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Transmission of rat hepatitis E virus infection to humans in Hong Kong: a clinical and epidemiological analysis

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List of abbreviations:

HEV: Hepatitis E virus
HEV-A: Hepatitis E virus species *A*
HEV-C: Hepatitis E virus species *C*
HEV-C1: Hepatitis E virus species *C* genotype 1
QMH: Queen Mary Hospital
DH PHLSB: Department of Health Public Health Laboratory Services Branch
qRT-PCR: quantitative real time reverse-transcriptase polymerase chain reaction
LFT: liver function tests
ELISA: Enzyme linked immunosorbent assay
ORF: open reading frame
RIR: rodent infestation rate
ALT: alanine aminotransferase

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Abstract

Background & aims

Hepatitis E virus (HEV) variants causing human infection predominantly belong to HEV species *A* (HEV-A). HEV species *C* genotype 1 (HEV-C1) circulates in rats and is highly divergent from HEV-A. It was previously considered unable to infect humans, but the first case of human HEV-C1 infection was recently discovered in Hong Kong. The aim of this study is to further describe the features of this novel zoonosis in Hong Kong.

Approach & results

We conducted a territory-wide prospective screening study for HEV-C1 infection over a 31-month period. Blood samples from 2,860 patients with abnormal liver function (n = 2,201) or immunosuppressive conditions (n = 659) were screened for HEV-C1 RNA. In addition, 186 captured commensal rats were screened for HEV-C1 RNA. Sequences of human-derived and rat-derived HEV-C1 isolates were compared. Epidemiological and clinical features of HEV-C1 infection were analyzed. HEV-C1 RNA was detected in 6/2,201 (0·27%) patients with hepatitis and 1/659 (0·15%) immunocompromised persons. Including the previously reported case, eight HEV-C1 infections were identified including five in immunosuppressed patients. Three patients had acute hepatitis, four had persistent hepatitis while one had subclinical infection without hepatitis. One patient died of meningoencephalitis and HEV-C1 was detected in cerebrospinal fluid. HEV-C1 hepatitis was generally milder than HEV-A hepatitis. 7/186 (3·76%) rats tested positive for HEV-C1. One HEV-C1 isolate obtained from a rat captured near the residences of patients was closely related to the major outbreak strain.

Conclusions

HEV-C1 is a cause of hepatitis E in humans in Hong Kong. Immunosuppressed individuals are susceptible to persistent HEV-C1 infection and extrahepatic manifestations. Subclinical HEV-C1 infection threatens blood safety. Tests for HEV-C1 are required in clinical laboratories.

Hepatitis E virus (HEV) is a major cause of viral hepatitis globally. (1) The clinical spectrum of acute hepatitis E includes asymptomatic infection, mild-to-moderate liver dysfunction and fulminant hepatitis. Persistent hepatitis E can develop in immunocompromised persons, which can progress to liver cirrhosis if left untreated. (2, 3)

HEV belongs to the family *Hepeviridae*, which includes two genera: *Orthohepevirus* (comprising variants which infect terrestrial vertebrates) and *Piscihepevirus* (cutthroat trout virus). (4) Hepatitis E in humans is mostly due to members of *Orthohepevirus* species *A* (HEV-A). HEV-A includes eight genotypes, which infect humans, pigs, wild boar, deer, rabbits, and camels. (5) Human hepatitis E infections in industrialized countries are due to either HEV-A genotype 3 (Europe, Japan, and the Americas) or HEV-A genotype 4 (China), and in developing countries are due to HEV-A genotypes 1 or 2. (6, 7) HEV-A genotypes 3 and 4 are usually acquired by consumption of undercooked pork or game meat, but can also be transmitted via contaminated blood products or organs. (8, 9)

Apart from HEV-A, the *Orthohepevirus* genus includes three other species: *B* (circulating in birds), *C* (HEV-C; circulating in rodents and ferrets) and *D* (circulating in bats). (4) HEV-C, discovered in German rats in 2010, has since been detected in wild rats in Asia, Europe, and North America. (10-13) Rats are susceptible to infection by HEV-C genotype 1 (HEV-C1) with other genotypes of HEV-C circulating in ferrets, shrews, voles etc. (14) Hitherto, HEV-C1 was considered to have minimal zoonotic risk due to wide phylogenetic divergence from HEV-A and failure of experimental infection of pigs and nonhuman primates. (11, 15) HEV-C only shares 50-60% nucleotide identity with HEV-A and has major differences in key epitopes of the putative receptor binding domain. (16) However, we recently demonstrated that HEV-C1 infected a liver transplant recipient even though the patient had pre-existing antibodies against HEV-A. (17) We

showed that commonly used HEV-A nucleic acid amplification tests would not be able to detect HEV-C1 infection due to significant sequence differences. (17) Another study subsequently identified an immunocompetent adult with acute HEV-C1 infection, likely acquired in Africa. (18) This raises the possibility that HEV-C1 is a globally prevalent zoonosis, which is routinely missed by existing assays that are specific for HEV-A. Therefore, we conducted this study to investigate the prevalence, clinical characteristics and molecular epidemiology of human HEV-C1 infection in Hong Kong.

Methods

Study setting

This study was conducted in the Queen Mary Hospital (QMH) microbiology laboratory and the Department of Health Public Health Laboratory Services Branch (DH PHLSB). The QMH laboratory provides molecular virology services for cancer units and organ transplant centers in Hong Kong. DH PHLSB is Hong Kong's reference laboratory and provides serological and molecular testing for patients with viral hepatitis throughout the territory (serving a population of 7·5 million people). The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Institutional Review Board of the University of Hong Kong/ Hospital Authority West Cluster (UW 18-074). We confirm that transplant centers in Hong Kong do not obtain organs from executed prisoners or other institutionalized persons.

Patient samples

Following identification of the first human HEV-C1 infection in 2017 (hereafter designated as the 'index case'), enhanced surveillance for HEV-C1 infections was carried out among patients with hepatitis and individuals with underlying immunosuppressive conditions. Two sample sets (A & B) were tested for HEV-C1 RNA using a quantitative real time RT-PCR (qRT-PCR) assay. Set A comprised sera from patients with liver function test (LFT) abnormalities sent to DH PHLSB for hepatitis E serological testing between January 1, 2017 and July 31, 2019. Set B comprised nucleic acid extracted from plasma or sera of transplant recipients and patients with solid organ malignancies, hematological malignancies, autoimmune disorders, and other immunosuppressive conditions sent to QMH between January 1, 2019 and June 30, 2019 for molecular virology

testing. For set B, abnormal LFTs in patients was not a prerequisite for HEV-C1 qRT-PCR testing. Sample processing schema for set A and set B samples are described further below.

Pig and commensal rat samples

Commensal rats (*Rattus rattus* and *Rattus norvegicus*) are regularly captured in Hong Kong by the Food and Environmental Hygiene Department as part of routine disinfestation practices. Rectal swabs collected from commensal rats captured in 12 districts between January 1, 2017 and June 30, 2019 were stored in virus transport medium. Rectal swabs were screened for HEV-C1 RNA. In addition, we screened liver tissue samples from 40 pigs and rectal swab samples from 172 pigs for HEV-C1. 177 pig samples were collected from Hong Kong and 35 were collected from the neighboring city of Shenzhen.

HEV-A & C1 qRT-PCR and sequencing

HEV-A qRT-PCR and HEV-C1 qRT-PCR were performed using an in-house validated nucleic acid amplification tests. Assay evaluation, protocols and primer/ probe sequences are described in the supplementary data. Samples testing positive for HEV-A or HEV-C1 RNA by qRT-PCR were subjected to sequencing. For commensal rat rectal swab samples which could not be sequenced due to presence of inhibitors, liver or spleen samples were used for sequencing if available. Sequencing and phylogenetic analysis methodology are described in the supplementary data. Primers used for complete genome sequencing are listed in supplementary tables 1 and 2.

Serological assays

Hepatitis E serological testing of human sera were performed using commercial HEV-IgM and IgG enzyme-linked immunosorbent assay (ELISA) kits (Wantai, Beijing, China). Although these kits use a HEV-A genotype 1 open reading frame (ORF) 2 fragment as the capture antigen, serum from the index case was strongly positive for HEV-IgM and IgG antibodies using this assay. (17) This provided grounds for further evaluation of the Wantai kits to screen for HEV-C1 infections. Interpretation of results was according to manufacturer instructions. Borderline HEV-IgM & IgG results were considered negative.

