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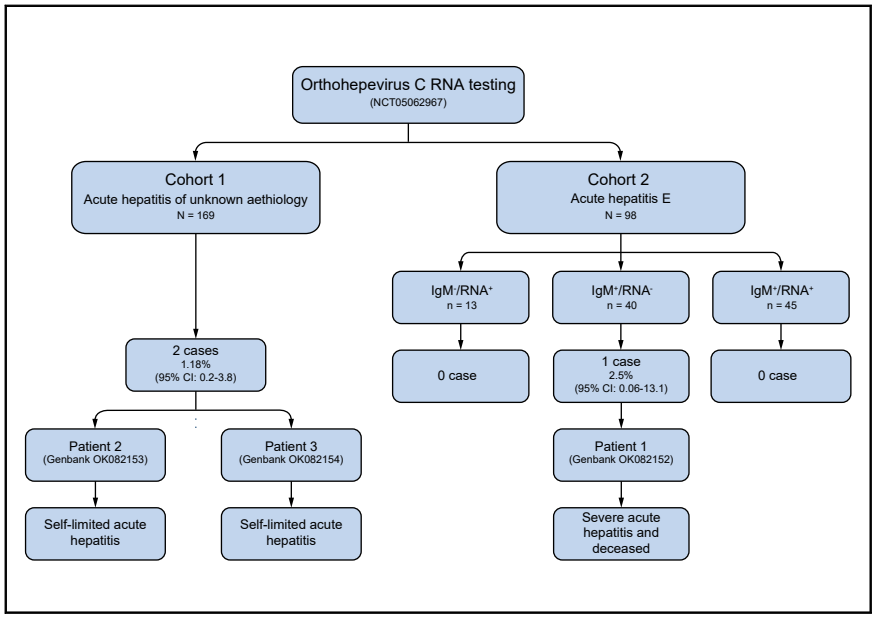
Orthohepevirus C infection as an emerging cause of acute hepatitis in Spain: First report in Europe

Graphical abstract



Highlights

- First cases of acute hepatitis related to *Orthohepevirus* C infection in Europe.
- Second registered death related to *Orthohepevirus* C infection worldwide in an immunosuppressed individual.
- Screening for *Orthohepevirus* C RNA should be evaluated in all patients with acute hepatitis.

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Lay summary

We describe the first cases of acute hepatitis related to rat hepatitis E virus in Europe. The prevalence found in our study suggest that rat hepatitis E virus could be considered an emerging disease in Europe.

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Orthohepevirus C infection as an emerging cause of acute hepatitis in Spain: First report in Europe

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Background & Aim: Hepatitis E virus (HEV) was considered the only member of the Hepeviridae family with zoonotic potential. Nevertheless, this consideration has been reassessed owing to several reported cases of acute and chronic hepatitis linked to the *Orthohepevirus* C genus. Because the circulation of *Orthohepevirus* C in rodents has been described worldwide, the risk of zoonotic transmission is plausibly global.

Methods: *Orthohepevirus* C RNA was retrospectively evaluated in 2 cohorts of patients in Spain. The first cohort included patients with acute hepatitis without etiological diagnosis after screening for hepatotropic virus infection. The second cohort included patients diagnosed with acute HEV infection, defined as positivity for anti-HEV-IgM antibodies and/or detectable HEV RNA in serum.

Results: Cohort 1 comprised 169 patients (64.4% male, median age 43 years) and cohort 2 comprised 98 individuals (68.3% male, median age 45 years). Of the individuals included in Cohort 1, two (1.18%; 95% CI 0.2-3.8) had detectable *Orthohepevirus* C RNA in serum. In Cohort 2, of the 98 included patients, 58 showed detectable HEV RNA, while 40 only showed positivity for IgM antibodies. Among those bearing only IgM antibodies, *Orthohepevirus* C RNA was detected in 1 (2.5%; 95% CI 0.06-13.1) individual. All strains were consistent with genotype C1. The infection resulted in mild self-limiting acute hepatitis in 2 patients. Infection caused severe acute hepatitis in the remaining patient who died as a result of liver and renal failure.

Conclusions: We described 3 cases of *Orthohepevirus* C in patients with acute hepatitis, resulting in the first description of this infection in Europe. The prevalence obtained in our study suggests that Orthohepevirus C could be an emerging disease in Europe.

