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CLINICAL RESEARCH

Relevance of Tacrolimus Trough Concentration and Hepatitis E virus Genetic Changes in Kidney Transplant Recipients With Chronic Hepatitis E

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Introduction: Hepatitis E virus (HEV) can cause chronic infection (≥3 months) and cirrhosis in immunocompromised patients, especially kidney transplant recipients. Low alanine aminotransferase (ALT) levels and high HEV intrahost diversity have previously been associated with evolution toward chronicity in these patients. We hypothesized that additional clinical and viral factors could be associated with the risk of chronic HEV infection.

Methods: We investigated a series of 27 kidney transplant recipients with HEV infection, including 20 patients with chronic hepatitis E.

Results: High tacrolimus trough concentration at diagnosis was the most relevant marker associated with chronic hepatitis E (9.2 vs. 6.4 ng/ml, $P = 0.04$). Most HEV genetic changes selected during HEV infection were compartmentalized between plasma and feces.

Conclusion: This compartmentalization highlights the diversity and complexity of HEV replication compartments. Tacrolimus trough concentration at diagnosis of HEV infection could allow an early identification of patients at high risk of chronic hepatitis E and guide treatment initiation.

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HEV is a major pathogen associated with acute viral hepatitis worldwide. In Europe and America, the most prevalent genotype is HEV-3, which causes zoonosis transmitted principally by the ingestion of pig or wild boar meat, or through direct contact with animals. HEV is a single-stranded positive-sense RNA virus with a genome of about 7.2 kb. This genome contains 3 open reading frames (ORFs). ORF1 encodes a multidomain replication polyprotein with 5 conserved functional domains and a hypervariable region.[1,2] ORF2 encodes the structural capsid protein, which plays an important role in viral attachment to the host cell and is the major target for neutralizing antibodies and T-cell responses.[3-8]

HEV infection is an emerging major concern, with an incidence of 3.2 cases/100 person-years in solid-organ transplant patients. There are currently no treatment guidelines for acute hepatitis E in immunocompromised patients. HEV infection progresses to chronicity in 47% to 66% of these patients, increasing the risk of severe fibrosis or cirrhosis.[9-13] Of note, the definition of chronic hepatitis E recently changed from persisting HEV replication beyond 6 months to persisting HEV replication beyond 3 months after infection.[14-16] The first-line treatment for chronic hepatitis E is a reduction of immunosuppression, resulting in viral clearance in 30% of patients.[10,15,17] The second-line treatment, 3 months of ribavirin monotherapy, results in a sustained virological response in 81% of patients, but with major adverse effects.[16] Early identification of patients at risk

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of chronic hepatitis E could contribute to improve viral response, reduce treatment duration and side effects. Main factors associated with evolution toward chronicity are low ALT and the use of tacrolimus at diagnosis of HEV infection.[10,18] Nevertheless, the relevance of this finding is limited because tacrolimus is used as first-line calcineurin inhibitor in the vast majority of solid organ transplant recipients.[19-21] In contrast, tacrolimus trough concentration could be a promising marker because it allows to distinguish between patients treated with low-dose and standard-dose tacrolimus.[19,22,23] Relevance of this biomarker at diagnosis of HEV infection is unclear, but it has been associated with the prognosis of HEV infection in patients who already developed chronic hepatitis E.[10,13,24]

Evolution of HEV genomes during infection could also contribute to evolution toward chronicity. This hypothesis is supported by the association between a high level of intrahost HEV diversity (in ORF1 and ORF2 regions) and evolution toward chronic hepatitis E.[18,25] In addition, a compartmentalization of HEV quasi-species was observed between the blood and cerebrospinal fluid in patients with chronic hepatitis E.[26,27] Such compartmentalization argue for extrahepatic HEV replication in the central nervous system, in line with other clinical and *in vitro* studies.[28-30] Recent studies suggested that HEV could also replicate in human intestines. Indeed, HEV can replicate in enterocytes *in vitro* and be detected in intestinal tissue samples from patients with chronic HEV infection.[31] Nevertheless, extrahepatic HEV replication in the human intestines remain to be fully characterized, along with the possible compartmentalization between blood and feces. These could contribute to explain the evolution toward chronicity and the prognosis of HEV infection.

In this retrospective clinical study, we proposed to identify clinical and viral factors at diagnosis of HEV infection associated with the evolution toward chronic HEV infection in kidney transplant recipients. We also aimed to identify HEV genetic changes selected in plasma and feces during chronic HEV infection to better understand the pathogenesis of chronic hepatitis E.