Sample processing

Samples in set A were processed based on their HEV-IgM antibody result. Sera testing positive for HEV-IgM antibodies during the study period were tested for HEV-A RNA. Any IgM-positive samples testing negative for HEV-A RNA were individually tested for HEV-C1 RNA by qRT-PCR. To capture HEV-C1 infections missed by the Wantai kit, HEV-IgM negative sera obtained from patients with liver dysfunction between March 1, 2018 to August 31, 2018 were screened for HEV-C1 RNA in pools. During this period, 20 µL of HEV-IgM negative serum samples were combined together into pools of ten for extraction followed by HEV-A and HEV-C1 qRT-PCR testing. As none of these pools tested positive during this 6-month period, starting from September 1, 2018, HEV-IgM negative sera were individually tested for HEV-C1 RNA only if specifically requested by clinicians or deemed appropriate by clinical virologists.

For set B samples, 4 µL of nucleic acid extracted from sera/ plasma sent to QMH over a 6-month period were combined together in pools of five followed by HEV-C1 qRT-PCR testing. For positive pools, individual samples constituting the pool were tested for HEV-C1 RNA. HEV antibody testing was not performed for set B samples.

This design enabled capture of the full spectrum of human HEV-C1 infection: patients with hepatitis who were HEV-IgM positive, patients with hepatitis who were HEV-IgM negative, and sub-clinically infected patients in whom hepatitis E was not suspected by clinicians.

Total nucleic acid was individually extracted from commensal rat rectal swab samples followed by HEV-C1 qRT-PCR testing.

Case definitions

Patients were defined as having persistent hepatitis E if hepatitis E virus species *C* (HEV-C) viremia persisted for more than three months as per Kamar et al. (19) Patients with HEV-C viremia lasting less than three months were designated as having acute hepatitis E. If sufficient archived samples were unavailable for retrospective viral load testing, duration of hepatitis was used to differentiate acute and persistent hepatitis E. Subclinical hepatitis E was defined as HEV-C viremia without abnormal liver function tests or clinical symptoms.

Patients were considered immunosuppressed if they a) had a hematological malignancy, b) were organ transplant recipients, c) were receiving disease modifying anti-rheumatic drugs/ marrow suppressive cancer chemotherapy, d) were taking steroids at doses above 0·5 mg/kg/day prednisolone-equivalent for at least one month, or e) were living with advanced HIV infection with CD4 T-lymphocyte counts < 200 cells/mm^3.

Epidemiological investigation

Patients confirmed to have HEV-C1 infection were questioned regarding living conditions, travel, occupation, and potential contact with rodents. Clinical and demographic data were retrieved from electronic patient records. Available archived samples were retrieved for HEV-C1 qRT-PCR testing. Disease course was classified into acute, persistent or subclinical hepatitis E infections according to definitions described above. Viral kinetics on ribavirin therapy was classified into monophasic, biphasic, triphasic, and flat-partial responses according to recent definitions. (20) Clinical data from the reported index case was included in the analysis. (17) District rodent infestation rates (RIR) were measured as described in the supplementary material. Age, sex, and clinical characteristics of HEV-A infected patients were retrieved for comparison with HEV-C1 infected patients.

Results

Set A and B HEV-C1 qRT-PCR screening results

Age distribution of the 2,201 patients in set A is presented in supplementary figure 1A. Hepatitis E screening results for set A are summarized in figure 1. Of the 169 individuals who were HEV-IgM positive, five (2·9%) samples were positive for HEV-C1 RNA. HEV-A RNA was detected in 82/169 (49·7%) of IgM positive samples: the majority of these were HEV-A genotype 4 (68/82; 82·9%) followed by HEV-A genotype 3 (9/82; 10·9%) and HEV-A genotype 1 (5/82; 6·1%). 2,000 HEV-IgM negative samples collected between March 1, 2018 and August 31, 2018 were negative for HEV-C1 RNA. A further 32 HEV-IgM negative samples were tested for HEV-C1 RNA between September 1, 2018 and July 31, 2019 due to unexplained hepatitis. One of these was positive for HEV-C1 RNA. Therefore, of the 2,032 HEV-IgM negative individuals, one (0·05%) was positive for HEV-C1 RNA. The higher proportion of HEV-C1 infection in HEV-IgM positive individuals (2.9%) compared to HEV-IgM negative individuals (0.05%) indicated an association between HEV-C1 infection status and HEV-IgM seropositivity ($P < 0.001$ by Fisher's Exact test).

Age distribution and clinical characteristics of patients in set B are presented in supplementary figure 1B and supplementary table 3 respectively. Set B comprised of 1,945 samples from 659 patients collected over a 6-month period, of which one (0·15%) tested positive for HEV-C1 RNA.

Including the previously reported index case, a total of eight patients with HEV-C1 infection were identified during the study period.

Demographic and epidemiological characteristics of human HEV-C1 infection

The mean age of HEV-C1 infected patients was 66·5 years while the mean age of HEV-A infected patients was 57·4 years (p = 0·072). 7/8 (87·5%) HEV-C1 patients were male compared to 51/82 (62·2%) HEV-A infected patients (p = 0·251). Demographic and epidemiological details for individual HEV-C1 infected patients are summarized in table 1.

Based on hepatitis onset or timing of HEV-C1 RNA detection in clinical samples, two patients were infected with HEV-C1 each year in 2017 & 2018, and four were infected in 2019 indicating ongoing sporadic transmission throughout the study period (supplementary figure 2).

5/8 (62·5%) patients resided in two neighbouring districts located in the east-central Kowloon part of the territory. These five patients resided close to each other within a 5 km radius (figure 2). The other three cases resided in non-adjacent districts. No correlation was observed between human HEV-C1 infection and district RIR (supplementary table 4). In fact, the RIR for affected districts were among the lowest in the territory. None of the patients reported seeing rats inside their homes or had consumed rat organs. Only the index case had definite evidence of rodent infestation in his housing estate.

Only the index case had received blood products within six months of diagnosis of HEV-C1 infection, but transmission from blood products had been excluded by testing of residual sera from blood donors.

Pig and commensal rat sample HEV-C1 screening results

186 rats (159 *R. norvegicus* and 27 *R. rattus*) collected during the study period were tested for HEV-C1 RNA. Commensal rats were captured from November to July (supplementary figure 3). Districts from which rats were collected and screening results are presented in supplementary table 4. None of the *R. rattus* and 7/159 (4·4%) *R. norvegicus* tested positive for HEV-C1 RNA and were confirmed by sequencing. Only two districts showed evidence of rat HEV-C1 epizootics, one of which was the Wong Tai Sin district where four human HEV-C1 cases resided (supplementary table 4). All pig samples tested negative for HEV-C1 RNA.

Sequencing and phylogenetic analysis

Sequencing of a partial HEV-C1 ORF1 nucleotide fragment was possible for all eight human cases and seven commensal rat samples. Additionally, complete HEV-C1 genomes were sequenced for patients 1, 2, 3, 5, and 8. Plasma viral load of patients 4, 6, and 7 were insufficient for complete genome sequencing. Complete genome sequencing was performed for one HEV-C1 isolate from a commensal rat captured in Wong Tai Sin whose partial genome aligned with isolates from human cases.

The HEV-C1 isolate infecting patient 1 had previously been assigned the strain name LCK-3110 (GenBank accession no.: MG813927.1). Other human HEV-C1 isolates were compared to LCK-3110 (Table 2). Isolates from patients 2, 3, 4, 6, 7, and 8 were closely related to LCK-3110 (Figure 3). Isolates from patient 2 and patient 7 were in the same subclade as LCK-3110; this represented a nucleotide identity > 99% (Table 2). Isolates from patients 3, 4, 6, and 8 formed another subclade (Figure 3), which corresponded to > 95% nucleotide identity to LCK-3110. The HEV-C1 isolate from patient 5 was highly divergent from LCK-3110 and other described complete HEV-C1 genomes (Table 2, supplementary figure 4). Therefore, phylogenetic analysis revealed two outbreak strains circulating in Hong Kong: seven patients were infected by LCK-3110-like strains while one patient was infected by a divergent strain.

One commensal rat-derived HEV-C1 isolate (strain name: WTSRN170519) shared 99% complete genome nucleotide identity to LCK-3110 (Table 2, supplementary figure 4). This rat was captured in May 2019 close to the residence of several HEV-C1 infected patients (Figure 2). Partial gene fragments of the other six rat-derived HEV-C1 isolates showed that they were very divergent from the human outbreak strains (Figure 3).