Lay summary: We describe the first cases of acute hepatitis related to rat hepatitis E virus in Europe. The prevalence found in our study suggest that rat hepatitis E virus could be considered an emerging disease in Europe.

Introduction

The *Orthohepevirus* genus comprises single-strain RNA viruses including 4 species: *Orthohepevirus* A, B, C and D.[1] *Orthohepevirus* A, known as hepatitis E virus (HEV), is one of the major causes of acute hepatitis worldwide.[2,3] According to genome sequences, HEVs present 8 major genotypes,[4] whose circulation can be limited to humans through consumption of fecal-contaminated water in Asian and African countries (genotypes 1 and 2) or present a worldwide distribution circulating among humans and a wide number of mammalian species with zoonotic transmission through the consumption of raw or undercooked meat (genotypes 3 to 8).[5,6] Therefore, HEV is a zoonotic disease that is considered a major public health issue in both high- and low-income countries.[7,8] In contrast, the other 3 *Orthohepevirus* species seemed to lack zoonotic threat, and their circulation seemed to be limited to their main host: *Orthohepevirus* B in birds,[9] *Orthohepevirus* C in mustelids and rodents,[10] and *Orthohepevirus* D in bats.[11] Nevertheless, this consideration was reassessed because of recent studies. In 2018, a case of rat HEV infection in a liver transplant recipient was reported in Hong Kong.[12] Thereafter, in a large screening population in the same setting, 7 additional cases of *Orthohepevirus* C infection were

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described.[13] Interestingly, in this study, the author demonstrated epizootic transmission because the virus was identified in rodents sampled from the same district. Despite most patients reported in these studies present underlying chronic conditions, most of them with immunosuppression, several cases were immunocompetent.[13] After that, a prospective screening program for *Orthohepevirus* C in Hong Kong has been set up, demonstrating that this virus is an emerging cause of acute hepatitis in this setting,[14] with the number of known cases rising to 16. Because the circulation of *Orthohepevirus* C in rodents has been described worldwide (supplementary information), the risk of zoonotic transmission is plausible globally and not restricted to a specific area. Notably, 1 case outside Hong Kong has been reported, and it showed severe acute hepatitis. This was an immunocompetent patient who presumably acquired the infection in central Africa.[15] This finding justifies the active search for *Orthohepevirus* C cases in other countries. A recent study conducted in France retrospectively analyzed samples from 224 individuals for *Orthohepevirus* C RNA.[16] They did not find any case, nevertheless most individuals included were immunocompromised and only the 63% had abnormal liver function tests.[16] Therefore, the aim of our study was to evaluate the prevalence of *Orthohepevirus* C infection in immunocompetent individuals with acute hepatitis from a country where *Orthohepevirus* C has been identified in rodents,[17] and to elucidate the role of this virus as an etiological agent of acute hepatitis.

Patients and methods

Patients and setting

The study population included 267 patients with acute hepatitis in follow-up at 7 reference hospitals in Spain and belonged to 2 different cohorts (ClinicalTrials.gov Identifier: NCT05062967). The study period encompassed 1 January 2018 (both cohort setups) to 1st September 2021 (study censored date).

The first cohort comprises 169 patients. The criteria for inclusion in this cohort were i) clinical and biological manifestations compatible with acute hepatitis, ii) alanine aminotransferase level 3x the upper limit of normal, and iii) no etiological diagnosis after screening for hepatotropic virus infection. This screening included serological and/or molecular markers for hepatitis A virus (IgM antibodies), hepatitis B virus (HBsAg, HBcAc, and viral DNA), hepatitis C virus (IgG antibodies and viral RNA), HEV (IgM antibodies and viral RNA), cytomegalovirus (IgM antibodies), and Epstein-Barr virus (IgM antibodies).

The second cohort included 98 patients diagnosed with acute HEV infection. Acute HEV infection was defined as i) clinical and biological manifestation compatible with acute hepatitis and ii) positivity for anti-HEV-IgM antibodies and/or detectable HEV RNA in serum.

