of the ORF2/ORF3 overlap (Hartard et al., 2021; Inoue et al., 2006) or whole genome (Nicot et al., 2018), all strains detected in patients belonged to the HEV genotype 3 (subtype 3f, 3c and 3ra), which correspond to the strains the most frequently found in France (Nicot et al., 2018). All the 766 re-

maining samples were tested for *Rocahepevirus* RNA detection and were negative.

### 3.2. HEV in water

Based on F-specific RNA phages detection, the recovery rates of the methods used to concentrate samples were between 0.6 % and 22.3 % for slaughterhouse water (median = 5.7 %) and between 32.1 % and 223.4 % for surface water (median = 62.2 %), in line with what is generally observed in this type of study (Chaqroun et al., 2024).

Urban WWTP collected between July and September 2023 were all positive for *Rocahepevirus* detection ( $n = 11$ ), while 91 % of samples (10/11) were positive for *Paslahepevirus* (Fig. 1). The *Rocahepevirus* concentration ranged between 3.8 and 5.7 log<sub>10</sub> genome copies (gc)/L and were in average 40-fold higher than that of *Paslahepevirus* (2.9–4.1 log<sub>10</sub> gc/L). No *Rocahepevirus* was detected in slaughterhouse water (SH,  $n = 14$ ), while all samples were positive for *Paslahepevirus* (2.9–4.4 log<sub>10</sub> gc/L). At last, surface waters (SW,  $n = 4$ ) were positive for both viruses with close concentrations, ranging between 2.8 and 3.4 log<sub>10</sub> gc/L for *Paslahepevirus* and 2.5–3.1 log<sub>10</sub> gc/L for *Rocahepevirus*, respectively.

Metagenomics applied to environmental water allowed to confirm the detection of HEV and to partially characterize the circulating strains. *Paslahepevirus* belonged to genotypes 3c and 3 m and were closed to strains previously detected in Europe (Table 1). *Rocahepevirus* sequences could all be compared with strains previously described in rodents (i.e. *Rattus norvegicus*, *Rattus rattus*, *Sorex* spp.), mainly detected for the first time in Asia, where most of the work concerning their emergence has been carried out. Unfortunately, low concentrations of HEV genome detected in other water samples than WWTP prevented the performance of metagenomics analyzes.

## 4. Discussion

Hepatitis E virus has recently been ranked 6th among viruses with high spillover risk, highlighting its zoonotic potential (Grange et al., 2021). The transmission of *Paslahepevirus* from animals (i.e. swine, boars, game) to humans is now well established, but a number of outstanding issues persist. Among them, the exploration of other animal reservoirs likely to hosting HEV strains able to infect human is a key issue. *Rocahepevirus*, initially isolated from rodents, is currently of concern.

In this study, no evidence of human infection with *Rocahepevirus* has been observed among 766 samples collected from patients followed for a suspected HEV hepatitis, between April 2019 and October 2023, in the Nancy university hospital. Similar results were previously observed among a patient's cohort followed in South-Western France, i.e. 224 patients mainly immunocompromised tested between December 2019 and May 2020, despite the presence of anti-HEV IgG (49 %) and IgM (9.4 %) (Parraud et al., 2021), as well as in Germany, i.e. 200 serum or plasma archived samples randomly selected between 2000 and 2020 (Faber et al., 2022). These results underline a relative poor involvement of *Rocahepevirus* as the etiology of human viral hepatitis. However in Spain, among 267 patients explored for an acute hepatitis between January 2018 and September 2021, 3 individuals were positive for *Rocahepevirus* RNA detection, meaning the first cases reported in Europe (Rivero-Juarez et al., 2022). These infections lead to 2 self-resolutions and 1 hospitalization associated to a severe acute hepatitis with a fatal issue 9 days after the admission. None of them reported any contact with suspicious animals or recent travel, while 2 patients have reported the consumption of undercooked pork meat. More recently, the first European case of chronic *Rocahepevirus* infection associated with cirrhosis was described in an immunosuppressed patient (Rodriguez et al., 2023). In this last report, the use of metagenomics and pan-pathogen detection method has solved the difficulty to diagnose emerging or un-

