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Prevalence of *Rocahepevirus ratti* (rat hepatitis E virus) in humans and rats in China

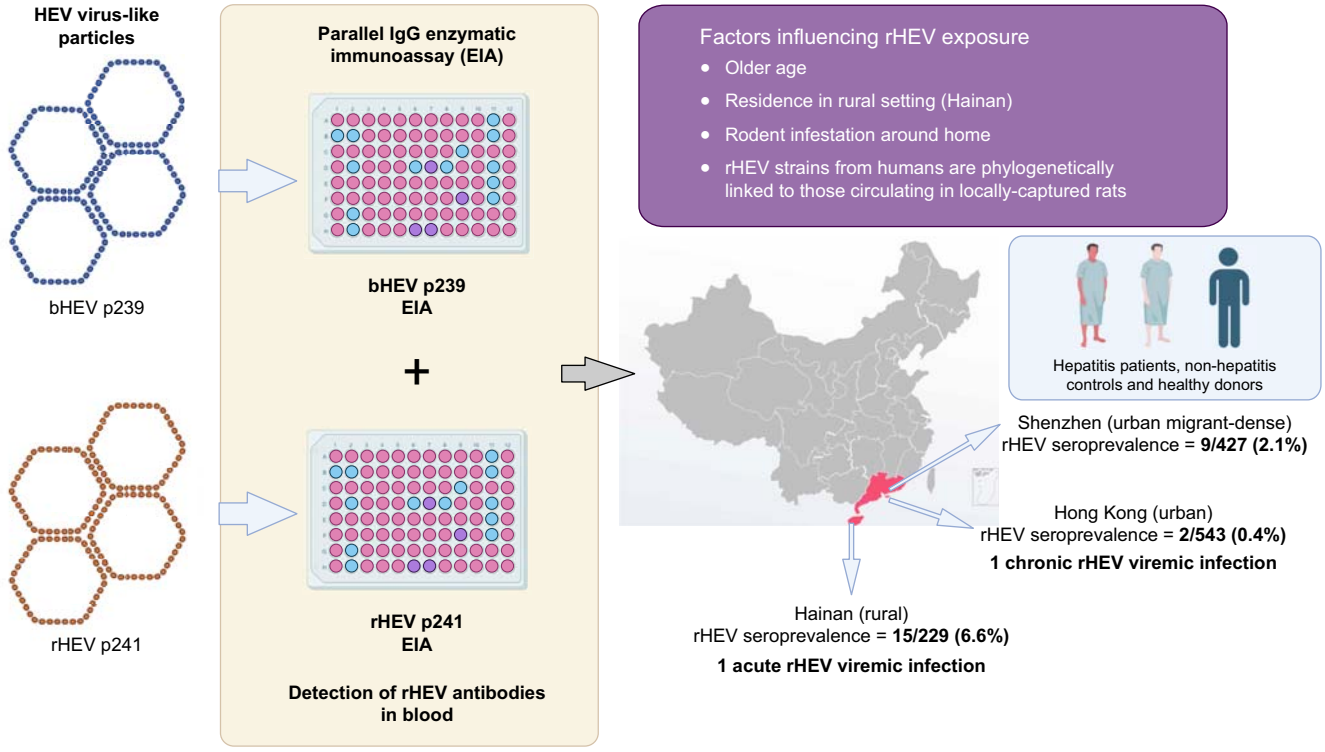
Authors

Xiuji Cui, Jianwen Situ, Ting Tang, …, Feifei Yin, Jasper Fuk-Woo Chan, Siddharth Sridhar

Correspondence

yinfeifeiff@163.com (F. Yin), jfwchan@hku.hk (J.F.-W. Chan), sid8998@hku.hk (S. Sridhar).

Graphical abstract



Highlights:

- Individuals are exposed to rat hepatitis E in rural and urban settings in China.
- Older age and rodent exposure in rural settings are risk factors for exposure.
- Overt hepatitis occurs in individuals with liver disease or immunosuppression.

Impact and implications:

Building on our previous work finding that *Rocahepevirus ratti* (rHEV) is a novel cause of sporadic viral hepatitis in humans, we studied rHEV exposures in humans from various epidemiological settings. We found intermittent exposure to rat hepatitis E in individuals living in both urban and rural settings with a markedly higher seroprevalence in the latter. Spillover viremic infections in individuals with underlying liver disease or immunosuppression were closely linked to epizootic rHEV strains circulating in rodents. Physicians and diagnostic laboratories should incorporate rHEV testing in algorithms for viral hepatitis while policymakers should consider rHEV surveillance in rodents to guide disinfestation efforts.

https://doi.org/10.1016/j.jhepr.2025.101370

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Prevalence of *Rocahepevirus ratti* (rat hepatitis E virus) in humans and rats in China

Xiuji Cui[1,†], **Jianwen Situ**[2,†], **Ting Tang**[1,†], **Zhiyu Li**[2,†], Dongzhui Chen[3], Stanley Siu-Fung Ho[2], Hiu-Laam Chung[2], Tsz-Chung Wong[2], Yonghao Liang[2], Chaowen Deng[4], Yongxian Su[4], Huijun Cai[4], Simon Kam-Fai Lo[4], Shiyao Huang[4], Sheng Zeng[4], Liyuan Zhang[5], Yunchun Chen[6], Shusheng Wu[2], Estie Hon-Kiu Shun[7], Nicholas Foo-Siong Chew[2], James Yiu-Hung Tsoi[2], Kelvin Hon-Yin Lo[2], Fanfan Xing[4], Vincent Chi-Chung Cheng[2], Kwok-Yung Yuen[2,7,8,9,10,11], Feifei Yin[1,*], Jasper Fuk-Woo Chan[1,2,7,8,11,*], Siddharth Sridhar[2,11,*]

JHEP Reports **2025**. vol. 7 | 1–8

Background & Aims: *Rocahepevirus ratti* (rat hepatitis E virus; rHEV) is a ubiquitous pathogen of rats that has recently emerged as a cause of hepatitis in humans. Although several rHEV cases have been detected worldwide, the extent of human exposure to this hepatitis agent is still poorly defined. We conducted a multicenter surveillance study in China examining rHEV exposures in demographically diverse human populations from a One Health perspective.