Serological and molecular screening for HEV

Information about all reagents, software and biological samples employed in the study can be found in the supplementary CTAT table. HEV molecular and serological markers for HEV infection were centrally evaluated at the Clinical Virology and Zoonoses laboratory of the Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC). Anti-HEV antibodies were evaluated by enzyme immunoassay using the HEV-IgM kit and HEV-IgG kit developed by Wantai (Beijing Wantai Biological Pharmacy Enterprise Ltd., Beijing, China) by an automated procedure using DYNEX DS2® (DYNEX Technologies. Sullyfield Circle Chantilly, VA, USA). For HEV RNA evaluation, we applied a protocol developed and validated by our group for *Orthohepevirus* A detection, with a detection limit set at 21 IU/ml.[18] Briefly, RNA was extracted from 400 μl of serum using the QIAamp Mini Elute virus spin kit (Qiagen, Hilden, Germany) and QIAcube (Qiagen, Hilden, Germany). The purified RNA was eluted in a 50 μl volume using 25 μl for reverse-transcription quantitative PCR using the One-Step PCR Kit (Qiagen, Hilden, Germany). The sensitivity of the ELISA for rat HEV-derived IgG and IgM employed at screening has been estimated to be 70% and 40%, respectively.[19]

Molecular evaluation for *Orthohepevirus* C infection

All patients were retrospectively evaluated for *Orthohepevirus* C infection using broad-spectrum nested PCR targeting the RdRp gene, as described previously.[20] Ten microliters of nucleic acid extraction were used in combination with the One-Step reverse-transcription quantitative PCR kit (Qiagen, Hilden, Germany) for the first round of PCR and 5 μl of the first PCR product using Promega master Mix (Madison, USA) for the second reaction. We used the 1st World Health Organization International Standard for HEV RNA nucleic acid amplification test-based assays – consistent with HEV genotype 3a and provided by the Paul-Ehrlich-Institut (PEI code 6219/10) – as a positive control. The lyophilizate material was reconstituted with 500 μl of diethyl pyrocarbonate-treated water (Thermo Fisher Scientific, Waltham, MA, USA) using 200 μl for nucleic acid extraction employing the same procedure used for serum samples.

The amplicons were examined on 1.5% agarose gels stained with RedSafe™ Nucleic Acid Staining solution. PCR products with the correct target size (approximately 330 nucleotides) were purified using Illustra™ ExoProStar™. Both sense strands were sequenced using a BigDye Terminator cycle sequencing ready reaction kit on an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Categorical variables were expressed as the number of cases (percentage), and continuous variables were expressed as the median (IQR). The prevalence of *Orthohepevirus* C infection was calculated in each cohort. For prevalence, the 2-sided 95% CI was calculated using the exact binomial distribution.

The *Orthohepevirus* species were assigned using the HEVnet genotyping tool (https://www.rivm.nl/mpf/typingtool/hev/) and confirmed by BLAST.[21] Sequence alignments were generated by the MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Phylogenetic trees were constructed using the maximum likelihood method using 68 *Orthohepevirus* C strains. Information about these strains can be found in Table S1. Four sequences belonging to *Orthohepevirus* A (M73218), *Orthohepevirus* B (GU954430), *Orthohepevirus* D (KX513953), and the proposed *Orthohepevirus* E (KF951328) were included as outgroups to root the tree. Two sequences previously isolated in 2 *Rattus rattus* specimens sampled in 2013 in South Spain were also included (KY938026 and KY938027).[17] The final tree was obtained with MEGA Software (Version 7) using the bootstrap method (bootstrapped with 1,000 replicates).

Ethics statement

This study was designed and conducted in accordance with the Declaration of Helsinki. The Ethics and Clinical Trials Committee

(CEIC) of Córdoba approved the study protocol (protocol reference number 5081), and informed consent was obtained from each patient. The Sistema Sanitario Público Andaluz (SSPA) Biobank coordinated the collection, processing, handling and assignment of the biological samples used in this study in accordance with the standard procedures established for this purpose.

Results

Population

Of the 169 patients included in Cohort 1, 109 (64.4%) were male, with a median (IQR) age of 43 years (36-55). This population showed a median (IQR) level of 157 U/L (101-601) for alanine aminotransferase, 95 U/L (23-366) for aspartate aminotransferase, 114 U/L (40-272) for gamma-glutamyltransferase, and 0.7 mg/dl (0.5-4) for bilirubin.