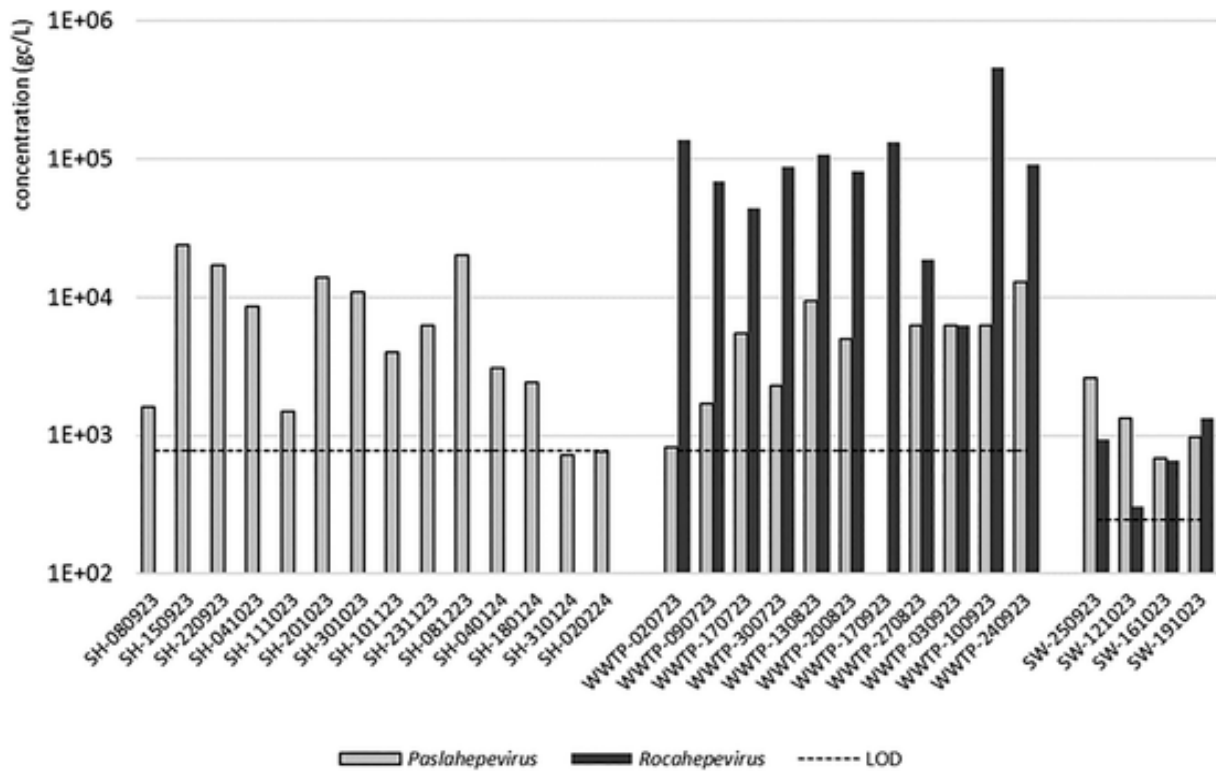


Fig. 1. Circulation of HEV (paslahepevirus and Rocahepevirus) in environmental waters. SH: slaughterhouse; WWTP: wastewater treatment plant; SW: surface water; LOQ: limit of quantification; LOD: theoretical limit of detection.

**Table 1**  
HEV strains identified in WWTP.

WWTP sample	HEV typing	Best sequence identity (Access number)	First detection		
			Host	Country	Year
020723	<i>Rocahepevirus ratti</i>	OP947207.1	<i>Rattus norvegicus</i> (liver)	China	2021
	<i>Paslahepevirus balayani</i> (gt3c)	MH504137.1	<i>Homo sapiens</i> (serum)	United Kingdom	2014
090723	<i>Rocahepevirus ratti</i>	OR500096.1	<i>Rattus norvegicus</i> (liver)	South Korea	2019
170723	<i>Paslahepevirus balayani</i> (gt3c)	MH504137.1	<i>Homo sapiens</i> (serum)	United Kingdom	2014
300723	<i>Rocahepevirus ratti</i>	MW795566.1	<i>Rattus norvegicus</i> (ileum)	Hungary	2010
	<i>Paslahepevirus balayani</i> (gt3m)	MZ289100.1	<i>Homo sapiens</i> (serum)	Spain	2016
130823	<i>Rocahepevirus ratti</i>	OP947209.1	<i>Sorex</i> spp. (lung)	China	2021
200823	<i>Rocahepevirus ratti</i>	OR500095.1	<i>Rattus norvegicus</i> (liver)	South Korea	2016
030923	<i>Paslahepevirus balayani</i> (gt3m)	MZ289100.1	<i>Homo sapiens</i> (serum)	Spain	2016
100923	<i>Rocahepevirus ratti</i>	AB847308.1	<i>Rattus rattus</i> (serum)	Indonesia	2012

determined origin disease, which is currently the case concerning *Rocahepevirus*. These recent data suggest that in the absence of routinely molecular techniques able to detect *Rocahepevirus*, an underdiagnosis may be suspected, both in patients carrying anti-HEV IgM (i.e. cross-reactivity), but also in the absence of IgM in the case of clinical manifestations (Rivero-Juarez et al., 2022; Sridhar et al., 2021b).