Methods: In this multicenter cross-sectional study, we used fully validated rHEV IgG enzymatic immunoassays and reverse transcription PCR in 1,199 individuals with (n = 655) or without hepatitis (n = 544) recruited from three centers in China (Hainan, Hong Kong, and Shenzhen). The patient population included both urban and rural populations. Characteristics of infected individuals and phylogenetic links with rat epizootics were described.

Results: rHEV IgG seroprevalence was higher in the rural Hainan cohort (15/229, 6.6%) compared with Shenzhen (9/427, 2.1%) and Hong Kong cohorts (2/543, 0.4%) (p <0.0001). This difference persisted on multivariable logistic regression with an adjusted odds ratio of 20.52 (95% CI: 13.86–30.39). rHEV exposure was also associated with increasing age and environmental rodent exposure. We observed rHEV viraemia in two hepatitis patients (2/655; 0.3%) in Hainan and Hong Kong with hepatitis B and renal transplantation, respectively. The latter developed chronic hepatitis E. 19/509 (3.7%) rats captured in Hainan harbored rHEV. Both human rHEV isolates were phylogenetically related to rodent-derived rHEV strains circulating in Hainan and Hong Kong, respectively.

Conclusions: Humans are intermittently exposed to rHEV, especially in rural settings with rodent exposure. Overt hepatitis occurs in individuals with liver disease or immunosuppression. Spillover strains are related to epizootics in rodents offering opportunities for targeted disinfestation.

Introduction

Hepatitis E virus (HEV) is a major cause of acute hepatitis globally. HEV variants causing human infection belong to two species within the family Hepeviridae: *Paslahepevirus balayani* (bHEV) and *Rocahepevirus ratti* (rHEV). Most human hepatitis E cases are caused by bHEV, which comprises eight genotypes of which five are capable of infecting humans.[1] rHEV, commonly known as rat hepatitis E, is a frequent pathogen of rats that is highly divergent to bHEV.[2] In 2018, we reported for the first time that rHEV can infect humans.[3] Since then, multiple studies by us and other investigators have detected rHEV in immunocompetent and immunocompromised patients with hepatitis in China, Canada, Spain, and France.[4–8] Diagnosis of rHEV infection requires specific assays. Nucleic acid amplification tests and antigen assays based on bHEV completely miss rHEV infection.[3,9,10] Commercial hepatitis E IgM assays are insensitive for rHEV and do not differentiate bHEV from rHEV infections.[9] Therefore, epidemiological characteristics of rHEV in human populations are poorly defined. We have previously reported a fully validated IgG enzymatic immunoassay (EIA) system capable of detecting and differentiating bHEV and rHEV serological responses.[11] Such assays are important for measuring seroprevalence in different populations.

There is a substantial rHEV natural reservoir in China: the virus has been detected in rats captured across the country.[12–18] However, aside from Hong Kong, dedicated rHEV

* Corresponding authors. Addresses: Hainan Medical University-The University of Hong Kong Joint Laboratory of Tropical Infectious Diseases, Key Laboratory of Tropical Translational Medicine of Ministry of Education, Academician Workstation of Hainan Province, School of Basic Medicine and Life Sciences, Hainan Medical University, Haikou, Hainan, 571199, China (F. Yin); Department of Microbiology, School of Clinical Medicine, Li Ka Shing Faculty of Medicine, The University of Hong Kong, 19-026, Block T, Queen Mary Hospital, 102 Pokfulam Road, Hong Kong, China. Tel.: +852-22552407; Fax: +852-28551241 (J.F-W. Chan); Department of Microbiology, School of Clinical Medicine, Li Ka Shing Faculty of Medicine, The University of Hong Kong, 19-026, Block T, Queen Mary Hospital, 102 Pokfulam Road, Hong Kong, China, Tel.: +852-22552408; Fax: +852-28551241 (S. Sridhar).
E-mail addresses: yinfeifeiff@163.com (F. Yin), jfwchan@hku.hk (J.F.-W. Chan), sid8998@hku.hk (S. Sridhar).
† Jianwen Situ, Xiuji Cui, Ting Tang, and Zhiyu Li are co-first authors.
https://doi.org/10.1016/j.jhepr.2025.101370

surveillance in humans is not performed in China. In this multicenter study, we used our real-time RT-PCR (qRT-PCR) and bHEV/rHEV serological assays to investigate rHEV exposure among patients with and without hepatitis from three regions in China: Hainan Island, Shenzhen, and the Hong Kong Special Administrative Region. The three sites were chosen for their diverse settings and demographics. Hainan Island is a tropical island with high biodiversity – approximately 40% of its population lives in rural areas. Shenzhen is an urban center with a large migrant population from provinces across China. Hong Kong is a globally connected city of 7.5 million individuals characterized by high population density and a relatively older population. In addition to investigating human rHEV exposure in these three locations, we parsed rHEV genomic surveillance data from human and non-human hosts in Hainan and Hong Kong to investigate spatiotemporal links between human and rodent-derived rHEV strains.