METHODS

Patients and Samples

Kidney transplant recipients followed-up at Tours University Hospital, France between January 1, 2011 and December 31, 2020 were considered for inclusion. At this center, the follow-up of kidney transplant recipients included periodic ALT or aspartate aminotransferase measurement for the early diagnosis of liver disease (from 20 times per year during the first year, to 2–3 times a year during late follow-up). High ALT or aspartate aminotransferase levels (>40 IU/l) on 2 consecutive samples prompted screening for HEV infection by reverse transcription polymerase chain reaction (PCR) (Realstar HEV RT-PCR kit 1.0/2.0, Altona Diagnostics, France). Kidney transplant recipients diagnosed with HEV infection during this period were retrospectively included in this study. Follow-up data for HEV infection were extracted from the clinical records of these patients. Patients with incomplete follow-up (<6 months of follow-up after the diagnosis of HEV infection) and patients treated with ribavirin during the acute phase (<3 months postdiagnosis) of HEV infection were excluded from the study. Spontaneous HEV clearance was defined as clearance of HEV RNA from plasma and feces without ongoing ribavirin treatment. Chronic HEV infection was defined as the persistence of HEV RNA in the plasma or feces for at least 3 months after diagnosis.[14-16] Sustained virological response was defined as undetectable HEV RNA in both plasma and feces at the end of ribavirin treatment and at least 6 months later. Treatment failure was defined as the absence of sustained virological response. Symptomatic hepatitis was defined as fatigue, diarrhea, arthralgia, weight loss, abdominal pain, jaundice, itching, fever and/or nausea. HEV IgM antibodies were detected by enzyme-linked immunosorbent assay (DiaPro, France). Biochemical analysis and blood counts were performed on Cobas c501 (Roche) and XN3100 (Sysmex) analyzers, respectively. Immunophenotyping was performed by flow cytometry on an Aquios/Navios flow cytometer (Beckman Coulter). Therapeutic drug monitoring of tacrolimus in EDTA-whole blood was performed by HPLC-MS/MS (Waters). Drug monitoring of mycophenolic acid plasma concentrations were performed using a kinetic enzymatic method with inosine-5′-monophosphate dehydrogenase inhibition of NADH,H+ production (Cobas Integra 400+, Roche Diagnostics). Plasma samples collected for routine care were stored at −20 °C after IgM or HEV RNA determinations. Samples obtained from patients on ribavirin were excluded because of the known mutagenic effect of this drug on RNA viruses.[32] HEV RNA quantification, genotyping, and analyses of intrahost diversity were performed retrospectively on these samples. This study was approved by the ethics committee of Tours University (no. 2019 071). Informed consent was obtained from all subjects involved in the study.

HEV RNA Quantification

Viral nucleic acid was extracted from 210 µl of plasma or clarified feces with the EZ1 Mini Virus Kit 2.0

(Qiagen) on an EZ1 instrument (Qiagen). HEV RNA was then quantified by ORF3 RT-qPCR, as previously described.[33]

HEV RNA Genotyping

HEV genotyping was performed by sequencing the ORF2 region encoding the M and P domains (1.1 kb), with the primers described in a previous study.[18] We first incubated 10 µl of nucleic acid extracts with 5 µM random hexamers and 1 mM dNTPs for 5 minutes at 65 °C. Reverse transcription was performed with 200 U of SuperScript III reverse transcriptase (Thermo Fisher Scientific), 5 mM DTT (dithiothreitol) and 40 U RNaseOUT Recombinant Ribonuclease Inhibitor (Thermo Fisher Scientific). Cycling reactions were performed on a T3000 thermocycler (Biometra) with the following cycle parameters: 5 minutes at 25 °C, 60 minutes at 50 °C, and 10 minutes at 70 °C. ORF2 (1.1 kb) was then amplified from the cDNA with 500 nM ORF2_s and ORF2_as primers (Supplementary Table S1), 5 µl cDNA and GoTaq Long PCR Master Mix (Promega), in a 25 µL reaction mixture. The cycling program was: 2 minutes at 95 °C followed by 45 cycles of 30 seconds at 94 °C, 30 seconds at 57 °C, and 90 seconds at 70 °C; with a final extension for 5 minutes at 70 °C. Sequencing reactions were performed on an ABI3130XL high-throughput capillary DNA analyzer (Thermo Fisher Scientific) with BigDye Terminator Mix 1.1 (Thermo Fisher Scientific) and the ORF2s2_seq and ORF2as_seq primers. The consensus amino acid (aa) sequences obtained by next-generation sequencing for each patient were aligned in CLC Main Workbench with reference sequences proposed by Smith *et al.*[34] and Nicot *et al.*,[35] updated with recently described HEV-3m subgenotype.[36] Genotype and subtypes were determined with a UPGMA phylogenetic tree constructed with CLC Main workbench. The sequence of HEV-3f isolate AB36987 was used as a reference for aa numbering,[35] with 5 domains considered for the ORF1 polyprotein.[1,2] Major mutations (present in ≥50% of the viral population) were identified by comparison with sequences from the National Center for Biotechnology Information virus database (ORF1 and ORF2 sequences from European and North America human isolates). Minor mutations (2%–49%) at the same positions were sought in other samples from the same patients, to characterize viral evolution over time and to compare diversity in the plasma and feces.