In the current state of knowledge, rodents are the main host of *Rocahepevirus*, and an overall seroprevalence of anti-HEV antibodies reaching 20 to 75 % is described worldwide in rats (Wang et al., 2020a). Despite growing interest in the subject, transmission routes to human is still unclear. A direct contact with rats or with rat droppings are suggested, but few cases are fully documented and exposure factors remains unknown (Sridhar et al., 2021b). Currently, among the twenty cases reported worldwide, the source of infection remained mostly still undetermined (Palombieri et al., 2023; Sridhar et al., 2021b). Thus, alternative sources of infection have to be explored, both from an alternate host and from any other transmission route (Reuter et al., 2005).

Despite the rare human infections by *Rocahepevirus* documented in Europe, even in retrospective studies employing appropriate molecular methodologies, our findings indicated significant prevalence of *Rocahepevirus* in the human environment, particularly in urban wastewater. The contamination of wastewater by a human excretion (supposing an asymptomatic carriage) must be raised, but the involvement of the natural host (i.e. rodents) in sewer is the most likely scenario. In all instances, the detection of *Rocahepevirus* in wastewater raises questions regarding its potential as a new way of human contamination (Casares-Jimenez et al., 2024; Palombieri et al., 2023; Wang et al., 2020b). If the waterborne transmission is the most frequent for *Paslahepevirus* in developing countries (Kamar et al., 2012), it is now increasingly recognized in developed countries (Lhomme et al., 2023) and the same assumption might be made concerning *Rocahepevirus*, especially in the light of their concentrations observed in this study, 40-fold higher than that of *Paslahepevirus*. Urban wastewater would thus be a significant

source of exposure, whether directly or through the spreading of viruses in environmental waters, which were also positive for *Rocahepevirus* detection. Elsewhere, the close concentrations of *Paslahepevirus* and *Rocahepevirus* in surface water assume either (i) a different behavior of the two pathogens during water treatment process, or (ii) other sources of environmental contamination (e.g. wildlife or livestock). Since surface water sampling occurred upstream of the urban area's entrance and the WWTP discharges, the second hypothesis is likely the most plausible in this case.

The absence of *Rocahepevirus* in slaughterhouse water supposed here that the pig would not be involved as an intermediate host in the transmission route from rats to humans. This observation was supported by the initial failure of experimental infections, suggesting that pigs are not susceptible to *Rocahepevirus* (Cossaboom et al., 2012). It is however challenged by recent studies showing the circulation of *Rocahepevirus* in farmed pig populations and a new success in cross species infection assays (Rios-Muñoz et al., 2024; Yadav et al., 2024).

Based on these data, wastewater could be a major source of human exposure (Casares-Jimenez et al., 2024; Palombieri et al., 2023; Wang et al., 2020b). This hypothesis has yet to be supported, especially by conducting larger-scale studies. Indeed, the first limitation of this work concerns the limited number of samples. This is a local exploratory study, but presenting the advantage of exploring a defined population catchment area and the sources of exposure in its direct environment.

The second limitation of this work concerns the little data provided by sequencing. Although the use of metagenomics has made possible the detection of *Rocahepevirus* in wastewater, the genome fragments identified corresponded to regions too heterogeneous to perform genetic comparison or to carry out an optimal source tracking. Wastewater monitoring has a long history but has been booming since the SARS-CoV-2 pandemic (Boní et al., 2023). It now allows the early detection of new viral agent such as monkeypox (Tiwari et al., 2023) or the monitoring of pathogen in an epidemiological context, as proposed for respiratory viruses (Ahmed et al., 2023). It benefits from advances in concentration techniques, virus isolation (Chaqroun et al., 2024; El Soufi et al., 2024), as well as new sequencing protocols compatible with such complex matrix, and applied to pathogens present at comparable concentrations in wastewater (e.g. SARS-CoV-2) (Barbé et al., 2022; Wurtzer et al., 2022). These new approaches will have to be applied to monitor viruses for which wastewater is not only an indicator of circulation, but also a direct source of exposure. Among them and in contrast to metagenomics, the use of specific amplifications before sequencing should increase sensitivity and such approaches have already been applied to the molecular characterization of HEV (Casares-Jimenez et al., 2024; Churqui et al., 2024; Palombieri et al., 2023; Rau et al., 2024).