Materials and methods

Patients

Archived serum or plasma samples from patients sent to departments of microbiology of Hainan Medical University (Hainan), The University of Hong Kong – Shenzhen Hospital (HKU-SZ, Shenzhen), and Queen Mary Hospital (QMH, Hong Kong) between 2019 and 2023 were used for this study. Patients with hepatitis were either hospitalized or hepatitis clinic attendees. Patients without hepatitis following up for other medical issues or checkups were included as controls. In addition, sera from healthy individuals sent to QMH for evaluation of fitness for potential organ donation were retrieved. Inclusion criteria were adults with age >18 years with serum or plasma sent for virological testing to any of the three centers within the study period with sufficient residual sample volume. Specific exclusion criteria included: (a) known recent blood product transfusion within 3 months and (b) known hemopoietic stem cell transplantation within 6 months of blood sampling. All samples were stored at -80 °C until testing. Basic demographic details such as age, sex, clinical diagnosis, and liver function tests of patients were retrieved. Additional occupational and rodent exposure history was obtained from study participants in Hainan. Ethics approval was obtained from Hainan Medical University (HYLL-2020-061), HKU-SZ (hkuszh2024244), and The University of Hong Kong/Hospital Authority West Cluster (UW 18-074). All research was conducted in accordance with both the Declarations of Helsinki and Istanbul. As all samples were anonymized and archived before testing, need for informed consent was waived by the Institutional Review Boards.

Rodent samples and rHEV sequences

Liver tissue samples were collected from rats from multiple counties in Hainan Province between 2015 and 2019. All rats were captured in live traps and humanely euthanized. Liver tissues from captured rodents were immersed in tubes (Yocon, China) containing maintenance medium, and temporarily stored at -20 °C. The samples were transported to the laboratory and stored at -80 °C. We have been conducting genomic surveillance in rats captured in Hong Kong since 2018 because of the emergence of a human outbreak in the territory.[18] Sequences from this surveillance were analyzed for the purpose of this study. Wang *et al.*[16] have conducted rHEV surveillance in Shenzhen and sequences from this study were incorporated in the phylogenetic analysis of this study.

Real-time RT-PCR assays, sequencing, and phylogenetic analysis

Plasma and sera were combined into minipools of five for total nucleic acid extraction using the Dongsheng Swab/Saliva Viral DNA/RNA Extraction Kit (V4001, Guangzhou Dongsheng Biotech Ltd, China). We have previously used this method for HEV screening in large sample sets.[18] Minipool-extracted nucleic acid was then tested for bHEV and rHEV RNA using our previously described validated qRT-PCR assays.[3] Constituent samples of minipools testing positive were individually extracted and tested to identify the positive sample. Partial *ORF2* gene fragments were amplified from specimens positive for rHEV RNA by RT-qPCR. Primers for sequencing rHEV ORF1 and ORF2 genes are listed in Table S1. Reverse transcription was performed using the HiScript III 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). Nested PCR was performed using High Fidelity PCR Kit (Novagen®, CA, USA). Each PCR product was gel purified using the TIAN gel Purification Kit (Tiangen, China). The amplified products were sequenced using the Sanger method. For variant analysis, complete rHEV genomes in the LCK-3110-like cluster were aligned with MAFFT (–genafpair –maxiterate 1,000; 7.526).[19] Tiled PCR primers were designed with varVAMP (v.1.2.1) with –opt-length value tested iteratively between 400 and 1,500 bp to maximize genome coverage. LCK2 total nucleic acid was reverse transcribed using PowerScript RT SuperMix (Guangzhou Dongsheng Biotech Ltd.). Amplification was performed with Platinum SuperFi PCR Master Mix (Thermo Fisher, USA) using 1 μl of template in a 25 μl reaction. PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN, Germany) and amplicons were pooled equimolarly before library preparation using the ligation sequencing gDNA (SQK-LSK109) protocol (Oxford Nanopore Technologies). The library was then loaded onto a FLO-MIN106D flow cell and sequenced on a MinION device for 48 h. Reads were base-called using dorado SUP accuracy mode (0.7.4). Consensus sequence was generated using the ARTIC (https://github.com/artic-network/artic-ncov2019) nanopolish workflow (1.1.0), with MN450852.1 set as the reference genome, and sequencing depth normalized to 400-fold coverage.[20,21] Normalized reads were then mapped to the consensus sequence with Minimap2 (2.28) and variants were called using Varscan2 (–min-var-freq 0.1; *p* value 0.05; 2.4.6).[22,23] Variants were visualized using trackViewer R/Bioconductor package (1.42.0).

rHEV genomes and corresponding metadata were downloaded from NCBI GenBank on 24 August 2024. Sequences we generated from the same isolate were concatenated together. Multiple sequence alignment was performed using MAFFT (–localpair –maxiterate 1,000; v7.526).[24] Rat Hainan 5–6 and 6 partial rHEV RdRp sequences from Wang *et al.*[16] were added to this backbone alignment using MAFFT (–addfragments –adjustdirectionaccurately).[16] Sites with ≥20% gaps were removed with TrimAl (–gt 0.2; v1.4.22).[25] A maximum likelihood phylogenetic tree was inferred using IQ-TREE, with the best-fit substitution model automatically selected by ModelFinder.[26,27] Branch support was assessed using the SH-like approximate likelihood ratio test with 10,000 bootstrap replicates. The final

Table 1. Demographic and clinical characteristics of patients included in this study.