Among the 98 who constituted Cohort 2, 67 (68.3%) were male, and the median (IQR) age was 45 (34-53) years. The median alanine aminotransferase, aspartate aminotransferase, gamma-glutamyltransferase, and bilirubin levels in this cohort were 131 U/L (37-435), 99 U/L (27-394), 128 U/L (41-289), and 0.7 mg/dl (0.6-4.4), respectively. Forty-five individuals (45.9%) were positive for both anti-HEV-IgM antibodies and HEV RNA, 40 (40.9%) exhibited positivity only for IgM antibodies, and 13 (13.2%) showed detectable HEV RNA but were negative for IgM antibodies. Globally, 85 (86.7%) patients were positive for IgM anti-HEV, and 58 (59.1%) showed detectable viral RNA in serum. The viral genotype was assigned in all individuals with detectable HEV RNA: 51 (86.4%) cases were genotype 3f, 3 (5.2%) were genotype 3 m, 1 (3.2%) was genotype 3e, and subtype could not be defined in 3 (5.2%) patients bearing genotype 3 infection.

Prevalence of *Orthohepevirus* C infection

The screening results for *Orthohepevirus* C are shown in Fig. 1. Of the individuals included in Cohort 1, 2 (1.18%; 95% CI 0.2-3.8) had detectable *Orthohepevirus* C RNA in the serum. In Cohort 2, of the 98 included patients, 58 had detectable HEV RNA, while 40 only showed positivity for IgM antibodies. *Orthohepevirus* C was not detected in any of the patients with detectable HEV RNA. Instead, *Orthohepevirus* C RNA was detected in 1 (2.5%; 95% CI 0.06-13.1) individual among those bearing only IgM antibodies.

The partial genome of the RdRp gene showed that the 3 strains were consistent with genotype C1 (Fig. 2). These sequences are available at GenBank under accession numbers OK082152, OK082153, and OK082154. By BLAST, we found high homology (99.1%-98.5%) with *Orthohepevirus* C strains isolated in *Rattus rattus (MH400712, MH400713 and MH400717) and Rattus norvegicus (MH400714 to MH400716)* specimens from Lithuania sampled in 2016,[22] and with the sequence GU345043, originating from a *Rattus norvegicus* in Germany in 2009. Additionally, the sequences identified in our study were closely related to *Orthohepevirus* C strains previously isolated in 2 *Rattus rattus* specimens from southern Spain (KY938026 and KY938027) (Fig. 2).[17]

Cases of *Orthohepevirus C*

In Table 1, we summarized the main clinical and epidemiological features of the 3 cases of *Orthohepevirus* C infection. All patients were male, Patient 1 and Patient 2 were inhabitants of southern Spain, and Patient 3 lived in northern Spain. None of them reported contact with animals (including pets, farm animals, wild animals, or hunting), conscious contact with rodents, or travel outside Spain in the previous months. As risk factors for HEV infection, Patient 1 and Patient 2 reported consumption of undercooked pork in the 2 weeks prior to diagnosis. Patient 3 reported being a cleaner. *Orthohepevirus* C infection in Patient 2 and Patient 3 resulted in mild acute hepatitis with self-resolution. Meanwhile, Patient 1 was admitted to the hospital because of severe acute hepatitis and died 9 days after admission because of liver and renal failure.

Discussion

We report the first cases of *Orthohepevirus* C infection in Europe. *Orthohepevirus* C was identified as the etiological agent of acute