HEV Intrahost Diversity

ORF1 (nuc 1689–3013, ORF1 aa 562–1003) and ORF2 (nuc 6067–7121, ORF2 aa 305–656) were amplified from the cDNA for next-generation sequencing analysis according to the PCR protocols described above. We used the HEVORF1_S1 and HEVORF1_A1 or HEV_3F and HEVpos5508r (Supplementary Table S1) primers for the amplification of ORF1. For ORF2, we used ORF2_s with ORF2_as (whole ORF2), ORF2_s with ORF2as_seq (M-domain) and ORF2S2_seq with ORF2var_R (P-domain). The PCR products were purified with NucleoSpin Gel and PCR Clean-up (Macherey-Nagel), according to the manufacturer's recommendations. Next-generation sequencing was performed on the Miseq platform (Illumina). Briefly, the sequencing library was prepared with the Nextera XT DNA sample preparation kit (Illumina), in accordance with the manufacturer's recommendations. Quality control was performed for these libraries in a capillary electrophoresis LabChip GX (Perkin Elmer, France) with a DNA High Sensitivity chip (Perkin Elmer). We obtained 150 bp paired-end sequencing reads with the Miseq platform. Illumina sequencer output files of up to 150-base pair sequencing reads were processed with the Galaxy platform (https://mississippi.sorbonne-universite.fr) after filtering and checks on read quality (FastQC algorithm). Trinity software was used for *de novo* assembly. The sequences constructed were checked with the Blast option from the National Center for Biotechnology Information. The Bowtie 2 tool was used to map reads onto the best-match sequence provided by the *de novo* sequence assembly. Nucleotide analysis was performed position-by-position with mpileup. Positions with a sequencing depth of over 100X were retained for further analysis. We compared the nucleotide entropy (%) and aa diversity (%) of ORF1 (HVR and X domain) and ORF2 (M and P domain). For each isolate, nucleotide entropy (%) and aa diversity (%) correspond to the mean diversity at each position in the consensus nucleic acid and protein sequences, respectively. Synonymous or nonsynonymous mutations at each position accounting for more than 2% of the HEV population were considered for analysis.

Evolution of HEV Genomes During Chronic HEV Infection

For each patient, sequences from the acute phase of infection were compared to sequences from the chronic phase of infection (≥3 months postdiagnosis, Supplementary Table S2). For 2 patients, samples collected 11 weeks postdiagnosis were considered as chronic phase samples because HEV viral loads were too low to generate sequence data during the chronic phase.

In silico *Analysis of ORF2 Mutations*

The 2ZTN crystal structure of ORF2 from genotype 3 HEV-like particles was obtained from the RCSB database and annotated with Pymol software v2.5.[37] The mutations identified by next-generation sequencing in our study were introduced into this 3D structure.

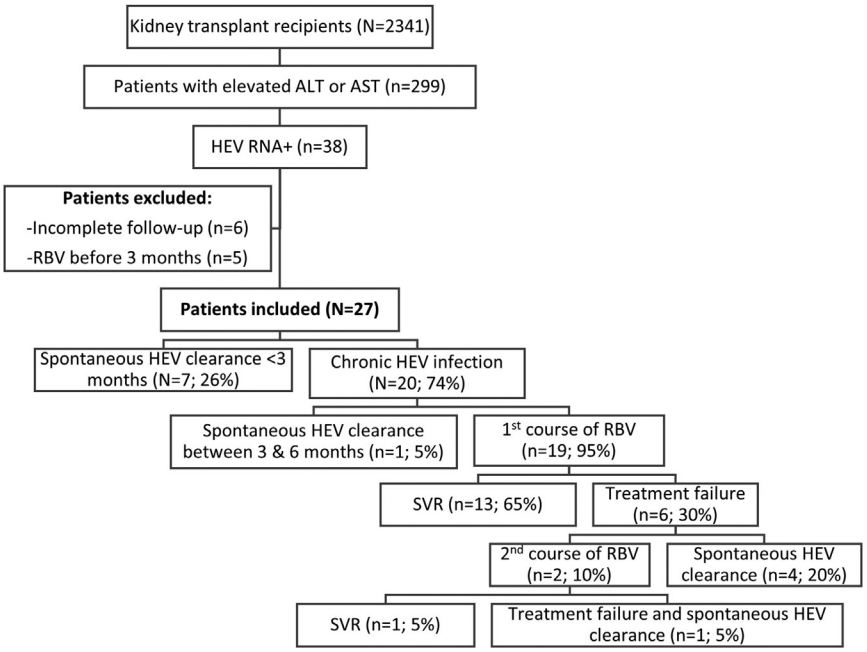

Figure 1. Outcomes of HEV infection in kidney transplant recipients. HEV, hepatitis E virus; RBV, ribavirin; SVR, sustained virological response.

Statistical Analyses

Statistical analyses were performed with GraphPad Prism software v9 (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com). Mann-Whitney (unpaired values for clinical factors) and Wilcoxon (paired values for longitudinal follow-up of HEV diversity) tests were used to compare quantitative variables. Fisher's exact tests was used to compare qualitative variables. P-values <0.05 were considered statistically significant.