	Hainan		Shenzhen		Hong Kong		
	Hepatitis (n = 134)	Non-hepatitis (n = 95)	Hepatitis (n = 302)	Non-hepatitis (n = 125)	Hepatitis (n = 219)	Non-hepatitis (n = 153)	Organ donors (n = 171)
Age (median; IQR)	46; 35–55	42; 33.5–52.5	40.5; 32–52	43; 33–51	60; 47–70.5	56; 47–63	39; (30–47)
Sex (male; %)	91; 67.9	66; 69.5	172; 57	91; 72.8	118; 53.9	84; 54.9	89; 52.0
ALT (median; IQR)	82.5; 47–169	N/A	112.6; 76.7–182.6	N/A	110; 59–254	N/A	N/A
AST (median; IQR)	60.5; 40.3–124.8	N/A	60.7; 41.3–124.2	N/A	92; 45–189	N/A	N/A

ALT, alanine aminotransferase; AST, aspartate aminotransferase; N/A, not applicable as liver function normal.

phylogenetic tree was visualized and annotated using TreeViewer.[28]

bHEV and rHEV antibody assays

We conducted bHEV- and rHEV-specific EIAs as described previously.[11] Briefly, samples were run in parallel in separate 96-well microtiter plates coated with either bHEV p239 or rHEV p241 with patients who were viremic serving as controls. These peptides are truncated fragments of the ORF2 protein of bHEV and rHEV, respectively; they comprise all major immunogenic epitopes and self-assemble into conformational virus-like particles.[9,29] We have previously calculated optical density (OD) cutoffs using this system that are capable of differentiating bHEV and rHEV serological signatures in people infected with rHEV with high accuracy.[11] Detailed methodology is presented in the Supplementary material.

Statistical analysis

Our previous pilot data found a seroprevalence of ~0.5% in the Hong Kong general population. To derive minimum required sample sizes, we assumed rHEV seroprevalence of 5% in the rural Hainan cohort at $\alpha = 0.05$ and power = 80%. All samples available at each center exceeding the minimum sample size (n = 206) were included in the study. Proportions were compared using the X^2 or Fisher's exact test as appropriate. Comparisons of mean/median ODs between groups were performed using Dunn's or Dunnett's multiple comparisons test in the context of parametric (Welch's ANOVA) or non-parametric (Kruskal–Wallis) assessments based on the normality of the underlying data distributions. Multivariable logistic regression was performed using a likelihood-based approach to identify the best model (XLStat, Lumivero (2024)). XLSTAT statistical and data analysis solution. https://www.xlstat.com/en). Graphs were charted using Prism 10 (v10.4.0, GraphPad Software, San Diego, CA, USA).

Results

Patient characteristics

Blood samples from 1,199 individual patients were tested for exposure to bHEV and rHEV in this study. Of these, 229 (19.1%) patients were from Hainan, 427 (35.6%) were from Shenzhen, and 543 (45.3%) were from Hong Kong. Each cohort was further classified based on the presence or absence of hepatitis (elevated alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST) as per cutoffs in Table S2). Their basic demographic characteristics are described in Table 1. Although the Hainan and Hong Kong cohorts mostly comprised of individuals of local origin, 162/427 (37.9%) of the Shenzhen cohort were migrants from other provinces across China. The degree of hepatitis was typically mild (Table 1).

rHEV seroprevalence in study cohorts

Serum or plasma samples were tested in parallel in bHEV p239 and rHEV p241 EIAs. We have previously validated these EIAs using samples from patients with confirmed bHEV and rHEV viremic infections.[11] This previous study established that a bHEV p239 IgG EIA OD cutoff of 0.437 differentiated bHEV-exposed individuals from rHEV-exposed and HEV non-exposed individuals with a sensitivity of 100% and specificity of 92.1%.[11] Similarly, a rHEV p241 IgG EIA OD cutoff of 0.864 differentiated rHEV-exposed individuals from bHEV-exposed and HEV non-exposed individuals with a sensitivity of 92.9% and specificity of 97.8%. In the current study, we applied these cutoffs to examine bHEV and rHEV exposure among study participants. Interpretative criteria are summarized in Table 2 and overall results by cohort are presented in Table 3. Viremic bHEV and rHEV control samples showed robust signals in the species-cognate EIAs confirming the specificity of the technique (Fig. S1).

Mean bHEV IgG ODs differed significantly between Hong Kong and both mainland cohorts (Fig. 1A). Overall bHEV seroprevalence in the Hainan cohort was 31/229 (13.5%) and 59/427 (13.8%) in the Shenzhen cohort compared with 31/543 (5.7%) in Hong Kong (p <0.0001 by X^2 test). The Hong Kong cohort also showed consistently lower mean rHEV EIA ODs than both the Shenzhen and Hainan cohorts irrespective of hepatitis status (Fig. 1B). rHEV exposures were highest in the Hainan cohort (15/229, 6.6%), followed by Shenzhen (9/427, 2.1%) and Hong Kong (2/543, 0.4%) (p <0.0001 by Fisher's exact test). Only 19 (1.6%) individuals across all cohorts reacted in both bHEV and rHEV EIAs confirming the high

Table 2. Interpretative criteria for bHEV and rHEV IgG enzymatic immunoassays.

Sample result pattern	bHEV p239 EIA OD	rHEV p241 EIA OD	Interpretation
1	<0.437	<0.864	No hepatitis E exposure
2	≥0.437	<0.864	bHEV exposure
3	<0.437	≥0.864	rHEV exposure
4	≥0.437	≥0.864	Dual exposure to bHEV and rHEV/cross-reactive responses

Modified from Situ *et al.*[11] bHEV, *Paslahepevirus balayani*; OD, optical density; rHEV, *Rocahepevirus ratti.*

Table 3. IgG seroprevalence of bHEV and rHEV in study cohorts.

