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Conflict of interest
The authors declare that they have no conflict of interest in regard to this research.
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Abstract
Aims: to determine the relative utility of *in situ* testing for hepatitis E virus (HEV) RNA and paraffin section PCR to diagnose HEV infection in paraffin-embedded clinical liver biopsies, and to correlate with clinico-pathological characteristics.
Methods and results: We evaluated *in situ* and quantitative PCR (qPCR)-based approaches to identifying HEV in clinical liver biopsies from infected patients from multiple centers, correlating with clinical setting (immunocompetent, allograft or immunosuppressed native liver) and histologic findings.
36 biopsies from 29 patients had histologic data, of which 27 and 23 biopsies had satisfactory material for *in situ* RNA testing and tissue qPCR respectively. Both approaches specifically identified HEV infection, but tissue qPCR was significantly more sensitive than *in situ* testing (P=0.035). In immunocompetent but not immunosuppressed patients the tissue qPCR yield correlated with the severity of lobular hepatitis (rho=0.94, P<0.001). qPCR viral yield was comparably high in allografts and immunosuppressed native livers and significantly greater than with native liver infection. Immunosuppressed patients showed reduced severity of hepatitis and cholestatic changes, compared with immunocompetent patients. Indeed, HEV-infected liver allografts could show minimal hepatitis for many months. In individual cases each technique was useful when serum was not available to retrospectively identify chronic infection (in biopsies taken 4-31 months before diagnosis), to identify persistent/residual infection when contemporary serum PCR was negative and to identify cleared infection.
Conclusions: qPCR is better than *in situ* RNA testing to identify HEV infection in paraffin-embedded liver biopsies and has diagnostic utility in selected settings.

Keywords
Liver, hepatitis E, allograft, biopsy, PCR, patients

Introduction

Hepatitis E virus (HEV) is the commonest cause of acute viral hepatitis worldwide and our understanding of the impact of HEV infection in developed countries has changed. Previously considered a traveller's disease acquired in endemic regions via contaminated water, locally acquired infection is now understood to be prevalent. HEV genotypes 1 and 2 are endemic in some developing regions, while genotypes 3 and 4 are established in zoonotic reservoirs from which sporadic or small cluster outbreaks of locally acquired infection originate. The predominant manifestation is self-limited subclinical or mild acute hepatitis that mimics drug-induced liver injury in 3-13% cases[1, 2]. Risk factors for severe hepatitis include pregnancy (genotype 1) or pre-

existing chronic liver disease[3, 4]. At least 5% patients present instead with neurological symptoms[5, 6]; other presentations include polyarthralgia and rash[7] or acute pancreatitis[8]. HEV infection can become chronic in immunosuppressed patients, including recipients of cancer chemotherapy or solid organ allografts [9]. Mixed HEV strain or genotype acute infections have been described[10, 11].

Pathologic studies in HEV-infected primates show a change from initial cytopathic liver injury (hepatocyte swelling, minor inflammation) to reactive hepatitis and transaminitis, which coincide with the appearance of anti-viral IgM and concomitant dwindling of serum viral RNA.[12, 13] In humans, the pathology of severe acute hepatitis E in endemic regions is described for epidemic outbreaks in Delhi 1955[14] and Kashmir 1978[15], while Morrow recorded the microscopic features from 158 liver biopsies of endemic sporadic acute hepatitis in Ghana[16]. Two broad histologic patterns were described: firstly, a classic non-specific acute hepatitis, including hepatocyte ballooning, portal inflammation, hepatocyte binucleation, Kupffer cell hyperplasia, hepatocellular and canalicular bile with highly variable degrees of necrosis; secondly, a more cholestatic acute hepatitis with dominant canalicular bile, pseudoglands and variable, sometimes rare, acidophil bodies[15, 16]. Agrawal made similar observations among 11 cases of fatal acute hepatitis E infection, finding also occasional lymphocytic cholangitis and noting that pseudoglands were more extensive among patients with higher bilirubin.[17] Morrow observed that the pathologic pattern tended to remain consistent across serial liver biopsies.[16]

Reports on the pathology of locally acquired hepatitis E naturally represent a severe subset, either because of pre-existing chronic liver disease that is reflected in the biopsy [4, 18] or intrinsically severe viral hepatitis. Again, cases fit a pattern of classic acute hepatitis[19] or cholestatic hepatitis[19-21], the latter with notable neutrophil cholangiolitis and portal neutrophils. Lymphocytic cholangitis is described[19, 21], occasionally severe[22]. Some cases show minimal or no hepatitis, with only sinusoidal lymphocytosis[18] or canalicular cholestasis in chronic liver disease as clues to a second injury[23].