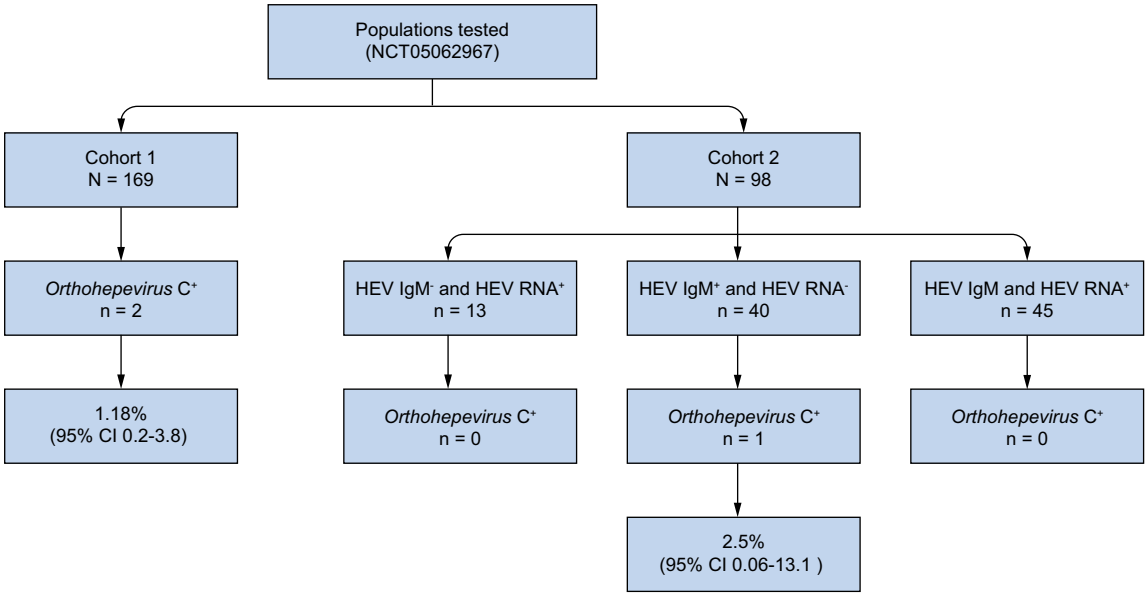

Fig. 1. Study diagram flow and screening results for *Orthohepevirus* C. HEV, hepatitis E virus.