At last, future studies will need to focus on the infectivity of *Rocahepevirus* detected in wastewater. Indeed, this last point is crucial to confirm the possible risk to human health since currently, molecular biology can only confirm the presence of the virus, not its infectious state. The detection of infectious viruses relies on cell culture, which still requires further refinement for application to wastewater. In addition, the use of alternative methods that can determine the integrity of the viral particle and/or its genome will also help to make progress on this point (Johne et al., 2024). Confirming that hypothesis, combined with a better characterization of *Rocahepevirus* circulation and the possible central role of water between the different reservoirs, could have major implications for public health.

## 5. Conclusion

For the first time, the circulation of *Rocahepevirus* has been identified in French wastewater. In addition to their detection in rodents worldwide, this suggests that the zoonotic risk is not confined to specific geographical regions and highlights the need for new diagnostic approaches for patient screening, including the use of serological or

molecular tests that cover all the HEV strains capable of infecting humans. As observed in France, human cases of documented infections remain anecdotally but many gaps concerning *Rocahepevirus* detection exist and studies targeting specifically this pathogen are too rare. Due to the inability of commonly used diagnostic tools to detect *Rocahepevirus*, the global prevalence of infection in the human population remains unknown.

The same applies around the surveillance of environmental reservoirs contributing to spread *Rocahepevirus* and especially the central role that water matrix could play in transmission routes. Further investigations integrating environmental, animal/food and human surveillance are thus crucial to provide a clearer picture of the pathways of *Rocahepevirus* infection, including source tracking and a focus on the infectivity of these strains exposing humans.

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## CRediT authorship contribution statement

**Achouak Rouba:** Writing – review & editing, Formal analysis, Data curation. **Thomas Ansmant:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Ahlam Chaqroun:** Writing – review & editing, Formal analysis. **Julie Challant:** Writing – review & editing, Formal analysis. **Thomas Josse:** Writing – review & editing, Formal analysis. **Evelyne Schvoerer:** Writing – review & editing. **Christophe Gantzer:** Writing – review & editing, Funding acquisition. **Isabelle Bertrand:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Cédric Hartard:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

## References

- Abbravanel, F., Sandres-Saune, K., Lhomme, S., Dubois, M., Mansuy, J.M., Izopeta, J., 2012. Genotype 3 diversity and quantification of hepatitis E virus RNA. *J. Clin. Microbiol.* 50, 897–902. <https://doi.org/10.1128/JCM.05942-11>.
- Abbravanel, F., Lhomme, S., El Costa, H., Schvartz, B., Peron, J.-M., Kamar, N., Izopet, J., 2017. Rabbit hepatitis E virus infections in humans. *France. Emerg. Infect. Dis.* 23, 1191–1193. <https://doi.org/10.3201/eid2307.170318>.
- Ahmed, W., Bivins, A., Stephens, M., Metcalfe, S., Smith, W.J.M., Sirikanthana, K., Kitajima, M., Simpson, S.L., 2023. Occurrence of multiple respiratory viruses in wastewater in Queensland, Australia: potential for community disease surveillance. *Sci. Total Environ.* 864, 161023. <https://doi.org/10.1016/j.scitotenv.2022.161023>.
- Andonov, A., Robbins, M., Borlang, J., Cao, J., Hattchete, T., Stueck, A., Deschaumbault, Y., Murnaghan, K., Varga, J., Johnston, B., 2019. Rat hepatitis E virus linked to severe acute hepatitis in an immunocompetent patient. *J. Infect. Dis.* 1–14. <https://doi.org/10.1093/infdis/jiz025>.
- Barbé, L., Schaeffer, J., Besnard, A., Jousse, S., Wurtzer, S., Moulin, L., Le Guyader, F.S., Desdouts, M., 2022. SARS-CoV-2 whole-genome sequencing using Oxford Nanopore Technology for Variant Monitoring in wastewaters. *Front. Microbiol.* 13, 1–14. <https://doi.org/10.3389/fmicb.2022.889811>.
- Baylis, S.A., O'Flaherty, N., Burke, L., Hogema, B., Corman, V.M., 2022. Identification of rabbit hepatitis E virus (HEV) and novel HEV clade in Irish blood donors. *J. Hepatol.* 118159. <https://doi.org/10.1016/j.jhep.2022.04.015>.