To investigate how the Wantai kit was able to detect IgM and IgG antibodies in HEV-C1 patient sera, we analysed the pE2 peptide region of HEV-C1 isolates. (21) pE2, which is the capture antigen used in the Wantai kits, corresponds to amino acid position 394 – 606 of the HEV-A genotype 1 ORF2 protein. By aligning two HEV-A genotype 1 reference strains with complete HEV-C1 genomes of patients 1, 2, 3, 5, and 8, we found that the amino acid sequence identity between HEV-A genotype 1 and HEV-C1 was only 53 – 54%. We then focused on immunogenic domains comprised within pE2: domain 4 and domain 5. Within these two domains, we examined the sequence alignments of several overlapping immunogenic 25-to-30-mer subdomains as described previously. (22). In two subdomains in domain 4 and domain 5, the sequence identity between HEV-A genotype 1 and the 5 HEV-C1 strains rose to 70% (aa 403 to 465 of HEV-1 ORF2) and 76% (aa 539 to 563 of HEV-1 ORF2) respectively. This increased identity in regions

associated with IgM immunoreactivity could have led to the sensitivity of Wantai kits for HEV-C1 infection diagnosis.

Clinical characteristics of HEV-C1 infection

All eight HEV-C1 infected patients had chronic medical conditions: three were solid organ transplant recipients, two had solid organ malignancies, one had rheumatoid arthritis, one was living with HIV (CD4 T-lymphocyte count 66 cells/mm^3 on antiretroviral therapy), and one had diabetes mellitus. Two patients were chronic hepatitis B virus carriers and were taking nucleo(s)tide analogues. 7/8 (87·5%) infected individuals had abnormal LFTs at time of HEV-C1 detection. 6/8 (75%) infected individuals were positive for HEV-IgM by the Wantai kit while 7/8 (87·5%) were positive for HEV-IgG. Infected individuals showed subclinical (1/8; 12·5%), acute (3/8; 37·5%) and persistent infection patterns (4/8; 50%) as per case definitions in this study.

Patients 2, 4, and 6 had acute self-limiting hepatitis: patients 2 and 4 required hospitalization due to malaise while patient 6 was asymptomatic with abnormal LFTs noted on routine blood testing. Their mean peak ALT was 469 U/L and bilirubin was normal. ALT reached nadir on day-7 and day-18 post-hepatitis onset for patients 2 and 4 respectively while patient 6 presented after ALT had already peaked. Hepatitis resolved within 4 weeks of presentation in all three patients (Figure 4A).

Patient 7 did not have either clinical symptoms or biochemical hepatitis. Infection was identified by HEV-C1 screening in a sample sent for routine diagnostic testing. Subsequent blood taking confirmed clearance of viremia and LFTs remained normal.

All three transplant recipients (patients 1, 3, and 5) and the HIV-infected patient (patient 8) developed persistent infection (Figure 4B). They were initially asymptomatic with abnormal LFTs discovered on routine blood testing. Mean peak ALT was 309 U/L and bilirubin remained within reference range. Once diagnosis of persistent HEV-C1 infection was established, patients 1 and 3 were started on oral ribavirin therapy. Patient 1 had a monophasic response to ribavirin therapy while patient 3 exhibited a biphasic response (Figure 4B). Patient 8 was persistently viremic 5 months after diagnosis.

After one year of persistent hepatitis, patient 5 was admitted for malaise and thoracic herpes zoster. He developed altered mental status after admission. LFTs had normalized (Figure 4B). Diagnosis of HEV-C1 was not yet confirmed at this stage. Magnetic resonance imaging of the brain was normal. Cerebrospinal fluid analysis showed total white cell count of 80/mm^3 (86%

lymphocytic pleocytosis) and elevated cerebrospinal fluid protein of 0·43 g/L. Bacterial and fungal cultures of cerebrospinal fluid were negative as were PCR tests for herpes simplex virus, varicella zoster virus, and enteroviruses. The patient eventually succumbed; once diagnosis of HEV-C1 infection was established, his cerebrospinal fluid was retrieved for HEV-C1 testing and contained $1{\cdot}65 \times 10^3$ copies/mL of HEV-C1 RNA.

Comparison of HEV-C1 and HEV-A infections

We compared the severity of liver dysfunction, rates of progression to chronicity, and fatality rates of the eight HEV-C1 infected patients and 69 HEV-A infected patients from set A for whom data was available. We found that the HEV-A group had higher mean peak ALT (1723 U/L vs 333 U/L, $p < 0{\cdot}0001$) and higher mean peak bilirubin (155·7 μmol/L vs 11·38 μmol/L, $p < 0{\cdot}0001$) than the HEV-C1 group (figure 5). However, the difference in prothrombin time between the groups did not achieve statistical significance (13·13 s vs 15·23 s, $p = 0{\cdot}190$). Four HEV-C1 infected persons progressed to persistent infection compared to 5/69 (7·25%) HEV-A infected persons ($p = 0{\cdot}005$). Two patients with HEV-A infection had extrahepatic manifestations (renal failure and Guillain-Barre syndrome) compared with one in the HEV-C1 group with suspected meningoencephalitis (P = 0.284). There was no significant difference in mortality rates between the two groups (P = 0.284).

Discussion

Hepatitis E virus species *C* comprises four putative genotypes, which infect rats, ferrets, mink, bandicoots, field mice, shrews, and voles. (4, 13, 14, 23, 24) Of these four genotypes, humans are most likely to be exposed to HEV-C1, which infects urban commensal rats. HEV-C1 has been documented in European, Asian, and North American rats. (11, 23, 25) Although there was some indirect serological evidence of HEV-C1 infection in German forestry workers and Vietnamese febrile inpatients, experimental studies initially concluded that HEV-C1 did not have zoonotic potential due to its inability to infect non-human primates. (11, 26, 27) However, we demonstrated that HEV-C1 infected a liver transplant recipient in Hong Kong. (17) This discovery was borne out by a Canadian study finding that an immunocompetent individual was infected by a HEV-C1 strain while working in Africa. (18) In the present work, we describe the epidemiology and clinical characteristics of this novel zoonosis.

We identified eight HEV-C1 infected patients in Hong Kong. Although less frequent than HEV-A genotype 4, which is the most common autochthonous HEV-A variant in China, (6, 28) HEV-C1 was approximately as frequent as HEV-A genotypes 1 and 3 in Hong Kong. We believe our findings cannot be explained simply by the one-off emergence of human-adapted HEV-C1 strains in Hong Kong. Three distinct HEV-C1 strains capable of infecting humans have been described by this and previous studies, suggesting that the virus is capable of routinely crossing the species barrier. (17, 18) As HEV-C1 is found in rats in many parts of the world, strains with enhanced zoonotic potential may be constantly emerging and accounting for a share of the global human hepatitis E burden. As most molecular tests targeting HEV-A variants cannot detect HEV-C1 RNA, public health laboratories around the world should develop HEV-C1 specific molecular assays for surveillance.

The exact route of HEV-C1 transmission to humans is elusive. Our finding of a genetically related isolate in a rat captured in close proximity to residences of five human cases is consistent with either direct infection or a common exposure. It is possible that humans may have been infected via contact with environmental surfaces contaminated by rat droppings. Although we could not detect HEV-C1 RNA in a small set of pig samples, this cannot entirely exclude that pigs are sporadically infected due to regular exposure to rat faecal material, but experimental studies suggest that pigs are not susceptible to HEV-C1 infection. (15) Furthermore, contamination of pork and other food products by rat droppings in abattoirs and wet markets remains possible. Three HEV-C1 patients were residing in other districts of Hong Kong where we could not detect HEV-C1 isolates in rats. However, rat sampling was limited and could not be performed for all districts. Furthermore, sampling was not always carried out in the same timeframe as the human infections. Therefore, we may have missed transient epizootics of HEV-C1. Continuous sampling and rigorous testing of commensal rats to identify HEV-C1 epizootics may be useful to identify areas with higher risk of human transmission. Such areas may benefit from pre-emptive rodent disinfestation drives. Our experience shows that conventional measures of rodent infestation such as the RIR do not reflect actual risk of HEV-C1 infection.