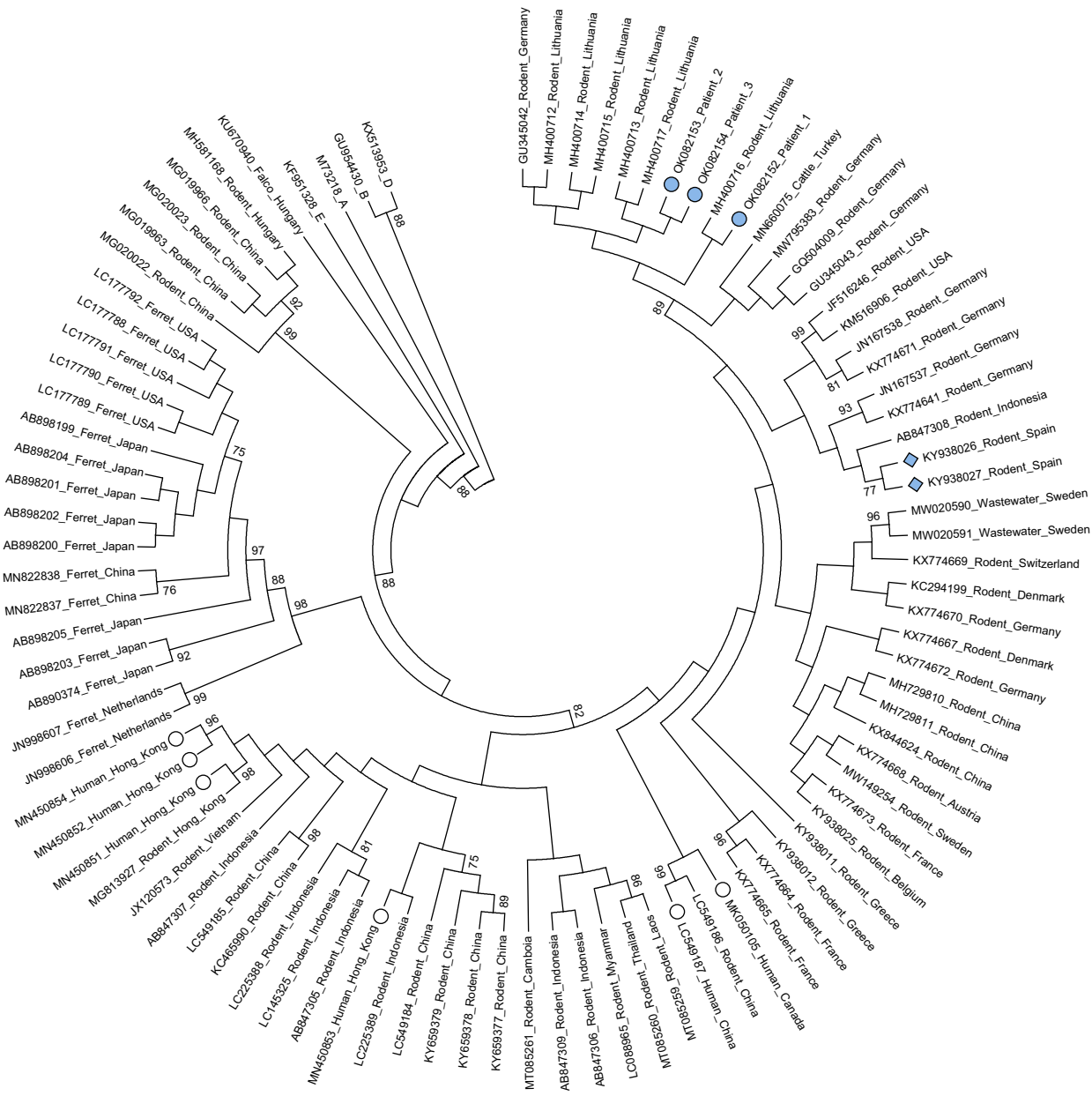

Fig. 2. The evolutionary history was inferred by using the maximum likelihood method based on the Jukes-Cantor model. The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach, and then selecting the topology with superior log likelihood value. The analysis involved 94 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. There were a total of 262 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. Light blue circle highlights the sequences identified in the present study. Light blue diamond highlights those sequences identified in Spanish *Rattus rattus* specimens.

hepatitis in 2.5% of patients with a diagnosis of acute HEV based only on the presence of IgM antibodies (1 out 40 cases) and in 1.18% of individuals with acute hepatitis not related to hepatotropic virus infection (2 out 169). Our findings suggest that *Orthohepevirus* C could be an emerging infectious disease.

In 2 cases, the infection course was symptomatic self-limiting hepatitis, while in the other case, the infection triggered fatal liver and renal failure. Our findings are in line with previous cases of *Orthohepevirus* C infection, showing that the clinical course of the infection ranges from mild and self-limiting acute hepatitis to severe fatal outcomes.[13,14] Although the first evidence of *Orthohepevirus* C infection was aimed at the context of underlying immunosuppression,[12,13] other cases were documented in an immunocompetent individual.[15] In this sense, 2 cases identified in our study did not suffer from immunosuppression, suggesting that this condition is not necessary to be susceptible to the infection and to present symptomatic infection. However, although the number of cases reported of *Orthohepevirus* C infection is low, the course of the infection appears to be worse in individuals with underlying

Table 1. Epidemiological and clinical characteristics of patients infected by rat HEV.

Variable	Patient 1	Patient 2	Patient 3
Cohort of origin	Cohort 2	Cohort 1	Cohort 1
Age (years)	62	30	54
Sex	Male	Male	Male
Year of the sample	2020	2018	2019
Region	Southern Spain	Southern Spain	Northern Spain
Location	Córdoba	Seville	Vitoria
Significant underlying disorder	Metastatic oral cancer	No	No
Concomitant medication	Amoxicillin and clavulanic acid* dexamethasone†	No	Atorvastatine‡
Exposure to domestic animals	No	No	No
Exposure to wild animals	No	No	No
Exposure to rodents	No	No	No
Consumption of raw or undercooked meat	Yes	Yes	No
Travel outside Spain	No	No	No
Blood transfusion	No	No	No
Other risk practice for HEV	No	Cleaning staff	Unknown
IgG anti-HEV antibodies	Positive	Negative	Positive
IgM anti-HEV antibodies	Positive	Negative	Negative
Peak ALT (U/L)	400	554	173
Peak AST (U/L)	340	549	137
Peak GGT (U/L)	630	72	536
Peak bilirubin (mg/dl)	8.4	0.7	1.09
Hospital admission	Yes	No	No
Intensive care unit admission	Yes	No	No
Infection outcome	Death by liver and renal failure	Self-limited	Self-limited

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyltransferase; HEV, hepatitis E virus.
*At a dose of 500 mg/125 mg every 8 hours.
†At a dose of 4 mg twice daily.
‡At a dose of 80 mg daily.

immunosuppression. In this sense, the only patient suffering from immunosuppression showed severe acute hepatitis with a rapid deterioration of liver function and death and is the second registered death related to *Orthohepevirus* C infection.[13] In this sense, the first registered death linked to *Orthohepevirus* C infection was recorded in a kidney transplant recipient. It should be noted that this patient also suffered from a severe underlying disease (metastatic cancer), which might have affected the clinical course of the hepatitis. Thus, close surveillance of *Orthohepevirus* C could be needed not only in patients with underlying immunosuppression, but also in patients with severe underlying comorbidities.