- Bertrand, I., Challant, J., Jeulin, H., Hartard, C., Mathieu, L., Lopez, S., Schvoerer, E., Courtois, S., Gantzer, C., 2021. Epidemiological surveillance of SARS-CoV-2 by genome quantification in wastewater applied to a city in the northeast of France: comparison of ultrafiltration- and protein precipitation-based methods. *Int. J. Hyg. Environ. Health* 233, 113692. <https://doi.org/10.1016/j.ijheh.2021.113692>.
- Bichet, M.C., Gardette, M., Das Neves, B., Challant, J., Erbs, A., Roman, V., Robin, M., La Carbona, S., Gantzer, C., Boudaud, N., Bertrand, I., 2024. A new understanding of somatic coliphages belonging to the Microviridae family in urban wastewater. *Water Res.* 249, 120916. <https://doi.org/10.1016/j.watres.2023.120916>.
- Boni, M., Wurtzer, S., Mouchel, J.M., Maday, Y., Le Guyader, S.F., Garry, P., Bertrand, I., Cluzel, N., Courbariaux, M., Wang, S., Gantzer, C., Maréchal, V., Moulin, L., 2023. Implementation of a National Wastewater Surveillance System in France as a tool to support public authorities during the Covid crisis: the Obepine project. *The Handbook of Environmental Chemistry*. 133–161. <https://doi.org/10.1007/978-2023-994>.
- Caballero-Gómez, J., Pereira, S., Rivero-Calle, I., Perez, A.B., Viciana, I., Casares-Jiménez, M., Ríos-Muñoz, L., Rivero-Juarez, A., Aguilera, A., Rivero, A., 2024. Acute hepatitis in children due to rat hepatitis E virus. *J. Pediatr.* 273, 114125. <https://doi.org/10.1016/j.jpeds.2024.114125>.
- Casares-Jimenez, M., Garcia-Garcia, T., Suárez-Cárdenas, J.M., Perez-Jimenez, A.B., Martín, M.A., Caballero-Gómez, J., Michán, C., Corona-Mata, D., Risalde, M.A., Perez-Valero, I., Guerra, R., Garcia-Bocanegra, I., Rivero, A., Rivero-Juarez, A., Garrido, J.J., 2024. Correlation of hepatitis E and rat hepatitis E viruses urban wastewater monitoring and clinical cases. *Sci. Total Environ.* 908, 168203. <https://doi.org/10.1016/j.scitotenv.2023.168203>.
- Chaqrour, A., El Soufi, G., Gerber, S., Loutrel, J., Cluzel, N., Delafoy, D., Sandron, F., Di Jorio, L., Raffestin, S., Maréchal, V., Gantzer, C., Olaso, R., Deleuze, J.-F., Rohr, O., Boudaud, N., Wallet, C., Bertrand, I., 2024. Definition of a concentration and RNA extraction protocol for optimal whole genome sequencing of SARS-CoV-2 in wastewater (ANRS0160). *Sci. Total Environ.* 952, 175823. <https://doi.org/10.1016/j.scitotenv.2024.175823>.
- Churqui, M.P., Ghaleb, M., Tunovic, T., Frankl, M., Enache, L., Nyström, K., Lagging, M., Wang, H., 2024. High prevalence of hepatitis E and rat hepatitis E viruses in wastewater in Gothenburg, Sweden. *One Heal.* 19, 100882. <https://doi.org/10.1016/j.onehlt.2024.100882>.
- Cook, N., van der Poel, W.H.M., 2015. Survival and elimination of hepatitis E virus: a review. *Food Environ. Virol.* 7, 189–194. <https://doi.org/10.1007/s12560-015-9196-2>.
- Cossaboom, C.M., Córdoba, L., Sanford, B.J., Piñeyro, P., Kenney, S.P., Dryman, B.A., Wang, Y., Meng, X.J., 2012. Cross-species infection of pigs with a novel rabbit, but not rat, strain of hepatitis E virus isolated in the United States. *J. Gen. Virol.* 93, 1687–1695. <https://doi.org/10.1099/vir.0.041509-0>.
- De Cock, M.P., De Vries, A., Fonville, M., Esser, H.J., Mehl, C., Ulrich, R.G., Joeres, M., Hoffmann, D., Eisenberg, T., Schmidt, K., Hulst, M., Van der Poel, W.H.M., Sprong, H., Maas, M., 2023. Increased rat-borne zoonotic disease hazard in greener urban areas. *Sci. Total Environ.* 896, 165069. <https://doi.org/10.1016/j.scitotenv.2023.165069>.