RESULTS

Follow-up of HEV Infection in Kidney Transplant Recipients

During the study period, 2341 kidney transplant patients were followed-up in our hospital. Median follow-up time was 5.48 person-years (interquartile range [IQR]: 2.59–9.61); total follow-up time was 13,334 person-years. During the study period, 299 (19%) of these patients experienced elevated ALT or aspartate aminotransferase levels and were subsequently screened for HEV RNA (Figure 1). The incidence of HEV infection among these 299 patients was 13% ($n = 38$). The overall incidence of HEV infection in kidney transplant recipients was 0.29 cases/100 person-years (95% confidence interval: 0.21-0.39). Six patients with incomplete follow-up and 5 patients treated with ribavirin during the acute phase of HEV infection were excluded from the study. Twenty-seven kidney transplant recipients with HEV infection were included in this study, including 20 patients (74%) with chronic HEV infection, defined as HEV replication extending beyond 3 months after infection (Figure 1 and Figure 2a).

Among these 20 patients with chronic HEV infection, the median duration of HEV detection was 5.6 months in plasma (IQR: 3.8–13.5) and 7.1 months in feces (IQR: 4.7–13.0). One patient with chronic hepatitis E experienced spontaneous HEV clearance 4 months postdiagnosis. The remaining 19 patients were treated with ribavirin, from a median of 3.7 months after the diagnosis of HEV infection (IQR: 2.5–6.0 months) for a median duration of 4.0 months (IQR: 2.5–5.0 months). HEV RNA was undetectable in the plasma and feces after a median duration of ribavirin treatment of 1.0 and 3.6 months (IQR: 0.8–6.0 and 1.2–7.6; Figure 2b), respectively. Sustained virological response to a first and second course of treatment were 65% (13/19) and 70% (14/19), respectively (Figure 2b). Six patients experienced treatment failures despite ribavirin exposure ≥3 months. One patient had to stop ribavirin, because of anemia, before HEV clearance. Two patients stopped ribavirin in the context of HEV clearance in plasma, despite HEV RNA still being detectable in feces. They experienced an HEV relapse in plasma. The remaining 3 patients had undetectable HEV RNA in plasma and feces at the end of treatment and experienced an HEV relapse afterward.

Factors Associated With Chronic HEV Infection

Patient characteristics were retrospectively analyzed to search for factors associated with evolution toward chronic HEV infection (Table 1). Twenty-one patients were treated with tacrolimus at diagnosis of HEV

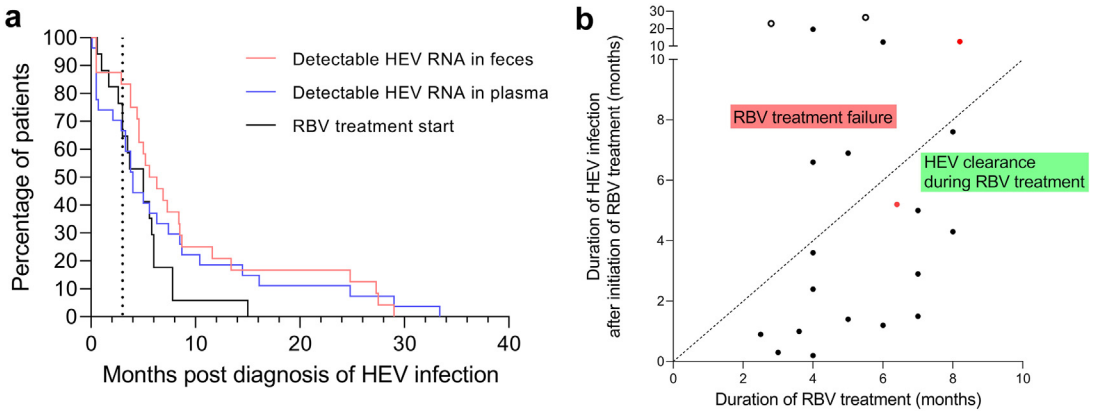

Figure 2. Follow-up of HEV infection. (a) Duration of HEV RNA detection in plasma and feces and date of ribavirin treatment start, in 27 kidney transplant recipients with HEV infection. (b) Duration of ribavirin treatment and duration of HEV infection since initiation of treatment in 20 kidney transplant recipients with chronic HEV infection. In black, first course of ribavirin in 19 patients, including 2 patients (empty circles) who were later re-treated (in red). HEV, hepatitis E virus; RBV, ribavirin;

infection. Among these, tacrolimus trough (i.e., residual) concentration at diagnosis of HEV infection was the main factor associated with evolution toward chronicity, with higher concentrations in the chronic group (median of 9.2 vs. 6.4 ng/ml, $P = 0.04$; Table 1 and Figure 3a). Receiver-operating characteristics analysis suggests that this marker could differentiate between patients with resolving and chronic HEV infections (area under the curve = 0.83, Figure 3b). Prompt reduction of tacrolimus trough concentrations was observed 2 to 4 months after diagnosis of HEV infection in the chronic group (9.2 vs. 5.6 ng/ml, $P = 0.001$, Figure 3). Of note, trough concentrations 1 year posttransplantation were comparable between both groups (8.0 vs. 6.1 ng/ml, $P = 0.20$, Figure 3).