Pathologic descriptions in immunosuppressed patients are less comprehensive. Most cases are genotype 3, although there are case reports of allograft infection with genotype 4 causing fulminant cholestatic hepatitis or accelerated cirrhosis[24, 25]. One report describes genotype 7 (camel) HEV infection of a liver allograft[26]. Early findings in liver allografts with accelerated fibrosis linked to chronic HEV infection included hepatocyte single cell death with no or minimal lymphocytic lobulitis, no ballooning or cholestatic features, and mild portal lymphocytic infiltration[27-30]. This evolved in some to mild-moderate lymphocytic portal, interface and lobular hepatitis with fibrosis, still without notable cholestatic features or ballooning[28-34]. Pathologic descriptions in other solid organ allograft recipients are similar[35, 36], sometimes with portal lymphoid aggregates and bile duct inflammation[37]. There are few pathologic descriptions in other immunosuppressed patients[38, 39]. Taken together, HEV infection of immunosuppressed patients can manifest only subtle or non-specific pathologic changes initially, which with mild changes to transaminases makes early clinical recognition a challenge.

We aimed to determine whether *in situ* testing for HEV RNA in routine liver biopsies was feasible and of comparable sensitivity to paraffin section PCR, to establish the relative utility of these approaches. Secondarily, we aimed to characterise examples of acute and chronic HEV, correlating the RNA findings with clinico-pathological characteristics. Molecular virological screens have not identified a viral virulence determinant for HEV, concluding that host factors are more relevant to clinical severity[40-42], so it seemed reasonable to group the cases by clinical setting.

Materials and methods

The study group comprised 36 paraffin-embedded indication liver biopsies (including one hepatectomy) from 29 HEV-infected patients. Clinical controls included indication biopsies of native liver from 4 uninfected (anti-HEV IgM and IgG seronegative) patients. The study biopsies were collected from 7 centers within the UK, Holland and France; the diagnosis was established locally on the basis of seropositivity for anti-HEV IgM and/or viraemia with serum PCR. We also included all pre-diagnosis allograft biopsies from one centre (Edinburgh). Biopsies were formalin-fixed, except one center that used Duboscq-Brasil fluid. Standard histological stains were reviewed by a pathologist (COCB) and scored semi-quantitatively (zero, minimal, mild, moderate, severe) for conventional metrics including portal, interface and lobular hepatitis, individual hepatocyte dropout (apoptosis/necrosis), specific infiltrating cell types (plasma cell, eosinophil, neutrophil), sinusoidal mononucleosis, ductular reaction, bile duct inflammation and atrophy (see supplementary methods for scoring details). The presence of confluent necrosis, large droplet steatosis (%), steatohepatitis, iron overload, cholate stasis, canalicular and ductular cholestasis were also recorded. Fibrosis was scored 0-6 as

described by Ishak et al[43]. The histologic features of two patient biopsies have been previously described by us[23]. The study has local ethics approval (REC07/S1102/21).

In situ hybridization

This was done on 4μm tissue sections using the RNAscope kit 2.0 HD (Advanced Cell Diagnostics, Hayward, CA, USA) with the recommended standard hybridization protocol on an automated stainer (Leica BondRX). Pooled oligonucleotide probes specific for Hepatitis E genotypes 1 to 4 were used (supplementary data 1).