- Eiden, M., Dähnert, L., Spoerel, S., Vina-Rodríguez, A., Schröder, R., Conraths, F.J., Groschup, M.H., 2020. Spatial-temporal dynamics of hepatitis E virus infection in foxes (*Vulpes vulpes*) in Federal State of Brandenburg, Germany, 1993–2012. *Front. Microbiol.* 11, 1–6. <https://doi.org/10.3389/fmicb.2020.00115>.
- El Soufi, G., Di Jorio, L., Gerber, S., Cluzel, N., Van Assche, J., Delafoy, D., Olaso, R., Daviaud, C., Loustau, T., Schwartz, C., Trebouet, D., Hernalsteens, O., Marechal, V., Raffestin, S., Rousset, D., Van Lint, C., Deleuze, J., Boni, M., Rohr, O., Villain-Gambier, M., Wallet, C., 2024. Highly efficient and sensitive membrane-based concentration process allows quantification, surveillance, and sequencing of viruses in large volumes of wastewater. *Water Res.* 249, 120959. <https://doi.org/10.1016/j.watres.2023.120959>.
- Faber, M., Wenzel, J.J., Erl, M., Stark, K., Schemmerer, M., 2022. No evidence for Orthohepevirus C in archived human samples in Germany, 2000–2020. *Viruses* 14, 742. <https://doi.org/10.3390/v14040742>.
- Fenaux, H., Chassaing, M., Berger, S., Jeulin, H., Gentilhomme, A., Bensenane, M., Bronowicki, J.P., Gantzer, C., Bertrand, I., Schvoerer, E., 2018. Molecular features of hepatitis E virus circulation in environmental and human samples. *J. Clin. Virol.* 103, 63–70. <https://doi.org/10.1016/j.jcv.2018.04.003>.
- Grange, Z.L., Goldstein, T., Johnson, C.K., Anthony, S., Gilardi, K., Daszak, P., Olival, K.J., O'Rourke, T., Murray, S., Olson, S.H., Togami, E., Vidal, G., Mazet, J.A.K., 2021. Ranking the risk of animal-to-human spillover for newly discovered viruses. *Proc. Natl. Acad. Sci.* 118, 1–8. <https://doi.org/10.1073/pnas.2002324118>.
- Hartard, C., Fenaux, H., Gentilhomme, A., Murray, J.M., Akand, E., Laugel, E., Berger, S., Maul, A., de Rougemont, A., Jeulin, H., Remen, T., Bensenane, M., Bronowicki, J., Gantzer, C., Bertrand, I., Schvoerer, E., 2021. Variability in molecular characteristics of hepatitis E virus quasipieces could modify viral surface properties and transmission. *J. Viral Hepat.* 28, 1078–1090. <https://doi.org/10.1111/jvh.13513>.
- Inoue, J., Takahashi, M., Yazaki, Y., Tsuda, F., Okamoto, H., 2006. Development and validation of an improved RT-PCR assay with nested universal primers for detection of hepatitis E virus strains with significant sequence divergence. *J. Virol. Methods* 137, 325–333. <https://doi.org/10.1016/j.jviromet.2006.07.004>.
- Johne, R., Scholz, J., Falkenhagen, A., 2024. Heat stability of foodborne viruses – findings, methodological challenges and current developments. *Int. J. Food Microbiol.* 413, 110582. <https://doi.org/10.1016/j.ijfoodmicro.2024.110582>.
- Kamar, N., Bendall, R., Legrand-Abravanel, F., Xia, N.-S., Ijaz, S., Izopet, J., Dalton, H.R., 2012. Hepatitis E. *Lancet* 379, 2477–2488. [https://doi.org/10.1016/S0140-6736\(11\)61849-7](https://doi.org/10.1016/S0140-6736(11)61849-7).
- Kamar, N., Izopet, J., Pavio, N., Aggarwal, R., Labrique, A., Wedemeyer, H., Dalton, H.R., 2017. Hepatitis E virus infection. *Nat. Rev. Dis. Prim.* 3, 17086. <https://doi.org/10.1038/nrdp.2017.86>.
- Lee, G.-H., Tan, B.-H., Chi-Yuan Teo, E., Lim, S.-G., Dan, Y.-Y., Wee, A., Kim Aw, P.P., Zhu, Y., Hibberd, M.L., Tan, C.-K., Purdy, M.A., Teo, C.-G., 2016. Chronic infection with camelid hepatitis E virus in a liver transplant recipient who regularly consumes camel meat and Milk. *Gastroenterology* 150, 355–357.e3. <https://doi.org/10.1053/j.gastro.2015.10.048>.