	Hainan		Shenzhen		Hong Kong		
IgG EIA result	Hepatitis (n = 134)	Non-hepatitis (n = 95)	Hepatitis (n = 302)	Non-hepatitis (n = 125)	Hepatitis (n = 219)	Non-hepatitis (n = 153)	Organ donors (n = 171)
bHEV exposure (no., %)	18, 13.4	13, 13.7	41, 13.6	18, 14.4	14, 6.4	13, 8.5	4, 2.3
rHEV exposure (no., %)	8, 5.9	7, 7.4	6, 1.9	3, 2.4	1, 0.5	1, 0.7	0, 0.0
Dual positive (no., %)	4, 2.9	4, 4.2	5, 1.7	4, 3.2	2, 0.9	0, 0.0	0, 0.0

bHEV, *Paslahepevirus balayani*; rHEV, *Rocahepevirus ratti*.

species-specificity of the EIAs. Most of these samples showed robust ODs in both EIAs (Fig. S2), which is typical of some rHEV-exposed individuals and likely indicates exposure to both bHEV and rHEV variants.[11] Characteristics of patients testing positive in the rHEV IgG EIA are summarized in Table S3. The mean age of these individuals was 52 and 14 (31.1%) of them were female; 12/18 (66.7%) rHEV IgG seropositive individuals in the Shenzhen cohort were migrants from the surrounding Guangdong province or from other areas in China.

As age is the most consistent risk factor for bHEV seropositivity from previous studies, we examined the effect of age on rHEV seroprevalence. As expected, both mean bHEV IgG EIA ODs and seroprevalence rates exhibited a clear rising trend with age (Fig. 2; Table S4). However, this was less apparent in the rHEV EIA with a non-significant trend towards higher seroprevalence in older individuals (Table S5). We then performed a multivariable regression analysis incorporating age, sex, hepatitis status, and place of origin as potential explanatory variables for rHEV EIA positivity (as a binary outcome variable based on Table 2 cutoffs). Age significantly predicted rHEV IgG seropositivity with an odds ratio of 1.03 (95% CI: 1.02–1.04; p <0.001). In contrast, residence in Hainan was a strong predictor of rHEV exposure with an odds ratio of 20.52 (95% CI: 13.86–30.39; p <0.001). Sex and hepatitis status were not significantly associated with rHEV IgG status. We then conducted another multivariable regression analysis on the Hainan cohort data using age, sex, hepatitis status, occupation (agricultural *vs.* non-agricultural), and environmental rodent exposure as potential explanatory variables for rHEV IgG positivity. For gauging environmental rodent exposure, participants were asked if they had seen rodents in or around their living environments. Apart from age, the only significant factor was rodent exposure. Individuals with no rodent exposure were less likely to be rHEV seropositive (odds ratio = 0.27; 95% CI: 0.14–0.51; p <0.0001).

bHEV and rHEV RT-PCR assays

None of the patients with hepatitis in the Hainan cohort, two (0.67%) in the Shenzhen cohort, and two (0.4%) in the Hong Kong cohort tested positive for bHEV RNA. Among individuals without hepatitis, one individual (in the Hong Kong cohort) tested positive for bHEV RNA. This was a patient convalescing from hepatitis E whose liver function tests had already normalized. Overall, two individuals tested positive for rHEV RNA – one in the Hainan cohort (0.4%) and one in the Hong Kong cohort (0.2%).

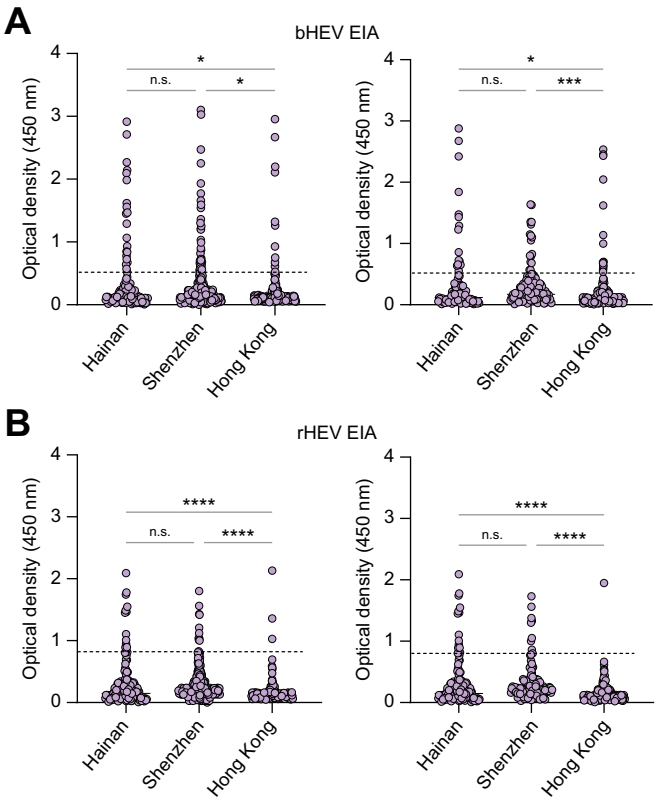

Fig. 1. Optical density values of study participants in the bHEV and rHEV EIAs. Cut-offs of respective EIAs are represented by dotted lines as per Table 2. Patients with hepatitis are represented on the left while patients without hepatitis are represented on the right. Intergroup comparisons of mean ODs were performed using Dunnett's multiple comparisons test. ***p* value <0.05, *****p* value <0.0001. n.s., not significant. bHEV, *Paslahepevirus balayani*; EIAs, enzymatic immunoassays; rHEV, *Rocahepevirus ratti*.