Consecutive sections were stained for HEV, positive control (the housekeeping PPIB gene product peptidylproyl isomerase B (cyclophilin B)) and negative control (bacterial dapB (dihydrodipicolinate reductase) gene product). A positive RNA signal manifested as crisp homogeneous dark-brown dots, lacking the golden refractile quality or irregularity of iron or lipofuscin granules when those were present, or zonal distribution. RNA dots in positive cases and controls were mostly cytoplasmic although could also overlie nucleus in cells with numerous cytoplasmic dots (probably representing cytoplasm overlying nucleus or some target RNA within the nucleus). Scoring was done by 2 observers independently at a double header microscope on the same 5 microscopic fields, viewed at x400 magnification. For PPIB control evaluations, the average number of cytoplasmic RNA dots per hepatocyte was categorized 0-5 (negative, sporadic single dot, 1-4 dots, 5-9 dots, 10-14 dots, 15+). Cases averaging less than 5 cytoplasmic PPIB dots per hepatocyte were excluded from further analysis.[44] For HEV and dapB, the number of hepatocytes per field with cytoplasmic RNA dots was recorded (specifying single/multiple dots per scored cell). Hepatocyte dapB cytoplasmic staining amounted to rare cells with single dots (average 0.25 cells per x400 field), confirming low background. Purely nuclear staining in a hepatocyte was also rare and similar in prevalence between test sections and negative controls (supplementary figure 1); this was usually a single nuclear dot, likely to reflect sporadic non-specific nuclear hybridization, which increases with tissue overdigestion.

Quantitative real time tissue PCR (qPCR) for HEV RNA

Three to five 10-micron-thick serial sections were cut from each block for PCR analysis. RNA was extracted using the RNeasy FFPE kit (Qiagen, Crawley, UK) then qPCR performed on 250ng RNA with the ceeram TOOLS® Hepatitis E virus detection kit (bioMerieux, Basingstoke, UK), whose detection limit was considered to be 0.2I.U./250ng RNA. Positive HEV samples were confirmed with replicates and quantified using the WHO nucleic acid standard for HEV (Paul Erlich Institute, Langen, Germany). The integrity of RNA samples was assayed using the 18srRNA control kit (Eurogentec, Southampton, UK).

Statistics

Data was analysed and charted according to data type using Minitab 17 statistical software as indicated in the text. Contingency table comparisons between categorical variables were evaluated with the Fisher exact test. Correlations with ordered categorical data used Spearman rank-order correlation. Comparisons of means were made by analysis of variance (ANOVA) with simultaneous 95% confidence limits, using Dunnett's method for multiple comparisons with a control, or Tukey's method for pairwise comparisons of means between groups. Joint 95% confidence limits (C.L.) or $P < 0.05$ were used to indicate significance.

Results

Patients and samples

The HEV-infected cases included 23 native liver biopsies from 22 patients (8 chronic infection) and 13 allograft liver biopsies from 7 patients with chronic infection (table 1). Histologic metrics were scored for all 36 biopsies. All 23 samples with tissue qPCR data had *in situ* RNA evaluation. HEV genotyping from serum (8 patients) or biopsy (7 patients, supplementary data 1) showed genotype 3c or 3f in fourteen patients (5 immunocompetent, 8 native immunosuppressed, 1 allograft) and genotype 1 in one immunocompetent patient (case 2, travel-acquired in India).

Twelve immunocompetent patients had an indication liver biopsy related to severe acute HEV hepatitis. All 7 patients with serum PCR testing at presentation were viraemic (1-16 and 25 days pre-biopsy). Three patients had significant liver fibrosis (Ishak stage >2), attributed to alcohol, metabolic syndrome and autoimmune hepatitis respectively.

Ten heavily immunosuppressed patients had native liver biopsies (4 renal transplant, 1 heart transplant, 5 haematologic malignancy – two with stem cell transplant). Eight were chronically viraemic at biopsy (case 4, 15, 16, 17, 22, 23, 25, 33), two with mild fibrosis (Ishak stage 1-2). The other two patients (cases 5, 7) had acute hepatitis, with viraemia and IgM sero-positivity 11 days pre-biopsy. Case 5 became serum PCR-negative 8 days before biopsy, suggesting cleared infection, in a background of alcohol-attributed steatohepatitis with moderate fibrosis. Case 7 became IgG-seropositive at next testing several months later.

Nine allograft biopsies from 5 patients preceded the diagnosis of HEV infection (by median 419 days, range 103-923). However, stored contemporary serum available for 4 biopsies from 3 patients showed for each that viraemia was already present.