Evolution of HEV Genomes During Chronic HEV Infection

We observed a considerable interhost diversity for HEV-3 in kidney transplant recipients. HEV-3f, HEV-3e and HEV-3c subtypes, associated with zoonotic transmission in France, were the most frequent in our study (Supplementary Figure S1).[38,39] Two ORF1 and 8 ORF2 major aa mutations (frequency >50 %) were detected in the acute and/or chronic phase samples from 8 patients (Figure 4a andSupplementary Table S2). Three of these mutations have never been reported (E749Q in ORF1, T414K; and L473F in ORF2) and 5 have been reported only rarely (<1%) in European and North American HEV isolates (ORF1 L828I; and ORF2 A477T, Q482K, M492V and A632V).

Characterization of HEV diversity in paired plasma and feces samples revealed that 6 of the 10 mutations were selected in only 1 compartment (feces or plasma) (Figure 4a andSupplementary Table S2). Among these, 1 mutation was selected during the chronic phase (ORF1 749Q in feces) and, surprisingly, 5 other mutations were already compartmentalized during the acute phase (ORF2 414K and 473F in feces; 477K, 482K and 614V in plasma). The remaining 4 mutations were either not compartmentalized (354Y) or could not be searched for in other compartments due to missing samples (828I in ORF1, 492M and 632V in ORF2).

Longitudinal follow-up of HEV diversity in patients revealed that both major ORF1 mutations were detected only in the chronic phase samples: E749Q (hypervariable region) and L828I (ADP ribose domain). In contrast, the 5 ORF2 mutations associated with compartmentalization during the acute phase were no longer detected in the chronic phase of infection: 414K (66% vs. 0%), 473F (63% vs. 0%), 477T (95% vs. 0%), 482K (96% vs. 0%), and 614V (99.7% vs. 0%) (Figure 4a andSupplementary Table S2). Interestingly, 3 of these mutations were localized in the E2s region containing the immunodominant epitopes (aa 455–602)[3–7] (Figure 4b). Mean HEV nucleotide and aa intrahost diversity were relatively stable over time in ORF1 (0%–0.8%) and ORF2 (0%–0.3%), both in plasma and in feces (Figure 5). There was no association between HEV viral load and nucleotide entropy ($P = 0.24$). Interestingly, during the chronic phase, aa diversity in the ORF1 region was more heterogeneous ($P = 0.04$) and tended to be higher (0.23 vs. 0.06, $P = 0.13$) than aa diversity in the ORF2 region (Figure 5b).

DISCUSSION

In this retrospective study, we identified tacrolimus trough concentration at diagnosis of HEV infection as an early factor associated with evolution toward chronic hepatitis E in kidney transplant recipients (9.2 vs. 6.4 ng/ml, $P = 0.04$). In addition, we revealed the emergence of HEV genetic changes during HEV

Table 1. Patient characteristics at the diagnosis of HEV infection

Characteristics	Resolving infection (n = 7)	Chronic infection (n = 20)	P
Age, yr	58 (43–64)	55 (49–61)	0.60
Sex, female/male	1 (14) / 6 (86)	7 (35) / 13 (65)	0.66
Symptomatic hepatitis	2 (29)	4 (18)	0.88
ALT, IU/l	82 (40–467)	84 (55–131)	0.72
AST, IU/l	53 (35–148)	48 (39–65)	0.43
γGT, IU/l	197 (107–260)	61 (38–150)	0.06
Total bilirubin, µmol/l	8.0 (5.0–18)	8.0 (7.0–11)	0.42
Platelet count /mm^3	226 (197–300)	213 (163–258)	0.65
Lymphocyte count /mm^3	1.6 (1.1–3.0)	1.1 (0.7–2.0)	0.24
Time after transplantation, mo	33 (21–176)	72 (26–106)	0.93
Induction therapy			
Basiliximab /Rabbit antithymocyte globulins / no data	4 (57) / 3 (43)	7 (35) / 11 (55) / 2 (10)	0.66
Maintenance therapy			
Calcineurin inhibitors	6 (86)	19 (95)	0.46
Tacrolimus/cyclosporine	4 (67) / 2 (33)	17 (90) / 2 (10)	0.29
Tacrolimus, mg/d	3.5 (3.0–7.0)	3.5 (3.0–5.5)	0.83
Tacrolimus trough concentration, ng/ml	6.4 (5.7–7.0)	9.2 (6.9–9.9)	0.04
MMF	7 (100)	18 (90)	1.00
Mycophenolate mofetil, mg/d	1500 (1000–2000)	1000 (1000–1500)	0.35
Sirolimus/everolimus	0 / 0	2 (10) / 0	1.00
Corticosteroids	5 (71)	14 (70)	1.00
Corticosteroids, mg/kg/d	7.5 (5.0–10.0)	10 (5.0–10.0)	0.74
HEV IgM detectable	5 (71)	14 (82)	1.00
HEV RNA in plasma or feces			
Detectable	7 (100)	17 (90)	0.55
Missing data / Not detected	0 / 0	1 / 2[a]	-
HEV RNA, $\log_{10}$ IU/ml in plasma[b]	5.1 (3.9–6.0)	5.7 (4.6–7.2)	0.44