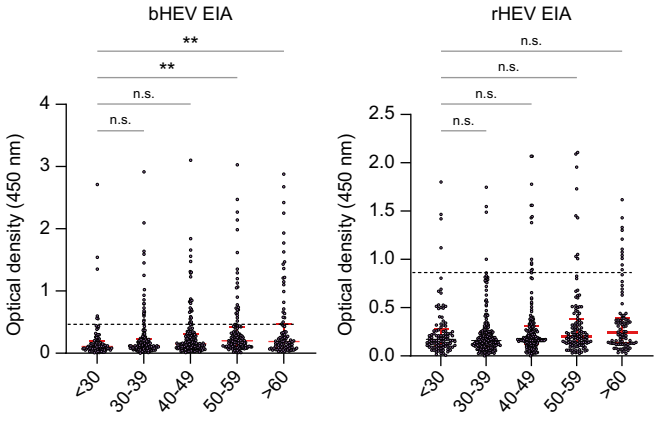

Fig. 2. Optical density values in bHEV and rHEV EIAs stratified by age (years) in the Hainan and Shenzhen cohorts. Mean optical density of each age band were compared with the <30 years old reference cohort using Dunnett's multiple comparisons test. ***p* value <0.01. n.s., not significant. bHEV, *Paslahepevirus balayani*; EIAs, enzymatic immunoassays; rHEV, *Rocahepevirus ratti*.

Other causes of ALT elevation in both patients were excluded. The Hainan patient was a 62-year-old man with a history of chronic hepatitis B under satisfactory control evaluated for acute hepatitis (ALT: 580 U/L, bilirubin 74.5 μmol/L and AST 920 U/L). The serum virus load was 8.4×10^5 copies/ml. The Hong Kong patient was a 75-year-old man with a history of renal transplantation 18 years previously taking sirolimus, prednisolone, and cyclosporine A. The patient had mild hepatitis of undetermined etiology for >3 years before his death from pneumonia. The plasma virus load was 2.5×10^6 copies/ml. Retrieval of multiple archived samples confirmed persistent viraemia over a period of >1 year suggestive of chronic rHEV infection in this patient (Fig. S3A). In view of the lack of intrahost genome diversity data in chronic rHEV infections, we examined variant alleles (>10% frequency) across the genome of this patient using deep nanopore sequencing. As shown in Fig. S3B, 41 variants were found in *ORF1* (of which 11 were non-synonymous), nine variants in *ORF2* (three non-synonymous), and four variants in *ORF3* (two non-synonymous). Interestingly, the patient was also noted to have persistent unexplained renal impairment during this period. The Hainan rHEV patient tested negative in the rHEV IgG EIA indicative of an acute infection, whereas the Hong Kong rHEV patient tested strongly positive in the rHEV IgG EIA (OD: 2.13). Neither patient had occupational or recreational exposure to rodents.

Rodent rHEV surveillance and phylogenetic comparison with human rHEV sequences

Because of the detection of a human rHEV case in Hainan, we screened for rHEV in 509 rats (*Rattus tanezumi*, *R. norvegicus*, *R. losea*) captured in various parts of the island (Fig. 3A). Detection breakdown by species is presented in Table S6. Overall, 19/509 (3.7%) of all rodents carried rHEV RNA in liver tissue (Table S7). We were able to sequence seven of these isolates (two *R. norvegicus* and five *R. tanezumi* derived). Phylogenetic analysis of human and rat-derived rHEV isolates from this study and strains on GenBank was conducted (Fig. 3B). The Hainan human-derived rHEV sequence was most closely related with Hainan *R. tanezumi* derived rHEV sequences (Rat Hainan 2–4; mean *p*-dist = 0.016). These rats were captured in Chengmai (November 2019) and Haikou (October 2016). This group was also monophyletic with Hainan rat-derived sequences Rat Hainan 5–7 as well as LC549184.1, a rHEV strain detected in Zhanjiang City, Guangdong, China (mean *p*-dist = 0.041). A more distantly related rHEV isolate (Rat Hainan 1) was also obtained in this study illustrating circulation of polyphyletic strains in Hainan. We then compared the human-derived Hong Kong rHEV sequence with sequences obtained in an ongoing rHEV surveillance project in Hong Kong (Fig. 3B). The new human sequence clustered monophyletically with previously described human-derived rHEV sequences infecting patients in Hong Kong (MN450852.1 and MN450854.1) as well as a rat-derived Hong Kong rHEV sequence (WCRN110620-2) sampled in 2020 (mean *p*-dist = 0.016), which was the same year that this patient contracted rHEV infection.[18] This cluster belongs to the LCK-3110 group that is the major circulating rHEV strain that caused human infections in Hong Kong from 2017 to 2024. Therefore, rHEV strains infecting humans are genomically related to concomitantly circulating local rHEV strains in commensal rodents. Additionally, rHEV sequences identified in a published study from Shenzhen were analyzed, even though we did not find any human rHEV cases in the city.[16] Isolates from Shenzhen belonged to both of the major clades within rHEV, but were rather distantly related to known human-infecting rHEV strains (Fig. 3B).

Discussion

rHEV is an emerging agent of hepatitis but its seroepidemiology has only been described in small studies of solid organ transplant recipients, persons who inject drugs, and persons living with HIV infection.[8,11,30] In this multicenter seroprevalence study, we find evidence for endemic rHEV exposure in the general population in China, which is suggestive of continuous low-level transmission from rats to humans. In contrast with bHEV, the association of rHEV seroprevalence with age was tenuous indicating that community rHEV exposures might be intermittent (possibly coinciding with epizootics in local rodent populations) in contrast to steady transmission via a foodborne route such as bHEV swine genotypes 3 and 4. This is consistent with rates of viremic infection from Hong Kong, which has varied widely year-to-year since systematic surveillance began in 2018.[5] Interestingly, rHEV seroprevalence differed significantly between study centers. Hainan residents tended to have a significantly higher risk of rHEV exposure, which might reflect increased rodent exposure in rural settings. This echoes a finding of higher rHEV exposure in German forestry workers.[31] Future studies of rHEV epidemiology should be aware of this urban–rural divide in exposure.