In situ RNA scoring and tissue qPCR testing for HEV

All formalin-fixed biopsies with tissue available for *in situ* testing had a satisfactory RNA control signal. However, Duboscq-Brasil-fixed biopsies gave no control signal despite testing different conditions, so were excluded, leaving 27 informative biopsies from 22 patients. There was no other evidence of a center effect on RNA quality (PPIB grade). There was also no correlation between an incrementally higher PPIB score and tissue qPCR yield for HEV (Spearman rho 0.047), suggesting the PPIB grade cutoff ensured adequate RNA quality to detect HEV (figure 1a).

In situ staining for HEV manifested in hepatocytes as discrete brown cytoplasmic dots (figure 1b, 1c). Infected hepatocytes tended to be randomly placed within lobules when few were apparent, with variably-sized loose clusters in cases with more prevalent positive-staining cells. Positive-stained hepatocytes were sometimes more numerous towards the periphery of the biopsy core, suggesting fixation-related effects on sensitivity. There was no consistent topographic relationship of positive-stained hepatocytes with lobular inflammation, although such areas could be identified (figure 1d). Positive-stained hepatocytes with cytoplasmic dots also showed some dots overlying the nucleus, but isolated nuclear staining of hepatocytes was rare, matching negative controls (supplementary figure 1). However, mononuclear inflammatory cells and biliary epithelium did show occasional non-specific isolated nuclear hybridization signals that were also apparent in negative controls (dapB). With this confounder, we did not identify convincing biliary epithelial cytoplasmic staining for HEV; the use of more aggressive digestion was unhelpful to increase sensitivity due to increased confounding non-specific nuclear hybridization signals within portal cells.

As the two observers differed in counts of HEV-positive hepatocytes per field by an average of only 0.88 cells/field (95% C.L. 0.096, 1.665), we represented each case by the median of the combined 10 field counts of the two observers, and separately by whether both observers agreed that at least one hepatocyte contained multiple cytoplasmic RNA dots. Alternative metrics (e.g. maximum field count) were positively correlated but did not add extra discriminatory information (data not shown).

Correlation of *in situ* HEV scores with clinical status and tissue qPCR

Figure 2a shows a boxplot of *in situ* HEV+ hepatocyte counts per biopsy according to the clinical group. The mean count per clinical group was significantly greater than uninfected controls ($p=0.001$, ANOVA with Dunnett multiple comparisons). Allografts had a higher mean than other groups ($p=0.001$), because individual biopsies more consistently scored high. Uninfected controls had consistently low counts (median 0.5, range for individual fields 0-6, always a single RNA dot per hepatocyte). Therefore we adopted a threshold of 6 (reference line on figure 2a) above which a median count across all scored fields was taken to suggest HEV infection, to confidently ensure specificity of 100%. In this way 13 of 27 (48%) study biopsies were designated HEV positive. The positive-scoring biopsies were most often allografts (8 of 9 biopsies, $p=0.02$), compared with immunosuppressed native (2 of 6) or immunocompetent patient biopsies (3 of 12). The highest scoring cases were distributed among all 3 groups and included the genotype 1 infection (case 2). If, alternatively, we selected as positive those cases where both observers agreed there was at least one hepatocyte with multiple cytoplasmic RNA dots, then all control cases were negative and 13 cases (48%) were again positive, including 9 also selected using the median count threshold above (figure 2a). Therefore we combined the two approaches, such that cases with inadequate median count were designated positive if agreed to have at least one hepatocyte with multiple dots. This improved sensitivity to 17 of 27 cases (63%) positive without compromising specificity.

Comparison of the *in situ* HEV scoring with the tissue qPCR was possible for 23 study biopsies from 19 patients (figure 2b). As expected, HEV was detected in the study groups but not uninfected controls (100% specificity). The high qPCR HEV yields from allografts and immunosuppressed native livers were significantly greater than from immunocompetent patients but not significantly different from each other (Tukey simultaneous 95%C.L.).