- Lhomme, S., Magne, S., Perelle, S., Vaissière, E., Abravanel, F., Trelon, L., Hennechart-Collette, C., Fraisse, A., Martin-Latil, S., Izopet, J., Fignon, J., Spacciferri, G., 2023. Clustered cases of waterborne hepatitis E virus infection, France. *Viruses* 15, 1–11. <https://doi.org/10.3390/v15051149>.
- Li, T.C., Yang, T., Yoshizaki, S., Ami, Y., Suzuki, Y., Ishii, K., Haga, K., Nakamura, T., Ochiai, S., Takaji, W., John, R., 2015. Construction and characterization of an infectious cDNA clone of rat hepatitis E virus. *J. Gen. Virol.* 96, 1320–1327. <https://doi.org/10.1099/vir.0.000072>.
- Mansuy, J.M., Gallian, P., Dimeglio, C., Saune, K., Arnaud, C., Pelletier, B., Morel, P., Legrand, D., Tiberghien, P., Izopet, J., 2016. A nationwide survey of hepatitis E viral infection in French blood donors. *Hepatology* 63, 1145–1154. <https://doi.org/10.1002/hep.28436>.
- Nicot, F., Jeanne, N., Roulet, A., Lefebvre, C., Carcenac, R., Manno, M., Dubois, M., Kamar, N., Lhomme, S., Abravanel, F., Izopet, J., 2018. Diversity of hepatitis E virus genotype 3. *Rev. Med. Virol.* 28, e1987. <https://doi.org/10.1002/rmv.1987>.
- Palombieri, A., Di Profio, F., Sarchese, V., Fruci, P., Suffredini, E., Martella, V., Veneri, C., Bonanno Ferraro, G., Mancini, P., La Rosa, G., Di Martino, B., 2023. Surveillance for rat hepatitis E in wastewater networks. *Italy. Microbiol. Spectr.* 11, 1–7. <https://doi.org/10.1128/spectrum.02675-23>.
- Parraud, D., Lhomme, S., Péron, J.M., Da Silva, I., Tavitian, S., Kamar, N., Izopet, J., Abravanel, F., 2021. Rat hepatitis E virus: presence in humans in South-Western France? *Front. Med.* 8, 6–10. <https://doi.org/10.3389/fmed.2021.726363>.
- Pischke, S., Hartl, J., Pas, S.D., Lohse, A.W., Jacobs, B.C., Van der Eijk, A.A., 2017. Hepatitis E virus: infection beyond the liver? *J. Hepatol.* 66, 1082–1095. <https://doi.org/10.1016/j.jhep.2016.11.016>.
- Rau, F., Elsner, C., Meister, T.L., Gömer, A., Kallies, R., Dittmer, U., Steinmann, E., Todt, D., 2024. Monitoring of hepatitis E virus in wastewater can identify clinically relevant variants. *Liver Int.* 44, 637–643. <https://doi.org/10.1111/liv.15842>.
- Reuter, G., Krisztalovics, K., Vennema, H., Koopmans, M., Szucs, G., 2005. Evidence of the etiological predominance of norovirus in gastroenteritis outbreaks—emerging new-variant and recombinant noroviruses in Hungary. *J. Med. Virol.* 76, 598–607. <https://doi.org/10.1002/jmv.20403>.
- Ríos-Muñoz, L., González, M., Caballero-Gomez, J., Castro-Scholten, S., Casares-Jimenez, M., Agulló-Ros, I., Corona-Mata, D., García-Bocanegra, I., Lopez-Lopez, P., Fajardo, T., Mesquita, J.R., Risalde, M.A., Rivero-Juarez, A., 2024. Detection of rat hepatitis E virus in pigs, Spain, 2023. *Emerg. Infect. Dis.* 30, 823–824. <https://doi.org/10.3201/eid3004.231629>.
- Rivero-Juarez, A., Frias, M., Perez, A.B., Pineda, J.A., Reina, G., Fuentes-Lopez, A., Freyre-Carrillo, C., Ramirez-Arellano, E., Alados, J.C., Rivero, A., 2022. Orthohepevirus C infection as an emerging cause of acute hepatitis in Spain: first report in Europe. *J. Hepatol.* 77, 326–331. <https://doi.org/10.1016/j.jhep.2022.01.028>.
- Robinson, S.J., Borlang, N., Himsforth, C.G., Pearl, D.L., Weese, J.S., Dibernardo, A., Osiowy, C., Naseri, N., Jardine, C.M., 2023. Rat hepatitis E virus in Norway rats, Ontario, Canada, 2018–2021. *Emerg. Infect. Dis.* 29, 2018–2021. <https://doi.org/10.3201/eid2909.230517>.
- Rodríguez, C., Marchand, S., Sessa, A., Cappy, P., Pawlotsky, J.-M., 2023. Orthohepevirus C hepatitis, an underdiagnosed disease? *J. Hepatol.* 79, e39–e41. <https://doi.org/10.1016/j.jhep.2023.02.008>.