Combined with our previous clinical observations, the background seroprevalence indicates that most rHEV infections in the general population are clinically silent, which is similar to the situation with bHEV.[5,18,32] However, infections still have a significant clinical impact on patients with underlying liver disease or immunosuppression as seen in the two patients who were viremic in this study. The renal transplant recipient in this study had persistent unexplained renal impairment; we postulate that this might have been another example of rHEV-related glomerulonephritis, which is a known extrahepatic manifestation of this novel zoonosis.[33] This patient also demonstrated high intrahost genomic diversity, a feature that has been linked to chronicity for bHEV infection.[34]

We have previously shown that strains from rats and humans are spatiotemporally linked.[18] In this study, we again demonstrated close identity between human rHEV isolates and locally captured rat counterparts. We also demonstrated that presence of rodents in the vicinity of households is a risk factor for acquiring rHEV. In Spain and Hong Kong, human rHEV sequences often form monophyletic clusters over multi-year periods, possibly indicating some degree of human adaptation in these strain groups.[5,35,36] Identifying linked sequences in rodent populations is a strong indication for heightened disinfestation efforts. Such surveillance may be more valuable than traditional indicators such as rodent infestation indices, which were inadequate for predicting rHEV zoonotic risk in Hong Kong.[18] Because of the substantial resource implications for running such programs, pilot studies incorporating rHEV and other rodent-pathogen surveillance (e.g. *Leptospira*, hantavirus) are required to justify this approach.

Although rats are almost certainly the definitive source of human infections, the exact route of transmission of rHEV is unknown. Increased seroprevalence in rural settings might

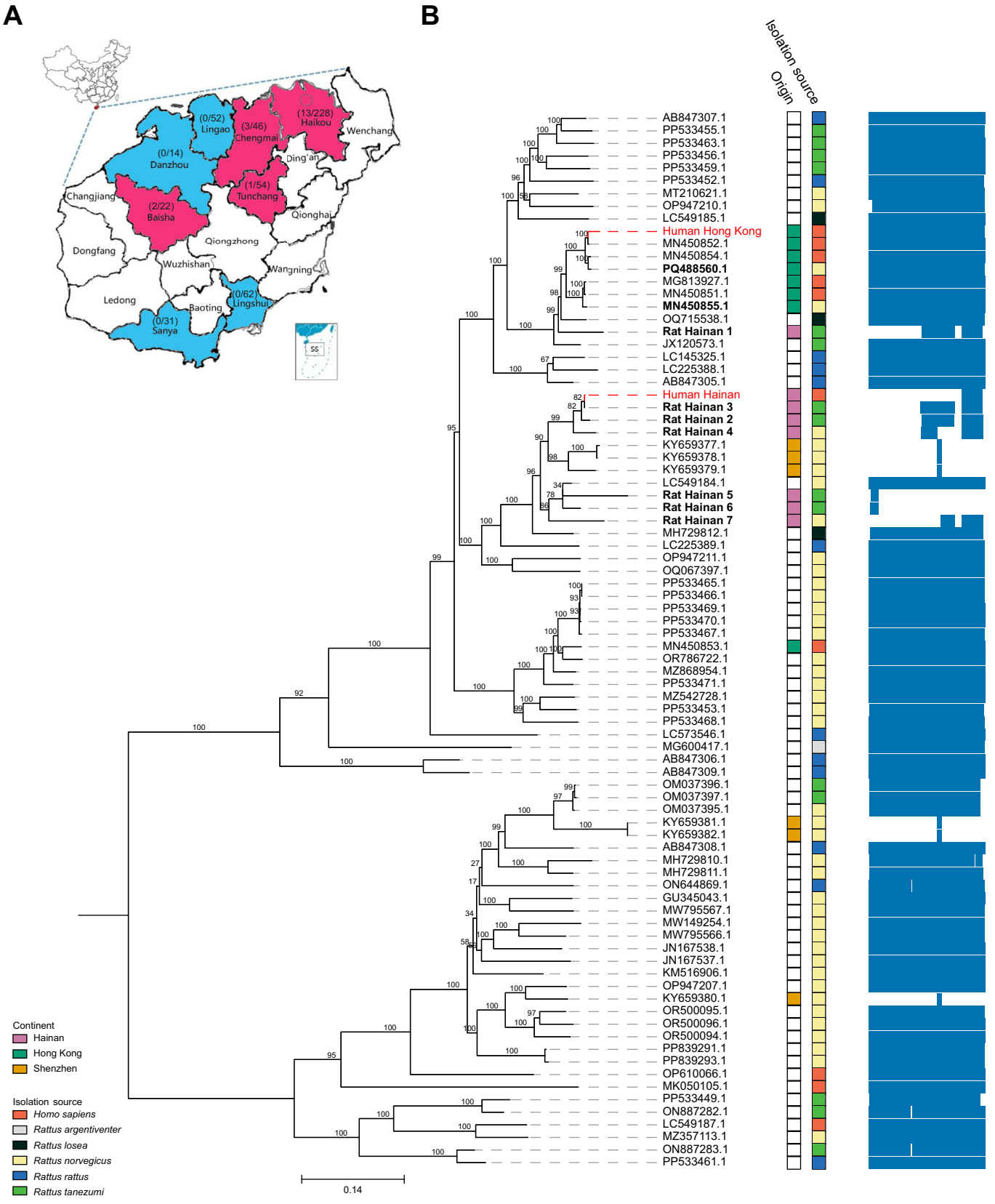

Fig. 3. Sampling map of rodents in Hainan Island. (A) Number of rodents testing positive. Counties with detected rodent infestation are marked in red. (B) Phylogenetic tree of complete rHEV genomes and ORF2 sequences from this study. Maximum likelihood phylogenetic tree was inferred from a multiple sequence alignment of complete rHEV genomes and ORF2 sequences from this study (branches highlighted in red/bold) using IQ-TREE. Region of GU345043.1 covered by our ORF2 sequences are appended to the sequence identifiers. The best-fit substitution model selected by ModelFinder was GTR+F+I+R4. Branch supports were assessed using the SH-like approximate likelihood ratio test with 10,000 bootstrap replicates. Branches with less than 70% support were collapsed. Scale bar represents nucleotide substitutions per site.