Like HEV-A, HEV-C1 can cause acute hepatitis, persistent hepatitis, and subclinical infection. HEV-C1 infections in individuals with intact immunity are self-limiting and less severe than HEV-A infections. However, infections in immunocompromised persons are problematic with progression to persistence observed in 50% of HEV-C1 infections compared to just 5/69 (7.25%) HEV-A infections. This may reflect the fact that immunocompromised persons are particularly

susceptible to HEV-C1 infections. We also observed meningoencephalitis in one immunocompromised patient even after hepatitis had resolved completely. Neurological manifestations have been documented in HEV-A infections and its association with HEV-C1 infection needs to be urgently investigated. (29) Subclinical HEV-C1 infection without deranged LFTs or clinical symptoms is particularly worrisome for transfusion safety. Given the high HEV-C1 viral loads we observed in plasma, transmission of HEV-C1 via contaminated blood products is possible. Currently used blood donor HEV RNA screening platforms probably cannot detect HEV-C1. However, as HEV-C1 is likely to be less common than autochthonous HEV-A variants, further epidemiological data is required to judge the cost-effectiveness of HEV-C1 donor screening.

Our study had several limitations. Pools were used for HEV-C1 screening among HEV-IgM negative and set B patients. While this approach enabled economical screening, it likely affected sensitivity for detecting samples with low viral load. However, maximal pool sizes for blood donor HEV screening are typically larger than pool sizes used in our study. (30) Furthermore, our prevalence estimate of HEV-C1 infection may be affected by the cessation of universal screening of HEV-IgM negative samples after the six month trial period. While this enabled us to optimize efficiency in screening for HEV-C1 infection, it is possible that this may have missed additional cases who were HEV-IgM negative. Another limitation was that we used a HEV-A based ELISA kit for screening sera in set A. The performance of commercial HEV antibody kits for diagnosing HEV-C1 infections is unknown due to the lack of human samples available for validation. However, this study preliminarily suggests that Wantai ELISA kits are reasonably sensitive for HEV-C1 infection. Until validated serological assays for HEV-C1 infection become available, the absence of HEV-A RNA in serum testing positive for HEV-IgM antibodies should trigger testing for HEV-C1 using a specific nucleic acid test. Like with HEV-A, underlying immunosuppression or subclinical infections impair sensitivity of serological tests. (31, 32)

Despite the above limitations, our study proves that HEV-C1 is a sporadic cause of human hepatitis. Efforts by reference laboratories around the world will be required to establish the global epidemiology of human HEV-C1 infection.

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Figure legends

Figure 1: **Summary of screening results of set A serum samples.**

Figure 2: **Distribution of human HEV-C1 infections and commensal rat carrying LCK-3110-like strain in east-central Kowloon.** Approximate location of the residence of human cases (circles) with relation to the LCK-3110-like HEV-C1 strain isolated from a rat (triangle). SK: Sai Kung district, KT: Kwun Tong district, KC: Kowloon City district, WTS: Wong Tai Sin district, YT: Yau Tsim district, SSP: Sham Shui Po district.

Figure 3: **Phylogenetic analysis using partial ORF1 nucleotide sequences of HEV-C1 strains identified in this study and other HEV strains**. The tree was constructed using neighbor-joining method with bootstrap values calculated from 1000 trees. Only bootstrap values > 70% are shown. HEV-C1 strains identified in patients in Hong Kong are highlighted in bold. Asterisks indicate HEV-C1 strains detected in commensal rats in Hong Kong. Year of infection for human patients or capture of rats is denoted next to the strain names.

Figure 4: **Natural course of HEV-C1 infection**. **(A)** Kinetics of alanine aminotransferase for acutely infected individuals 2, 4, and 6. HEV-C1 viral load among these individuals declined rapidly after diagnosis. Therefore, their viral load kinetics was not depicted. **(B)** Kinetics of alanine aminotransferase and HEV-C1 plasma viral load for persistently infected patients 1, 3, 5, and 8. X-axis crosses the Y-axes at the upper limit of normal of the reference range for alanine aminotransferase and limit of quantitation of the in-house HEV-C1 qRT-PCR assay.

Figure 5: **Comparison of hepatitis severity of HEV-A and HEV-C1 infections. (A)** Alanine aminotransferase (ALT), **(B)** Bilirubin and **(C)** Prothrombin time. Bar represents the mean of measurements, * indicates statistically significant difference between mean of HEV-A and HEV-C1 groups assessed by Welch's *t* test, and ns indicates that the difference between means was not statistically significant. For ALT and bilirubin, data from 69 HEV-A and 8 HEV-C infected patients was included. For prothrombin time, data from 66 HEV-A and 7 HEV-C infected patients for whom data was available was included.

Table 1: **Demographic and clinical features of HEV-C1 infected patients**

	Patient 1 [a] **(index case)**	**Patient 2**	**Patient 3**	**Patient 4**	**Patient 5**	**Patient 6**	**Patient 7**	**Patient 8**
Age	56	71	67	81	74	73	67	43
Gender	M	F	M	M	M	M	M	M
Chronic medical conditions/ organ transplants	Liver transplant (2017)	Rheumatoid arthritis	Kidney transplant (1999)	Prostate cancer	Kidney transplant (2003)	Hypertension, Diabetes mellitus	Metastatic cancer	HIV infection
Hepatitis B carrier/ treatment	Yes/ Entecavir	No	No	No	Yes/ Tenofovir	No	No	No
Immunosuppressive medication	Prednisolone Mycophenolate Tacrolimus	Methotrexate Sulfasalazine	Prednisolone Cyclosporin A Sirolimus	None	Prednisolone Mycophenolate Everolimus	None	None	No
Exposure to rodents or rodent feces	Yes	No	No	No	No	No	No	No
Travel outside Hong Kong	No	No	Yes (Japan)	No	No	No	No	No
Infection type	Persistent	Acute	Persistent	Acute	Persistent	Acute	Subclinical	Persistent
Reason for presentation	Abnormal LFTs	Malaise	Abnormal LFTs	Malaise	Abnormal LFTs	Abnormal LFTs	N/A	Abnormal LFTs
Hepatitis onset time	July 2017	April 2017	May 2018	April 2019	May 2018	June 2019	N/A	May 2019
HEV IgM/IgG [b]	+/+	+/+	-/+	+/+	+/+	+/+	-/+	+/-
Peak ALT (U/L)	279	498	141	410	133	498	20	685
Peak bilirubin (μmol/L)	21	10	12	14	11	< 3	6	14
Peak prothrombin time (s)	13.8	12.7	10.9	12.6	20.4	NA	9.9	11.6
Liver imaging findings	Normal	Parenchymal disease	Fatty liver	Normal	Fatty liver	NA	Normal	NA
Received ribavirin	Yes	No	Yes	No	No	No	No	No
Mortality	No	No	No	No	Yes	No	No	No

ALT: alanine aminotransferase, LFT: liver function test

[a] previously reported in reference xx

[b] performed using Wantai HEV-IgM and IgG kits

Table 2. Nucleotide and deduced amino acid sequence identities of HEV-C1 strains from this and other studies compared to HEV strain LCK-3110

HEV strain (GenBank accession number)	HEV strain LCK-3110 (patient 1)						
	Entire genome	Nucleotides, %			Amino acids, %		
		ORF1	ORF2	ORF3	ORF1	ORF2	ORF3
Patient 2	99.6	99.7	99.8	99.7	99.8	99.7	100
Patient 3	95.1	95	95.9	97.7	98.2	98.9	96.1
Patient 5	84.2	83.2	86.7	89.3	93.7	95.8	77.5
Patient 8	95	94.8	95.9	97.7	98.2	98.9	96.1
WTSRN170519-1	99	99	99.1	98.7	99.3	99.4	98
Vietnam-105 (JX120573)	93.7	93.3	95.2	96.4	98.2	98	95.1
ratESUMBAWA-140L (LC225389)	84.7	83.7	87.8	89.3	94.3	96.1	81.4
HEV 17/1683 (MK050105)[a]	77	76.2	79.1	75.1	86.4	91.6	60.8

ORF1: open reading frame 1; ORF2: open reading frame 2; ORF3: open reading frame 3; [a] Isolate infecting patient in reference [13]