γGT, gamma-glutamyl transferase; ALT, alanine aminotransferase; AST, Aspartate aminotransferase; HEV, hepatitis E virus; MMF, mycophenolate mofetil.
Results are presented as the median (interquartile range) or as absolute values (percentages). Sex was defined as different biological and physiological characteristics of males and females. In bold, P-value < 0.05.
[a]HEV RNA was not detected in plasma and feces from these patients until several weeks after diagnosis (HEV IgG+ and IgM+).
[b]Data were available for 4 and 9 patients with resolving and chronic HEV infection, respectively.

infection, most of which were compartmentalized between plasma and feces.

Treatment of HEV infection in solid organ transplant recipients is based on reduction of immunosuppressive therapies and antiviral ribavirin treatment. Ribavirin treatment is only recommended in patients who develop chronic hepatitis E, on the basis that spontaneous HEV cure is possible before 3 months.[15] This strategy has several limitations, especially a risk of relapse after ribavirin treatment in 10% to 18% of patients and anemia during treatment.[16] It could be relevant to identify early, patients who will not spontaneously cure HEV and who could potentially benefit from early ribavirin treatment. Such early intervention could increase virological response rates, thus limiting ribavirin exposure and side effects. To date, only low ALT levels at diagnosis of HEV infection have been associated with an increased risk of chronic hepatitis E in solid organ transplant recipients.[10,18] In this study, we identified tacrolimus trough concentration at diagnosis as the most relevant marker associated with chronic hepatitis E in kidney transplant recipients. Our findings are consistent with *in vitro* studies demonstrating that calcineurin inhibitors (tacrolimus and cyclosporine) stimulate HEV replication[40] and decrease T-cell activation.[41] Our findings are also consistent with a previous study which demonstrated that tacrolimus trough concentration at last follow-up during the chronic phase of HEV infection has been associated with the duration of HEV viremia in solid organ transplant recipients.[13] Of note, a previous study suggested that the use of tacrolimus at diagnosis of HEV infection, but not its trough concentration, was associated with evolution toward chronic hepatitis E in solid organ transplant recipients.[10] The recent change

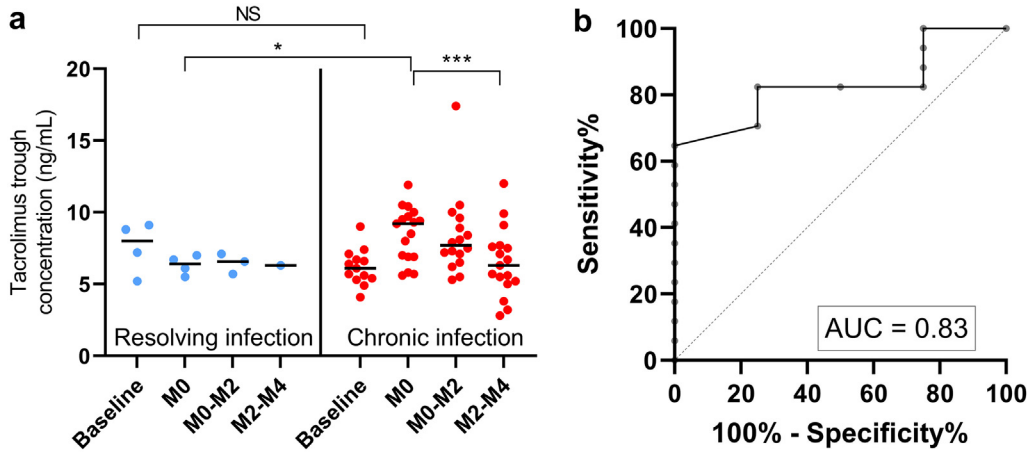

Figure 3. Tacrolimus trough concentrations in patients with HEV infection. (a) Results are represented as individual values with median at baseline (1 year posttransplantation), at diagnosis of HEV infection (M0), between diagnosis and 2 months postinfection (M0–M2) and between 2 to 4 months postdiagnosis (M2–M4). *, $P < 0.05$; ***, $P \leq 0.001$. (b) Receiver-operating characteristics (ROC) curve and area under the curve (AUC) value of tacrolimus trough concentration at diagnosis of HEV infection for the prediction of patients evolving toward chronic HEV infection. HEV, hepatitis E virus; M, month; NS, non-significant difference.