Based on our current knowledge, from a clinical point of view, *Orthohepevirus* C and HEV infection seem to be indistinguishable, and cross-reactivity antibodies between *Orthohepevirus* C and HEV have been observed.[19] Therefore, 1 case in our study and 6 cases in the Hong Kong study exhibited HEV-IgM antibodies with undetectable HEV RNA. In the same way, the proportion of *Orthohepevirus* C-infected individuals among those carrying HEV-IgM antibodies was similar between our study (2.5%) and the study conducted in Hong Kong (2.9%).[13] Therefore, based on both studies, the evaluation of *Orthohepevirus* C RNA in all patients with HEV-IgM antibodies with undetectable HEV RNA should be recommended, independent of the origin. In contrast, in both studies, 8 individuals lacking HEV-IgM antibodies were identified.[13,14] These findings suggest that the presence of HEV-IgM antibodies has a low sensitivity for *Orthohepevirus* C diagnosis, and therefore, its use as a diagnosis-based algorithm for *Orthohepevirus* C might be very limited.[19] Thus, based on this, screening for *Orthohepevirus* C RNA in patients with acute hepatitis should be evaluated independently of HEV serological patterns.

The main hosts of *Orthohepevirus* C are rodents, which have a global distribution and circulation.[23–25] For this reason, close contact with this species or with its droppings could be the main transmission route.[12] In our study, similar to the study conducted in Hong Kong, we found high homology between human viral sequences and those identified in rodents from the same area, suggesting that the route of infection is related to direct or indirect contact with rodents.[13,14] However, none of the cases described in our study and only 1 of the cases described in Hong Kong reported contact with rodents.[13] Thus, alternative transmission routes need to be evaluated.

Several limitations should be noted. First, the HEV serological pattern of the patients included in the study was assessed using only 1 assay. Because of the variation in the sensitivity of the available HEV commercial assays for *Orthohepevirus* C-derived antibodies, the categorization of the population could change depending on the assays employed. Second, although patients were included prospectively in both cohorts, because of the retrospective character of the analysis, epidemiological investigations were limited. Consequently, it is possible to fail to detect other risk factors associated with *Orthohepevirus* C acquisition. Finally, a survey of *Orthohepevirus* C in rodents from the same setting was not performed, so we only included 2 viral sequences isolated in rodents from Spain obtained in a previous study. This point has an evident negative impact on the phylogenetic traceability of the cases. Prospective *Orthohepevirus* C surveys in both rat and human populations from our setting are warranted.

In conclusion, the prevalence found in our study suggests that *Orthohepevirus* C may be an emerging cause of acute hepatitis in Europe, which could be misdiagnosed because of cross-reactivity with HEV-derived antibodies and the lack of molecular screening.

Abbreviations
HEV, hepatitis E virus.

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Conflicts of interest
The authors declare that there are no competing interests. Neither the authors nor their institutions have at any time received payment or services from a third party for any aspect of the submitted work (data monitoring board, study design, manuscript preparation, statistical analysis, and so on).

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions
ARJ was involved in the study design and conception, interpretation of the data, drafting of manuscript and study supervision. MF was involved in the serological and molecular determination. ABP, JAP, GR, JCA, AF, ERA and CF and were involved in the data acquisition and critical review of the manuscript. AR was involved in the study design and conception, interpretation of the data and drafting of manuscript.

Data availability statement
All data generated or analyzed during the study are included in this published article. The datasets used and/or analyzed during the present research project are available from the corresponding author upon reasonable request. Sequences are available in GenBank under accession numbers OK082152, OK082153, and OK082154.

Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2022.01.028.

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Author names in bold designate shared co-first authorship.

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