Tissue qPCR was more sensitive than *in situ* counting to detect HEV (21/23 (91%) versus 14/23 (61%) with combined median/multidot count (p=0.035, Fisher exact test), or 11/23 (48%) biopsies with either median or multidot count alone (p=0.0031)). The reduced sensitivity of *in situ* counting was clear for immunocompetent patients (2/10 versus 8/10, P=0.023, Fisher exact test) but not for allografts (7/8 versus 8/8, P=1); immunosuppressed native liver numbers were too small for a confident conclusion (2/5 versus 5/5, P=0.16). Reviewing the two qPCR-negative biopsies, one (case 21) was acute hepatitis biopsied 25 days after diagnosis with viraemia, and likely reflected clearance of infection. The other (case 24) was severe acute hepatitis in which the biopsy showed collapse and ductular reaction with only rare surviving hepatocytes, so may have reflected a sampling deficit for hepatocytes or immune clearance. The hepatectomy with a very low HEV yield (sample 6b), also had multiple hepatocyte RNA dots in some cells on *in situ* testing but a low median count. This patient had cleared viraemia on serum retesting, one month after diagnosis and biopsy (sample 6a), so the low yield might reflect almost cleared infection. By contrast, another patient (case 5) showed HEV with both *in situ* and qPCR analysis, despite having cleared viraemia on serum testing 6 days earlier. This patient had chronic leukaemia, so was significantly immunosuppressed and still harboring HEV within the liver, although not viraemic on point testing of serum.

All 7 allograft biopsies (4 patients) taken before diagnosis of HEV infection were HEV-positive with at least one tissue test, including all 6 tested with qPCR (figure 2a and 2b). The biopsy without material for qPCR (27a), had a positive *in situ* score similar to the subsequent qPCR-positive biopsy (27b). The biopsy scoring negative with *in situ* testing (8a) had similarly high qPCR yields to the other cases, including a follow-up biopsy (8b) with a positive *in situ* count. Thus tissue RNA analysis showed that all 4 of these patients carried chronic HEV infection many months (4, 14, 14, 31 months) before diagnosis. These findings were corroborated with retrospective serum testing in the 2 patients with stored serum.

Correlation with histologic analysis

While not a cohort, the different clinical settings merit description of the range of histologic appearances (see also supplementary figures 2-4). Immunocompetent patients biopsied during acute HEV infection typically showed moderate/severe interface and lobular lymphocytic hepatitis with some admixed portal and lobular plasma cells (supplementary figure 2), often with mild biliary features, including ductular reaction and duct epithelial reactivity, but no more than minimal duct inflammation or portal oedema, and without periduct oedema. Canalicular cholestasis was common (9 of 13 biopsies; moderate/severe in 6), including all 4 patients with significant chronic liver disease (Ishak fibrosis >2). Indeed, cholestasis was the only prominent manifestation in one patient with pre-existing chronic steatohepatitis. The biopsies after recently cleared infection (21) and almost cleared infection (6b) also showed canalicular cholestasis (not present in an earlier biopsy (6a)), with hepatocyte dropout and portal, but not lobular, hepatitis. The immunocompetent patient biopsies had a strong positive correlation between lobular hepatitis severity and the tissue qPCR yield for HEV (Spearman rho 0.936, P<0.001). This correlation was absent for immunosuppressed patients, whose biopsies almost all had high HEV qPCR yields (figure 2b). Contemporary serum alanine aminotransaminase (ALT) values in immunocompetent (but not transplanted) patients also correlated significantly with lobular hepatitis severity (Spearman rho 0.771, P=0.015) and had a marginally significant correlation with the *in situ* HEV count (Spearman rho 0.643, P=0.08); however, correlation with tissue qPCR yield was not significant (Pearson correlation coefficient 0.51, P=0.29).

Lobular hepatitis was usually more severe in immunocompetent patients compared with others (moderate-severe in 8/13 (62%) versus 4/23 (17%), P=0.011, Fisher exact test). Canalicular cholestasis was also common in HEV-infected immunocompetent patients but was not a feature of allograft HEV infection (9/13 vs 0/13; P=0.013, Fisher exact test) (table 2/figure 3). While allografts at diagnosis of HEV infection usually showed prominent portal hepatitis (moderate or severe in 3 of 4), allografts biopsied before HEV was diagnosed usually showed minimal or mild portal hepatitis, no or minimal interface and lobular hepatitis, only minimal hepatocyte dropout, no prominence of plasma cells or cholestatic features and usually minimal or mild sinusoidal mononuclear cell infiltration (figure 3, table 2, supplementary figure 4).