point to additional intermediate hosts. This has been an active area of investigation recently. We and others have shown rHEV antibodies in sera from cats and dogs.[37,38] rHEV RNA was detected in fecal rectal swabs of farmed pigs in Spain.[39] One group has successfully infected chickens and pigs with rHEV infectious clones.[40,41] These studies add nuance to previous experimental work finding that rHEV strains have a restricted host range.[42,43]

The main limitation of this study was its retrospective nature. All work was done on archived samples, therefore detailed dietary exposures could not be elicited from study participants. Rodent samples were also archival with rather limited sample size, therefore precise linking of human seroprevalence and rodent epizootics was not possible. Rodent sampling in Shenzhen was not performed because of the lack of human rHEV cases there and also because this work has been performed previously.[16] Sequences from this previous effort were incorporated in this study.

In conclusion, rHEV exposure is relatively common in the general population in China with a marked urban–rural divide. Clinical hepatitis was observed in patients who were viremic with underlying liver disease and immunosuppression. Sequences were phylogenetically linked to locally circulating rodent rHEV isolates indicating zoonotic transmission.

Affiliations

[1]*Hainan Medical University-The University of Hong Kong Joint Laboratory of Tropical Infectious Diseases, Key Laboratory of Tropical Translational Medicine of Ministry of Education, Academician Workstation of Hainan Province, School of Basic Medicine and Life Sciences, Hainan Medical University, Haikou, Hainan, China;* [2]*Department of Microbiology, Queen Mary Hospital, Pokfulam, Hong Kong Special Administrative Region of China;* [3]*The First Affiliated Hospital of Hainan Medical University, Hainan Medical University, Haikou, Hainan, China;* [4]*Department of Infectious Diseases and Microbiology, The University of Hong Kong-Shenzhen Hospital, Shenzhen, Guangdong, China;* [5]*Department of Infectious Disease, the Second Affiliated Hospital of Hainan Medical University, Haikou, Hainan, China;* [6]*Department of Laboratory Medicine, Haikou Hospital of Traditional Chinese Medicine, Haikou, China;* [7]*Centre for Virology, Vaccinology and Therapeutics, Hong Kong Science and Technology Park, Hong Kong Special Administrative Region of China;* [8]*State Key Laboratory of Emerging Infectious Diseases, Carol Yu Centre for Infection, Department of Microbiology, School of Clinical Medicine, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Pokfulam, Hong Kong Special Administrative Region of China;* [9]*Guangzhou Laboratory, Guangzhou, Guangdong Province, China;* [10]*Guangdong Provincial Key Laboratory of Virology, Institute of Medical Microbiology, Jinan University, Guangzhou, China;* [11]*Pandemic Research Alliance Unit at The University of Hong Kong, The University of Hong Kong, Hong Kong Special Administrative Region of China*

Abbreviations

ALT, alanine aminotransferase; AST, aspartate aminotransferase; bHEV, HEV variant *Paslahepevirus balayani*; EIA, enzymatic immunoassay; HEV, hepatitis E virus; OD, optical density; qRT-PCR, real-time RT-PCR; rHEV, HEV variant *Rocahepevirus ratti*.

Financial support

This work was supported by the Health and Medical Research Fund (HMRF), Food and Health Bureau, Government of the Hong Kong Special Administrative Region under grants 20190362 and 22210772; the HKUMed Research Fellowship Scheme for Clinical Academics, the Partnership Programme on Enhancing Laboratory Surveillance and Investigation of Emerging Infectious Diseases and Antimicrobial Resistance for Department of Health; Lo Ying Shek Chi Wai Foundation Award for Young Investigator 2022-23; Health@InnoHK, Innovation and Technology Commission, the Government of the Hong Kong Special Administrative Region, The Key Research and Development Plan of Hainan Province (grant no. ZDYF2024SHFZ119, ZDYF2022SHFZ098); The National Natural Science Foundation of China (grant no. 82060378); and Hainan Provincial Natural Science Foundation of China (grant No. 822RC870).

Conflicts of interest

JS, K-YY, and SS have filed a provisional patent application covering the utilization of hepatitis E virus-like particles described in this paper for serodiagnosis and vaccines. The other authors declare no conflicts of interest.

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

Authors' contributions

Designed the study and drafted the manuscript: JS, SS. Coordinated rodent collection, testing, and funding for the Hainan part of this project: XC, TT, DC, JFC, FY. Coordinated collection of human samples from Hainan: LZ, YC. Coordinated sample collection and data retrieval from Shenzhen: CD, YS, HC, SH, SKL, SZ, FX. Coordinated validation and implementation of ELISA assays and genomic surveillance of rat hepatitis E in Hong Kong rodents: JYT, SW, KHL. Performed all ELISA surveillance for Hong Kong patients: ZL. Performed statistical analysis and data curation: YL, TCW, HLC. Enabled human sample collection for the Hong Kong cohort: VCC. Performed all phylogenetic analyses for this study: SSH. Critically reviewed the manuscript: NFC, EHS, KYY, VCC.

Data availability statement

The raw data for this manuscript is available upon request.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2025.101370.

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Author names in bold designate shared co-first authorship

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Keywords: Hepatitis E virus; HEV; Rodents; Epidemiology; Zoonosis; One Health.

Received 22 November 2024; received in revised form 19 February 2025; accepted 21 February 2025; Available online 4 March 2025