Figure 1

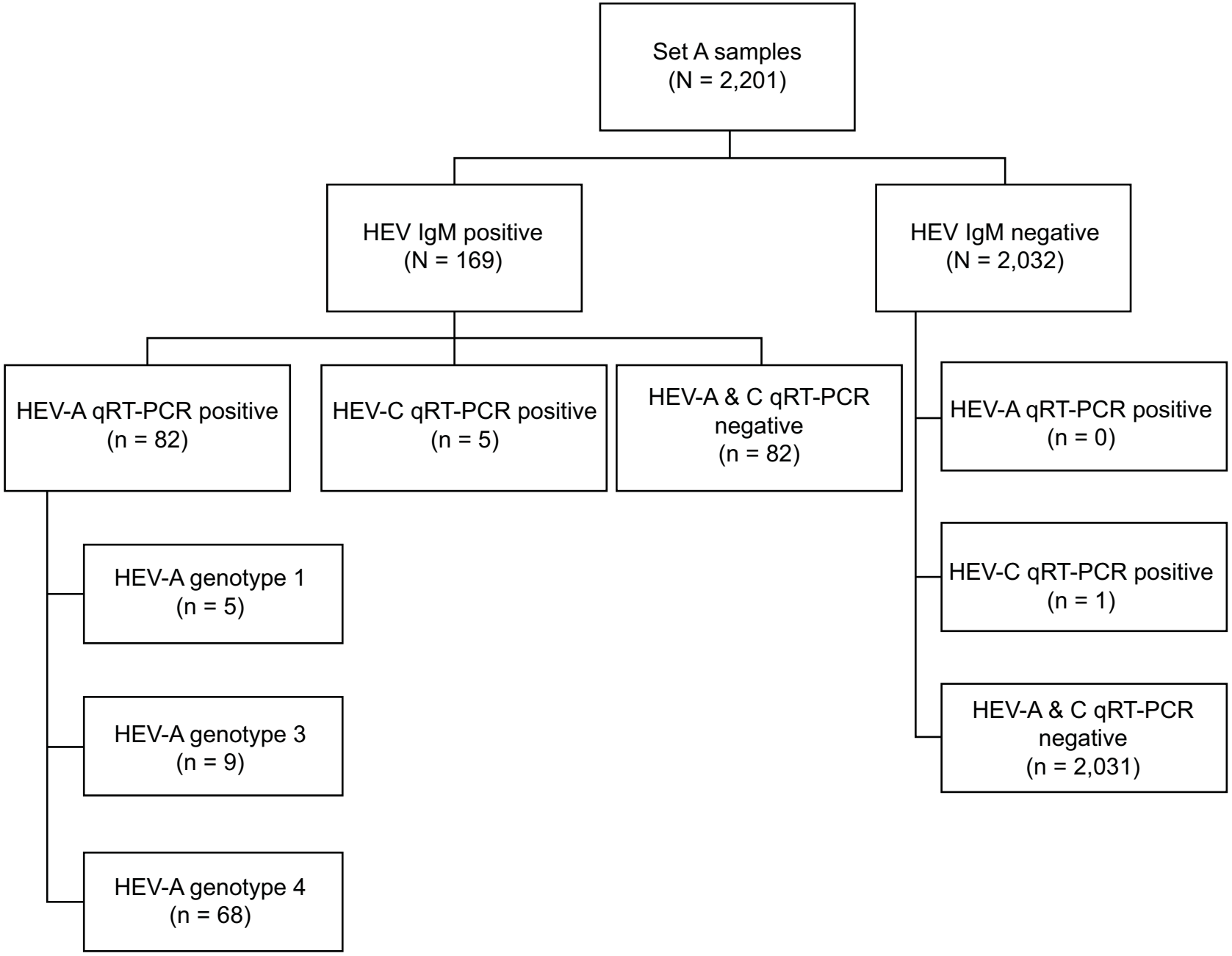

Set A samples
(N = 2,201)
HEV IgM positive
(N = 169)
HEV IgM negative
(N = 2,032)
HEV-A qRT-PCR positive
(n = 82)
HEV-C qRT-PCR positive
(n = 5)
HEV-A & C qRT-PCR
negative
(n = 82)
HEV-A qRT-PCR positive
(n = 0)
HEV-A genotype 1
(n = 5)
HEV-C qRT-PCR positive
(n = 1)
HEV-A genotype 3
(n = 9)
HEV-A & C qRT-PCR
negative
(n = 2,031)
HEV-A genotype 4
(n = 68)