Lobular plasma cells were associated with moderate-severe lobular hepatitis in acute HEV infection (8 of 10 biopsies), and perhaps as a result were apparent in native more often than allograft biopsies (9/23 versus 1/13, P=0.059 Fisher's exact test). Portal bile duct changes (epithelial reactivity, inflammation) and other infiltrating cell types (eosinophils, neutrophils) showed no particular correlation.

Discussion

The present results show that *in situ* RNA staining is less sensitive than qPCR to detect HEV in routine clinical liver biopsies. Nevertheless, *in situ* staining specifically identified HEV, as determined by comparisons against the tissue qPCR, contemporary serum PCR and uninfected control biopsies. *In situ* testing can identify virus when retrospective serum testing is not possible (as for 2 liver transplant patients here), or if there is insufficient tissue for PCR (as with one pre-diagnosis allograft biopsy). This has particular value in allografts to establish the timelines of chronic infection for research or clinical purposes.

The occurrence of HEV RNA-staining in hepatocyte cytoplasm, either in scattered single cells or sometimes in loose clusters but otherwise without a particular pattern agrees with observations in experimentally infected primates[45, 46] and clinical biopsies[47-49] using immunolabelling or older *in situ* RNA detection methods. While those and the present study did not identify convincing non-hepatocellular HEV staining, the virus is excreted in bile and some animal studies have suggested viral replication in biliary epithelium[45], while virus-like particles have been described in ductular-lining cells in one patient[50].

It is not clear why the *in situ* counts did not correlate better with the tissue qPCR yield for virus, even allowing that the PCR sample was several-fold larger than a 5 micron tissue section. Stronger digestion protocols did not increase *in situ* scores. The RNAscope probe-set was designed to detect HEV genotypes 1-4, and did identify both genotype 1 and 3 here; moreover, successive allograft biopsies from one patient (8a, 8b) had similar high qPCR yields, yet only one scored positive with *in situ* median counting, suggesting the issue is not wholly with genotype or subtype detection. We cannot exclude that some variable aspect of fixation (delay, duration) is prejudicing viral RNA detection by the *in situ* probe, even though the positive control PPIB probe counts and the qPCR method did not seem affected. Nevertheless, routine formalin-fixed paraffin-embedded clinical samples from different centers provided positive staining, suggesting that such samples are usually adequate for *in situ* detection, although importantly, other fixatives such as Duboscq-Brasil fluid here, can be unsuitable for both PCR and *in situ* evaluation.

In addition to the present data, PCR of archival liver biopsies has recently been shown to identify unrecognized HEV infection of small numbers of patients previously categorized as hepatitis of unknown origin[19], drug-induced liver injury[51] and among archival liver transplant biopsies[30]. Taking all factors into account, tissue PCR testing is robust, more sensitive, easier and cheaper than *in situ* RNA testing when sufficient tissue is available. The present correlation of tissue viral load (qPCR) with the degree of lobular hepatitis in immunocompetent patients, and in turn the correlation of lobular hepatitis with serum ALT, was perhaps intuitive, but affirms that histologic activity directly reflects the underlying disease biology and immune response[12]. The data also highlight how this correlation is unlocked with immunosuppression, where tissue viral loads were extreme and hepatitis subdued. Clearance of viraemia did not here necessarily imply clearance of infection: the present identification of viral RNA in the liver by both tests in one immunosuppressed patient when contemporary point testing of serum was negative, re-emphasizes the need for follow-up testing where the clinical setting suggests a potential for prolonged infection, particularly as serologic testing is insensitive in such patients. Protzer also noted 2 aviraemic liver allograft patients with contemporary HEV-RNA positive liver biopsies, although both appeared biochemically to eventually clear the infection[30]. In typical self-limited infection of immunocompetent patients, HEV is detectable in stool for 2-4 weeks after viraemia subsides[52], presumably reflecting dwindling hepatic viral release into bile, in which the temporary discrepancy with serum testing does not usually have a longer term significance. However, HEV recurrence after apparent clearance and seroconversion is also documented months after leukaemia chemotherapy (genotype 4)[53] or stem cell transplantation (genotype 3)[54] and we have encountered a similar case. Such examples indicate that aviraemic low level hepatic persistence of HEV is possible in certain clinical settings linked to immunosuppression; while extrahepatic reservoirs are possible, liver is the intuitive harbor, as observed here.