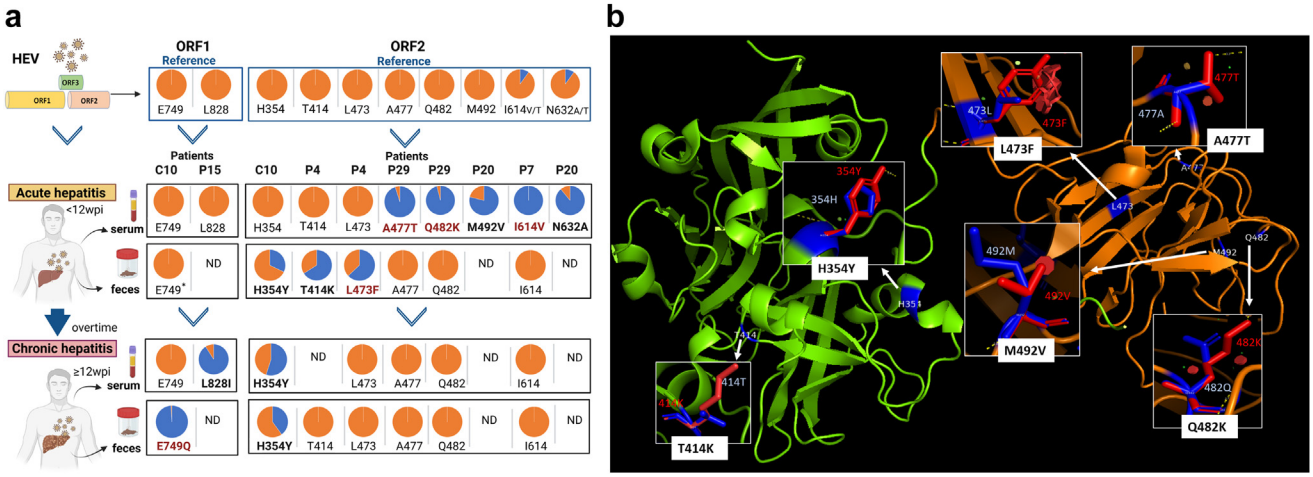

Figure 4. HEV intrahost diversity at positions associated with genetic changes selected during chronic HEV infection. (a) Mutations selected in the ORF1 (aa 650–999) and ORF2 regions (aa 305–656) from paired HEV clinical isolates collected from 8 patients during the acute and chronic phase of HEV infection. The reference (on top) represents the diversity at each position in published sequences from European and North American human HEV isolates. Relevant mutations (in bold), including mutations associated with compartmentalization (in red) are listed along with their respective prevalence in HEV quasi-species (pie charts: aa mutation in blue, wild type aa in orange). HEV-3f isolate AB36987 was used as a reference for aa numbering. *, sequencing depth (24 reads) was below the 100x cut-off for this sample. Missing samples or amplification failures are labeled "ND". (b) Crystal structure of HEV3 ORF2 (2ZTN) (aa 129-606). In blue, aa from the reference sequence; in red, mutations detected in clinical HEV isolates from kidney transplant recipients. In orange, E2s region containing the immunodominant B-cell epitopes (aa 455-602).[3-7] Aa, amino acid; HEV, hepatitis E virus; ORF, open reading frame.

in the definition of chronic HEV infection (≥6 to ≥3 months), the trend toward a reduction in calcineurin inhibitors dosage,[19] or the small number of patients in our study could have contributed to these discrepancies. The association between tacrolimus trough concentration at diagnosis and evolution toward chronicity should be further explored in other solid organ and hematopoietic stem cells transplant patients with HEV infection. If confirmed, this could contribute to improving patient care. Indeed, first-line maintenance immunosuppressive treatment in solid organ transplant recipients is based either on low-dose tacrolimus (trough concentration 3–7 ng/ml) or standard-dose tacrolimus (5–15 ng/ml).[19,22,23] Opting for low-dose tacrolimus regimen could contribute to better prevent chronic hepatitis E. When this is not feasible, mitigation strategies such as early ribavirin treatment, could be evaluated.

Extrahepatic HEV replication has been demonstrated in the central nervous system of patients with chronic HEV infection. There is growing evidence that extrahepatic HEV replication could also occur in the intestines of these patients. Indeed, HEV is able to infect human enterocytes *in vitro*,[31] can be detected in the