- Ryll, R., Bernstein, S., Heuser, E., Schlegel, M., Dremsek, P., Zumpe, M., Wolf, S., Pépin, M., Bajomi, D., Müller, G., Heiber, A.C., Spahr, C., Lang, J., Groschup, M.H., Ansoorge, H., Freise, J., Guenther, S., Baert, K., Ruiz-Fons, F., Pikula, J., Knap, N., Tsakmakidis, I., Dovas, C., Zanet, S., Imholt, C., Heickel, G., John, R., Ulrich, R.G., 2017. Detection of rat hepatitis E virus in wild Norway rats (*Rattus norvegicus*) and black rats (*Rattus rattus*) from 11 European countries. *Vet. Microbiol.* 208, 58–68. <https://doi.org/10.1016/j.vetmic.2017.07.001>.
- Sridhar, S., Situ, J., Cai, J.P., Yip, C.C.Y., Wu, S., Zhang, A.J.X., Wen, L., Chew, N.F.S., Chan, W.M., Poon, R.W.S., Chan, J.F.W., Tsang, D.N.C., Chen, H., Xia, N.S., Yuen, K.Y., 2021a. Multimodal investigation of rat hepatitis E virus antigenicity: implications for infection, diagnostics, and vaccine efficacy. *J. Hepatol.* 74, 1315–1324. <https://doi.org/10.1016/j.jhep.2020.12.028>.
- Sridhar, S., Yip, C.C., Wu, S., Chew, N.F., Leung, K., Chan, J.F., Zhao, P.S., Chan, W., Poon, R.W., Tsoi, H., Cai, J., Chan, H.S., Leung, A.W., Tse, C.W., Zee, J.S., Tsang, O.T., Cheng, V.C., Lau, S.K., Woo, P.C., Tsang, D.N., Yuen, K., 2021b. Transmission of rat hepatitis E virus infection to humans in Hong Kong: a clinical and epidemiological analysis. *Hepatology* 73, 10–22. <https://doi.org/10.1002/hep.31138>.
- Terio, V., Bottaro, M., Pavoni, E., Losio, M.N., Serrano, A., Giacometti, F., Martella, V., Mottola, A., Di Pinto, A., Tantillo, G., 2017. Occurrence of hepatitis a and E and norovirus GI and GII in ready-to-eat vegetables in Italy. *Int. J. Food Microbiol.* 249, 61–65. <https://doi.org/10.1016/j.ijfoodmicro.2017.03.008>.
- Tiwari, A., Adhikari, S., Kaya, D., Islam, M.A., Malla, B., Sherchan, S.P., Al-Mustapha, A.I., Kumar, M., Aggarwal, S., Bhattacharya, P., Bibby, K., Halden, R.U., Bivins, A., Haramoto, E., Oikarinen, S., Heikinheimo, A., Pitkänen, T., 2023. Monkeypox outbreak: wastewater and environmental surveillance perspective. *Sci. Total Environ.* 856, 159166. <https://doi.org/10.1016/j.scitotenv.2022.159166>.
- Wang, B., Harms, D., Yang, X., Lou, Bock, C.T., 2020a. Orthohepevirus C: an expanding species of emerging hepatitis E virus variants. *Pathogens* 9, 1–23. <https://doi.org/10.3390/pathogens9030154>.
- Wang, H., Neyvaldt, J., Enache, L., Sikora, P., Mattsson, A., Johansson, A., Lindh, M., Bergstedt, O., Norder, H., 2020b. Variations among viruses in influent water and effluent water at a wastewater plant over one year as assessed by quantitative PCR and metagenomics. *Appl. Environ. Microbiol.* 86, 1–14. <https://doi.org/10.1128/AEM.2073-20>.

Wurtzer, S., Levert, M., Dhenain, E., Accrombessi, H., Manco, S., Fagour, N., Goulet, M., Boudaud, N., Gaillard, L., Bertrand, I., Challant, J., Masnada, S., Azimi, S., Gillon-Ritz, M., Robin, A., Mouchel, J.-M., Sig, O., Moulin, L., 2022. From alpha to omicron BA.2: new digital RT-PCR approach and challenges for SARS-CoV-2 VOC monitoring and normalization of variant dynamics in wastewater. *Sci. Total Environ.* 848, 157740.

<https://doi.org/10.1016/j.scitotenv.2022.157740>.

Yadav, K.K., Boley, P.A., Lee, C.M., Khatiwada, S., Jung, K., Laocharoensuk, T., Hofstetter, J., Wood, R., Hanson, J., Kenney, S.P., 2024. Rat hepatitis E virus cross-species infection and transmission in pigs. *PNAS Nexus* 3, 1–14. <https://doi.org/10.1093/pnasnexus/pgae259>.

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