Figure 2

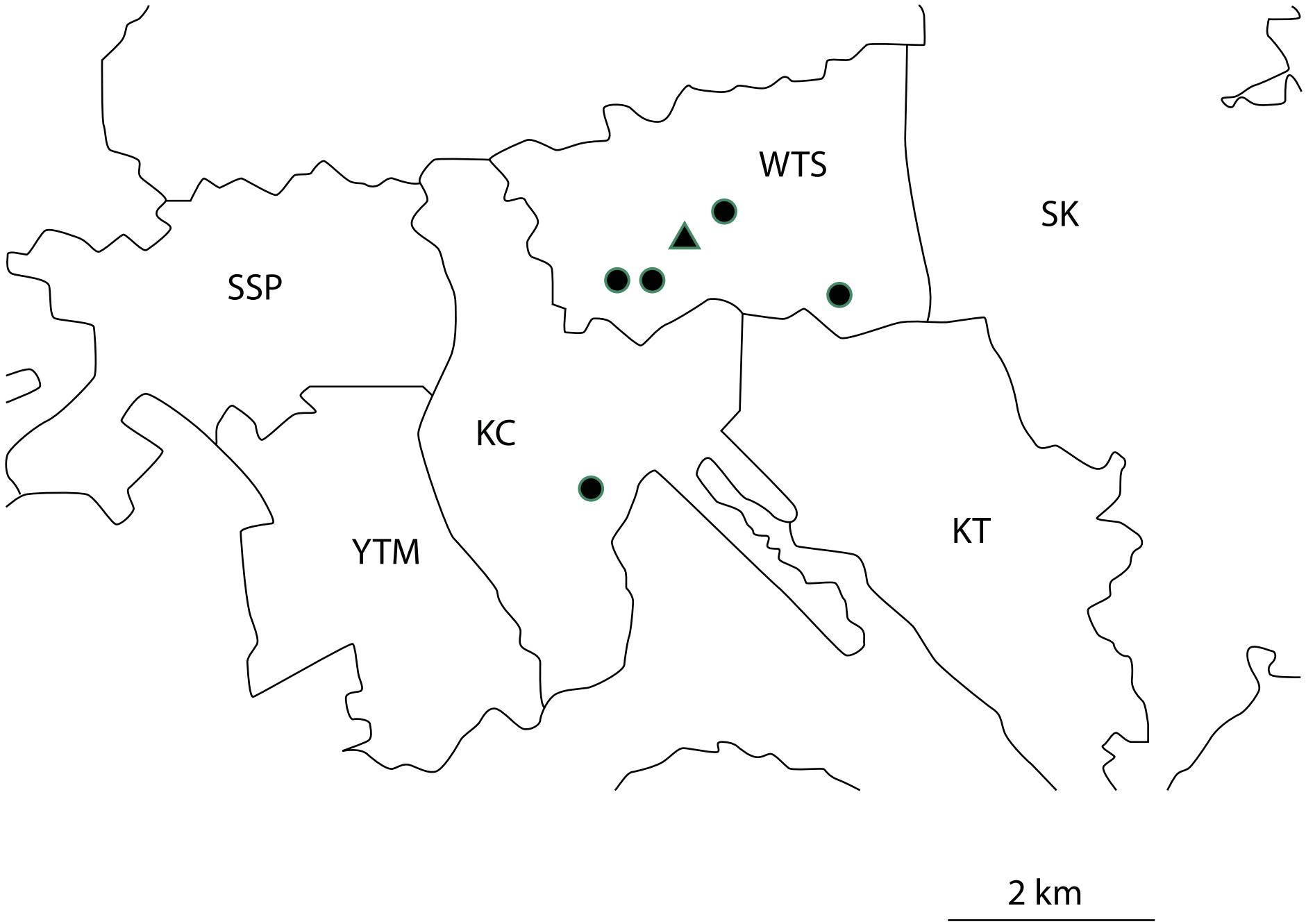


Figure 3

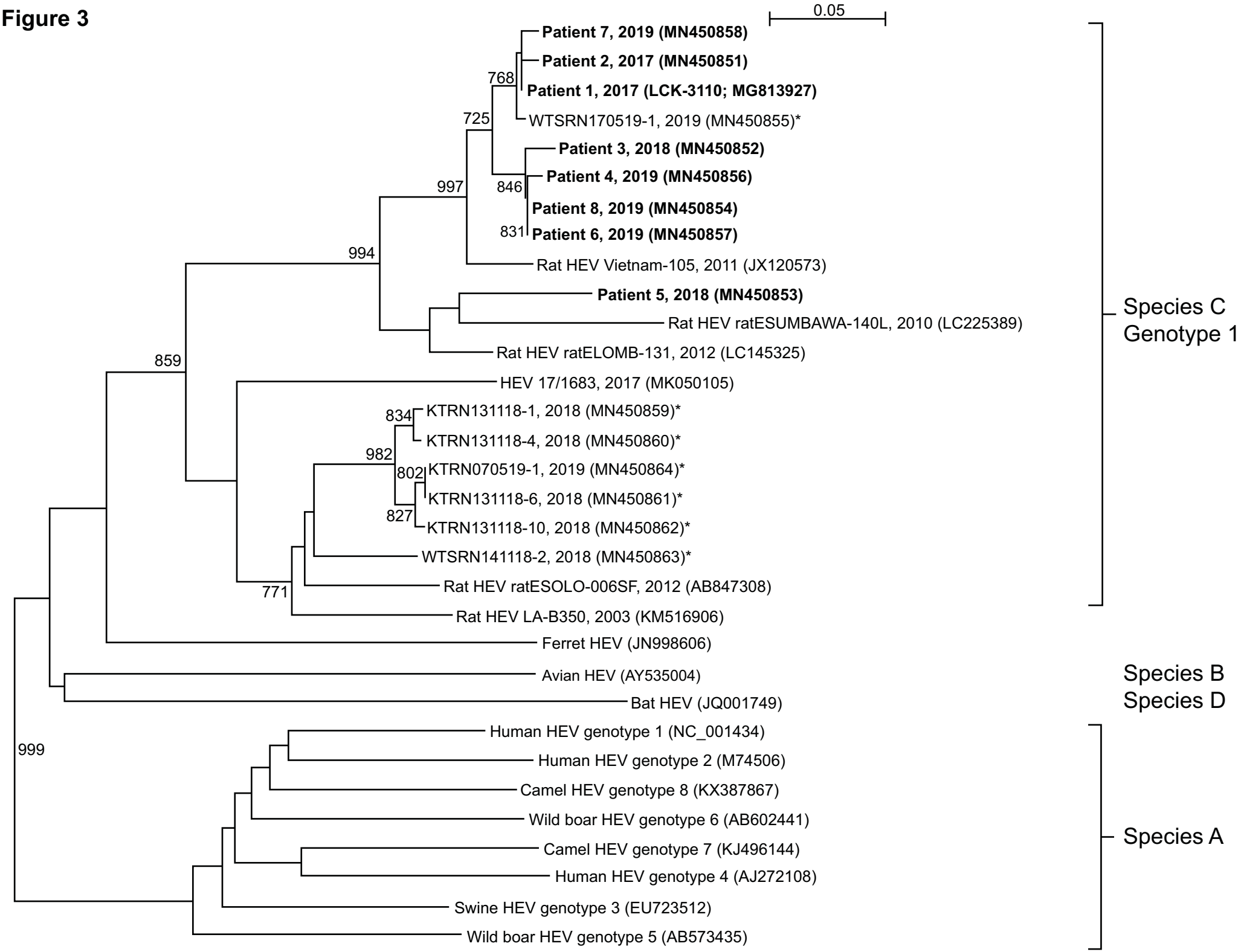

0.05
Patient 7, 2019 (MN450858)
Patient 2, 2017 (MN450851)
768
Patient 1, 2017 (LCK-3110; MG813927)
725
WTSRN170519-1, 2019 (MN450855)*
Patient 3, 2018 (MN450852)
Patient 4, 2019 (MN450856)
997
846
Patient 8, 2019 (MN450854)
831
Patient 6, 2019 (MN450857)
994
Rat HEV Vietnam-105, 2011 (JX120573)
Patient 5, 2018 (MN450853)
Rat HEV ratESUMBAWA-140L, 2010 (LC225389)
Rat HEV ratELOMB-131, 2012 (LC145325)
859
HEV 17/1683, 2017 (MK050105)
834
KTRN131118-1, 2018 (MN450859)*
KTRN131118-4, 2018 (MN450860)*
982
802
KTRN070519-1, 2019 (MN450864)*
KTRN131118-6, 2018 (MN450861)*
827
KTRN131118-10, 2018 (MN450862)*
WTSRN141118-2, 2018 (MN450863)*
Rat HEV ratESOLO-006SF, 2012 (AB847308)
771
Rat HEV LA-B350, 2003 (KM516906)
Ferret HEV (JN998606)
Avian HEV (AY535004)
Bat HEV (JQ001749)
999
Human HEV genotype 1 (NC_001434)
Human HEV genotype 2 (M74506)
Camel HEV genotype 8 (KX387867)
Wild boar HEV genotype 6 (AB602441)
Camel HEV genotype 7 (KJ496144)
Human HEV genotype 4 (AJ272108)
Swine HEV genotype 3 (EU723512)
Wild boar HEV genotype 5 (AB573435)
Species C
Genotype 1
Species B
Species D
Species A

Figure 4A

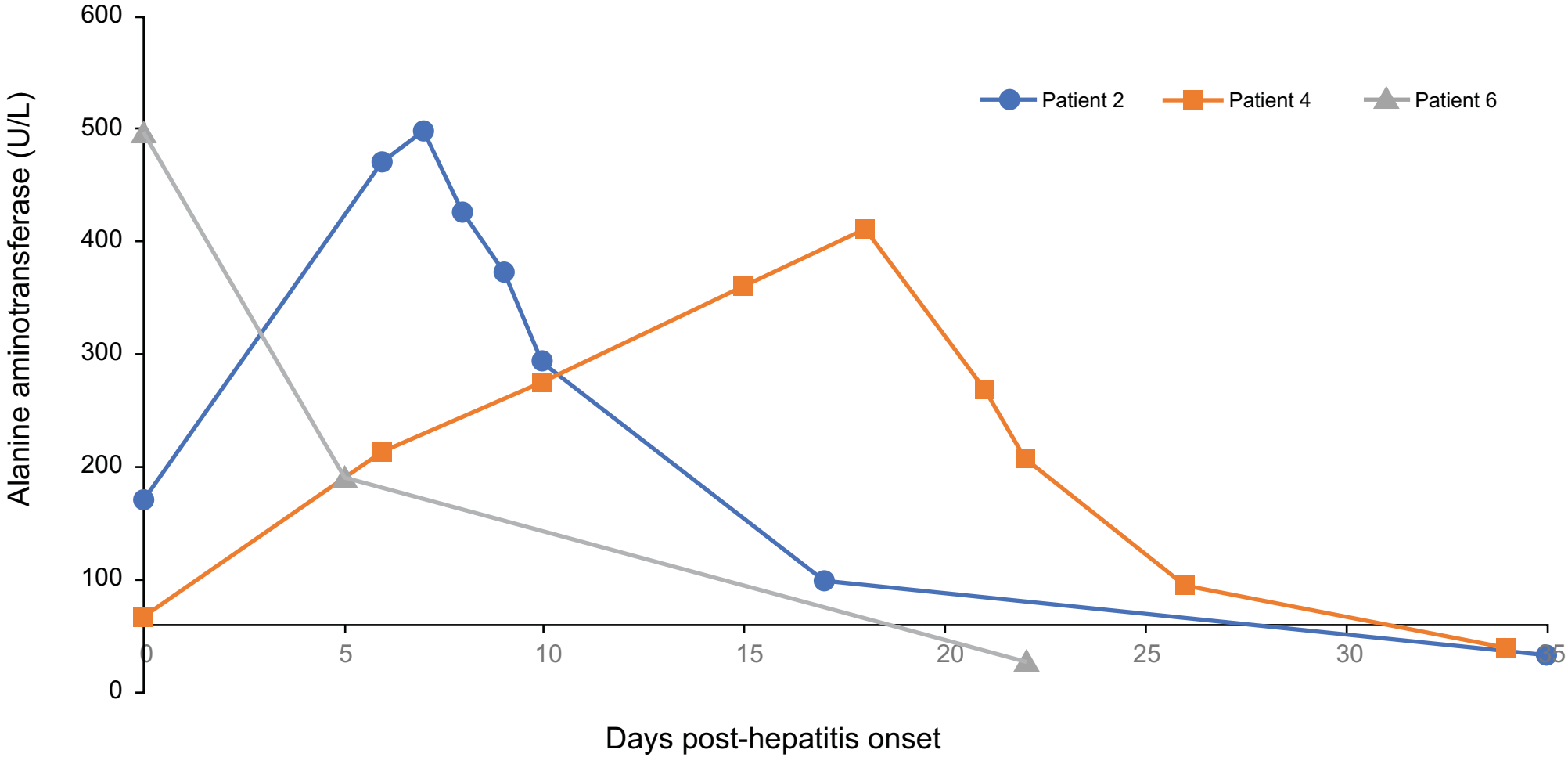

Alanine aminotransferase (U/L)
600
500
400
300
200
100
0
Patient 2
Patient 4
Patient 6
0
5
10
15
20
25
30
35
Days post-hepatitis onset