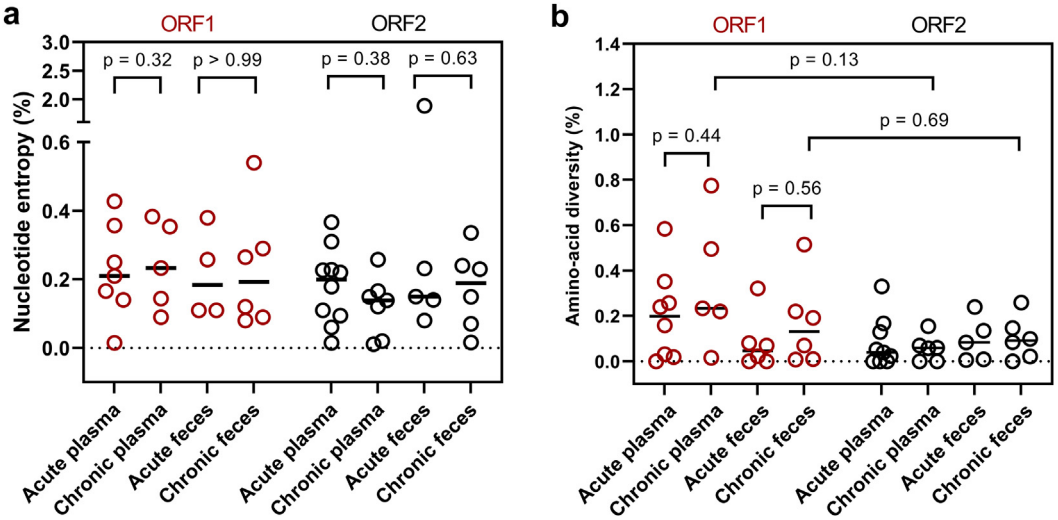

Figure 5. Changes in HEV intrahost diversity in the plasma and feces during HEV infection. (a) Nucleotide entropy (%) and (b) amino acid sequence diversity (%) for the ORF1 (red) and ORF2 regions (black) in the plasma and feces. HEV, hepatitis E virus; ORF, open reading frame

intestinal crypts of patients with chronic HEV infection,[31] and replicates in the intestines of pigs.[42,43] In our study, prolonged HEV fecal shedding was associated with relapse after ribavirin treatment, confirming previous studies.[44,45] Importantly, we observed for the first time a compartmentalization of HEV genomes between the plasma and feces in kidney transplant recipients with chronic hepatitis E, in the absence of ribavirin treatment. Current *in vitro* models of HEV infection only partially explain this compartmentalization. Indeed, these models suggest that both hepatocytes and enterocytes allow HEV replication and release HEV particles primarily toward the intestinal lumen and secondarily toward the plasma.[31,46] This should result in almost no compartmentalization of HEV genomes between the plasma and feces. HEV genetic changes could have contributed to this compartmentalization by increasing viral shedding from hepatocytes or enterocytes specifically toward the plasma or the feces. HEV replication in other cell types or physiological hypoxia could have also contributed to this compartmentalization. It has been suggested that liver stem cells, intestinal stem cells, or cholangiocytes could also be infected by HEV. HEV release in these cells could differ from hepatocytes and enterocytes, thus contributing to compartmentalization of HEV genomes.[47–49] Physiological hypoxia in the liver and intestinal mucosa differs from normoxia conditions considered for most *in vitro* cell culture models of HEV infection.[50–52] Hypoxia has an impact on the infectivity of other hepatitis viruses and could have an impact on HEV release.[53] Lastly, subpopulations of hepatocytes or enterocytes could be topologically closer to the blood than they are to feces, thus shedding more viral particles toward the blood. Overall, the compartmentalization of HEV genomes between the plasma and feces highlights the complexity and diversity of HEV replication compartments. Further studies are required to identify these compartments and characterize their roles in the pathogenesis of chronic hepatitis E.

HEV intrahost diversity in plasma samples was comparable to that found in paired feces samples from the acute and chronic phases of infection. Despite this apparent overall stability of HEV intrahost diversity, 4 highly conserved positions in ORF2 (aa 414, 473, 477, and 482) were associated with the transient selection of mutations during the acute phase of hepatitis E. One of these mutations (477T) has been associated with impaired B-cell responses *in silico*.[54] Three of these mutations (473F, 477T, and 482K) affected the P domain (aa 453–660), which is known to be associated with conformational neutralizing epitopes[55] for the monoclonal antibodies mAb 8C11, 8H3, MAB1323, MAB272, and Fab224.[4,37,56,57] These ORF2 mutations may have contributed to immune escape during the acute phase, either directly, through the alteration of immune epitopes; or indirectly, through conformational changes to the capsid protein and the modulation of HEV egress from cells.[58,59] Further functional studies are required to confirm the role of these ORF2 mutations and the role of the immune responses in the pathogenesis of chronic hepatitis E.

In conclusion, a higher tacrolimus trough concentration at diagnosis of HEV infection could contribute to the early identification (i.e., before 3 months of infection) of patients at high risk of chronic hepatitis E. This would allow early intervention to prevent the evolution toward chronicity. This study also revealed that HEV genetic changes selected during infection tend to be compartmentalized between the plasma and feces. This highlights the complexity and diversity of HEV replication compartments which should be considered for future antiviral strategies.

DISCLOSURE

All the authors declared no competing interests.

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Data Availability

All sequences were deposited in the National Center for Biotechnology Information database (Accession numbers: PRJNA777584 for MiSeq data and OL364988-OL365912 for Sanger sequencing data). The data that support the findings of this study are openly available in Mendeley Data at https://doi.org/10.17632/9tfd7gthh7.2

SUPPLEMENTARY MATERIAL

Supplementary File (PDF).

Figure S1. Phylogenetic analysis of HEV clinical isolates (ORF2 region) from kidney transplant recipients.

Table S1. Primers and probes used for HEV RNA amplification.

Table S2. List of samples and sequences for the analysis of HEV diversity during chronic infection.

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