Figure 4B

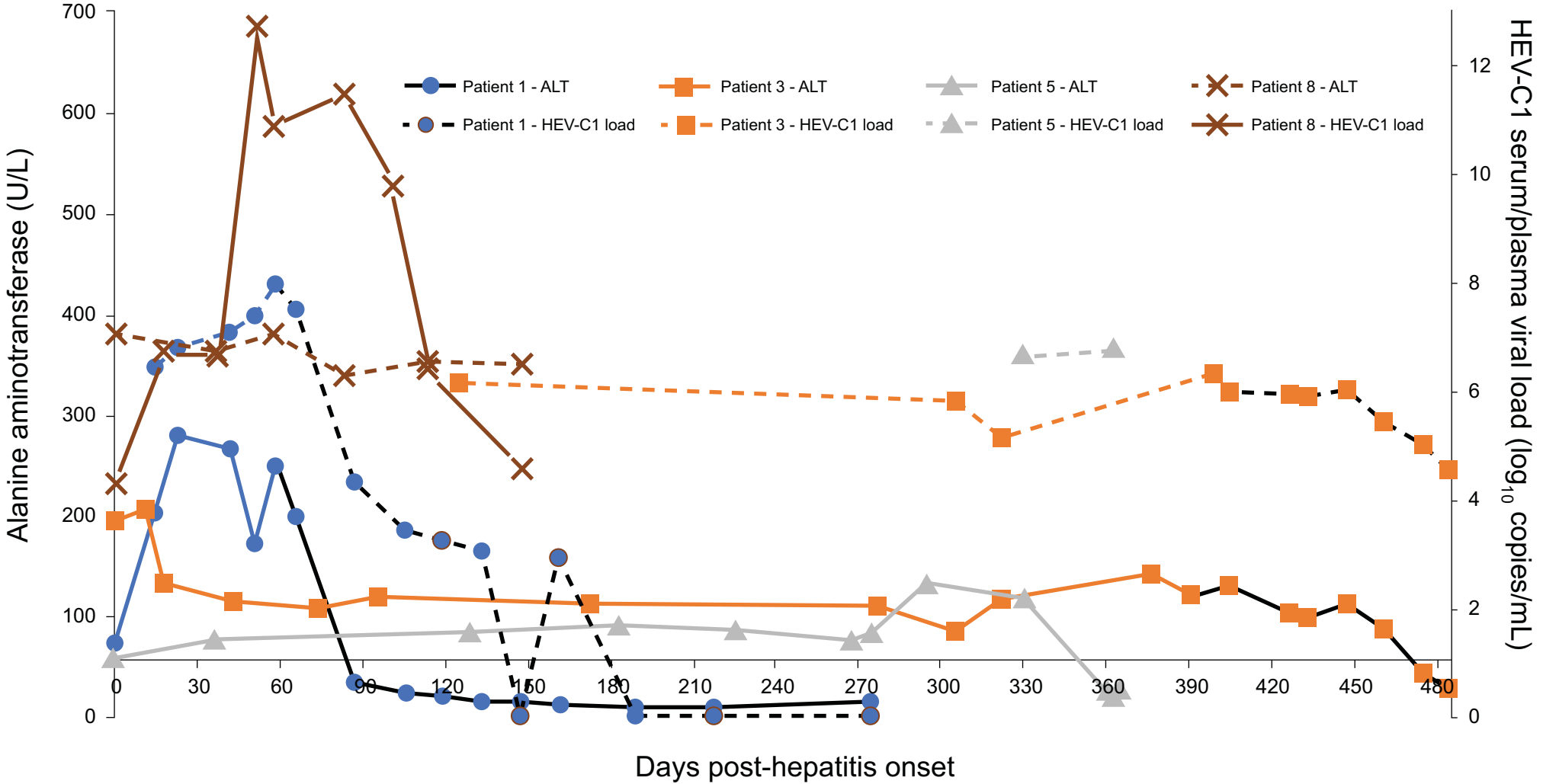

Patient 1 - ALT
Patient 1 - HEV-C1 load
Patient 3 - ALT
Patient 3 - HEV-C1 load
Patient 5 - ALT
Patient 5 - HEV-C1 load
Patient 8 - ALT
Patient 8 - HEV-C1 load
Alanine aminotransferase (U/L)
HEV-C1 serum/plasma viral load (log10 copies/mL)
Days post-hepatitis onset
0
100
200
300
400
500
600
700
0
2
4
6
8
10
12
0
30
60
90
120
150
180
210
240
270
300
330
360
390
420
450
480

Figure 5A

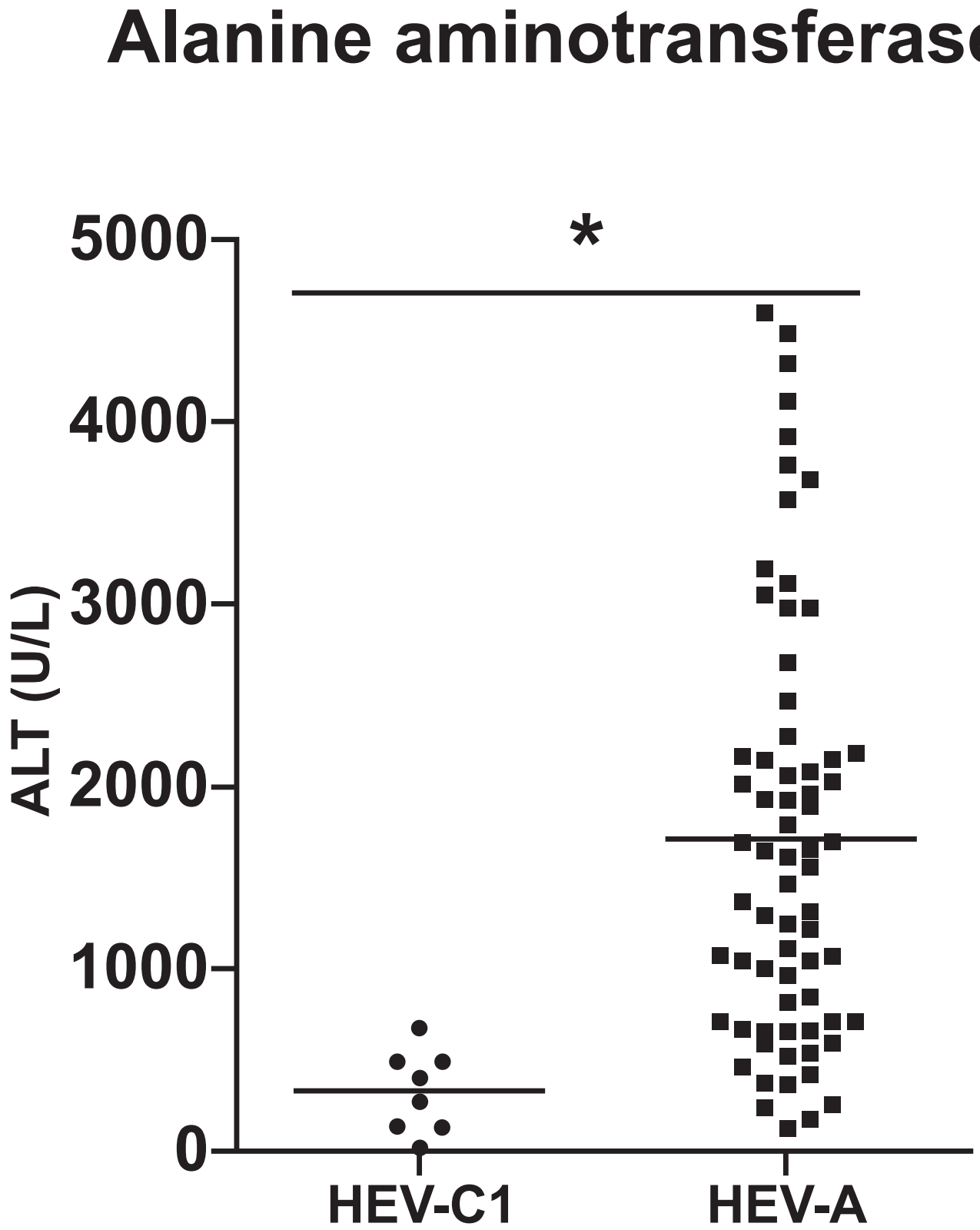

Alanine aminotransferase
5000
4000
3000
2000
1000
0
ALT (U/L)
*
HEV-C1
HEV-A

Figure 5B

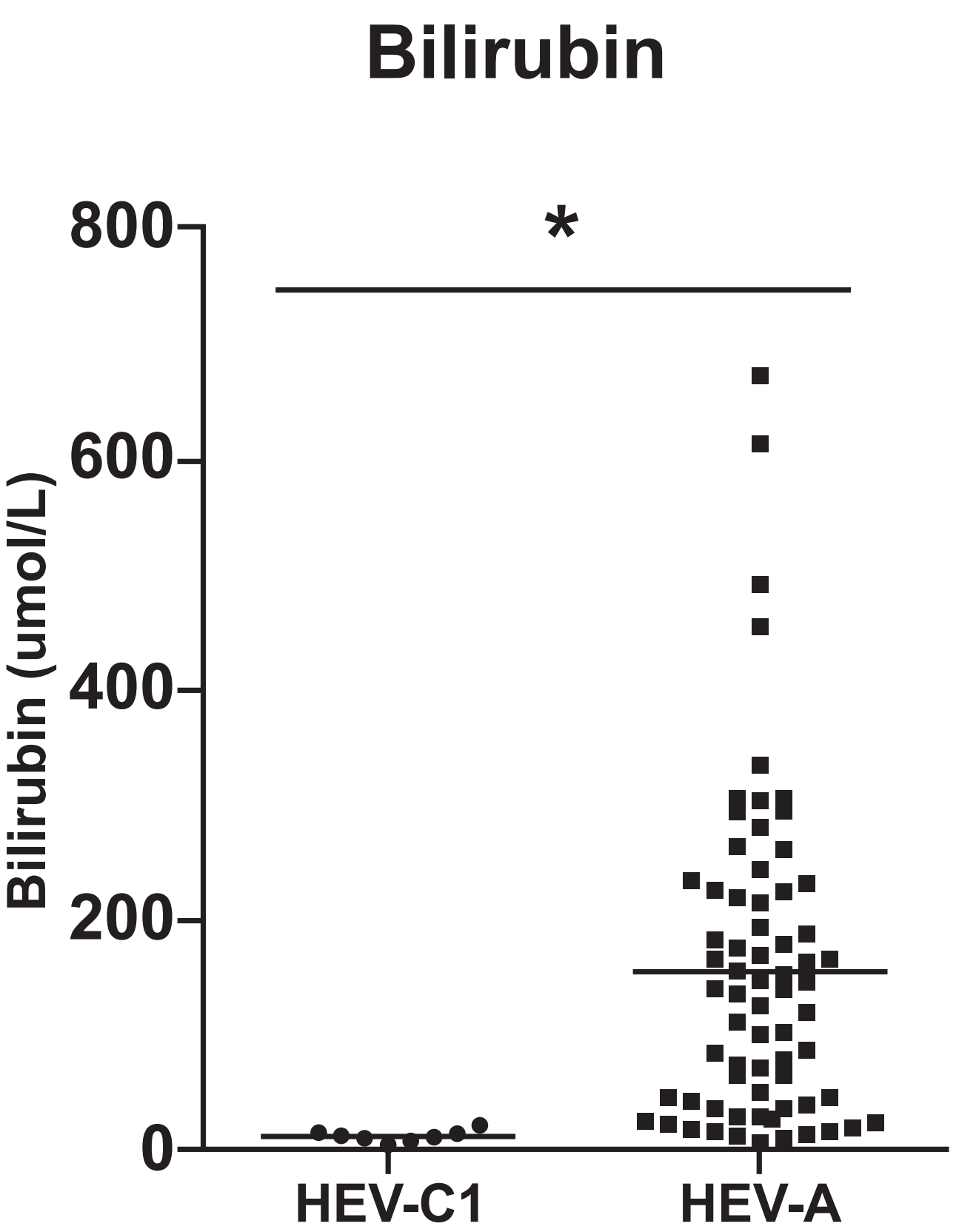
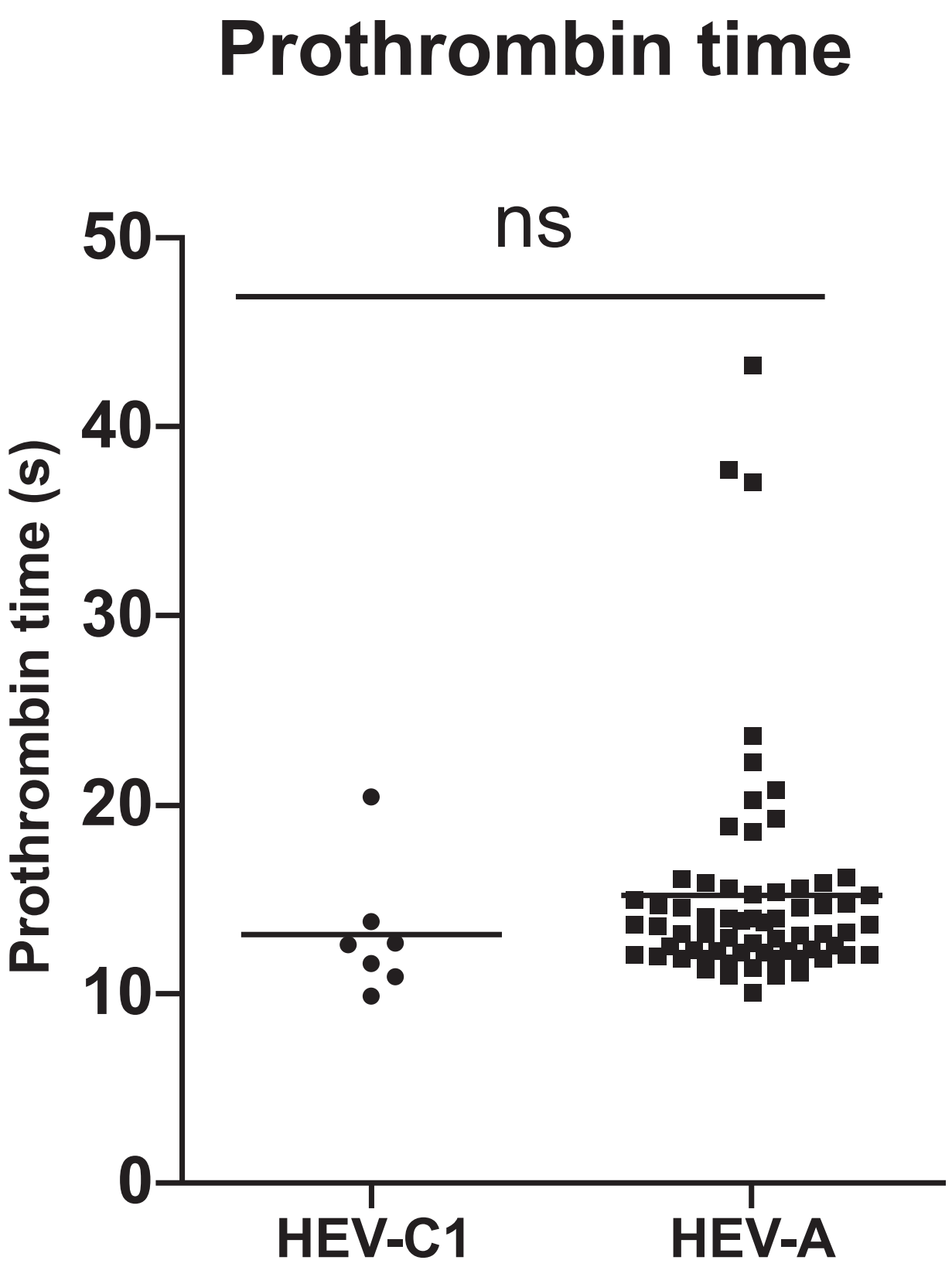


Figure 5C


Prothrombin time
ns
50
40
30
20
10
0
Prothrombin time (s)
HEV-C1
HEV-A