The present histological findings differed between clinical groups of HEV infection, although all but one patient genotyped had type 3 infection. Firstly, a cholestatic pattern of hepatitis was relatively common in severe

acute hepatitis E subject to biopsy, as previously reported[15-17, 19-21]. In 3 different patients here, cholestasis was also, respectively, the dominant manifestation (in prior chronic liver disease) or developed during HEV clearance, or persisted shortly after viral clearance. Pre-existing chronic liver disease was also common, in agreement with indication biopsy-based series of locally-acquired acute infection[4, 18]. Cholestatic features were less common in immunosuppressed patients, agreeing with the lack of mention of this feature in previous descriptions, and presumably reflecting the muted inflammatory disruption of liver cyto-architecture and function, together with an acquisition bias compared with immunocompetent patients, in whom such biochemically mild disease would not trigger a biopsy. Indeed, the histologic findings attributable to HEV in liver allografts with persistent infection could be minimal for many months here, as also recently found by Protzer and colleagues among their 4 cases[30]. In such a setting, where the histology is not specific and potentially falsely reassuring for 'significant' hepatitis, it may be for the pathologist to articulate the need to exclude HEV infection with specific serum PCR testing. The present allograft cases were biased towards early infection by the inclusion of pre-diagnosis biopsies. However, progressive fibrosis is well documented with chronic HEV infection of liver allografts and in other severely immunosuppressed patients[24, 28, 32-34, 37, 39].

In conclusion, testing of clinical biopsy material can directly identify HEV infection. This is useful for archival material when stored serum is unavailable, including for research purposes, retrospective diagnosis, determining chronic infection timelines and potentially to more sensitively identify persistent low-level infection under immunosuppression. Tissue qPCR can rapidly provide in-house diagnosis of HEV infection in difficult cases on current or past biopsies where pathologic or clinical suspicion is aroused.

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COCB designed the study, identified cases, performed the pathologic and in situ staining reviews, analysed the data and wrote the paper
SP designed and managed the study, performed the in situ staining reviews and analysed the data
LS and CLC performed the qPCR and the genotyping on tissue sections and analysed the data
CD analysed and quantified the RNAscope staining
HRD, RADM, NK, JS identified cases and samples, contributed to study design and reviewed the manuscript.

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Table 1. Numbers of study biopsies in different clinical groups of hepatitis E infection

Group	Hepatitis histology	*In situ* RNA	Tissue qPCR
Allograft liver, pre-HEV diagnosis	9 (5)	7 (4)	6 (4)
Allograft liver, index biopsy	4 (4)	2 (2)	2 (2)
Native liver, immunocompetent	13 (12)	12 (11)	10 (9)
Native liver, immunosuppressed	10 (10)	6 (6)	5 (5)
Total study group biopsies*:	**36 (29*)**	**27 (22*)**	**23 (19*)**

The number of biopsies with available tissue evaluated by each modality is shown, with the number of patients indicated in parentheses. In all, five patients (4 with liver allografts) had multiple (2 or 3) biopsies.
***** Some patients are represented in both pre- and index biopsy groups, so the column totals differ from the summed column cells.

Table 2. Prevalence of selected histologic features in the HEV-infected study groups

Histologic feature (>minimal degree unless specified)	Native liver, immunocompetent n=13; (%)	Native liver, immunosuppressed n=10; (%)	Allograft liver; n=4; (%)	Allograft liver pre-diagnosis n=9; (%)
Lobular hepatitis	10 (77)	7 (70)	3(75)	1 (11)
Portal hepatitis	10 (77)	7 (70)	4(100)	5 (55)
Confluent necrosis (any)	5 (38)	1(10)	1 (25)	0
Hepatocyte dropout	11 (85)	9(90)	3 (75)	1 (11)
Lobular plasma cells	4 (31)	0	0	0
Portal plasma cells	4 (31)	2 (20)	0	0
Canalicular cholestasis	9 (69)	1 (10)	0	0
Ductular reaction	7 (54)	3 (30)	1 (25)	1 (11)
Bile duct inflammation	2 (15)	2 (33)	1 (25)	0
Fibrosis (>mild)	4 (31)	1 (17)	0	0

The table shows the occurrence of different histologic features according to the clinical group. Each cell shows the number of biopsies with the feature, with the corresponding % in that clinical group in parenthesis.

Figure Legends

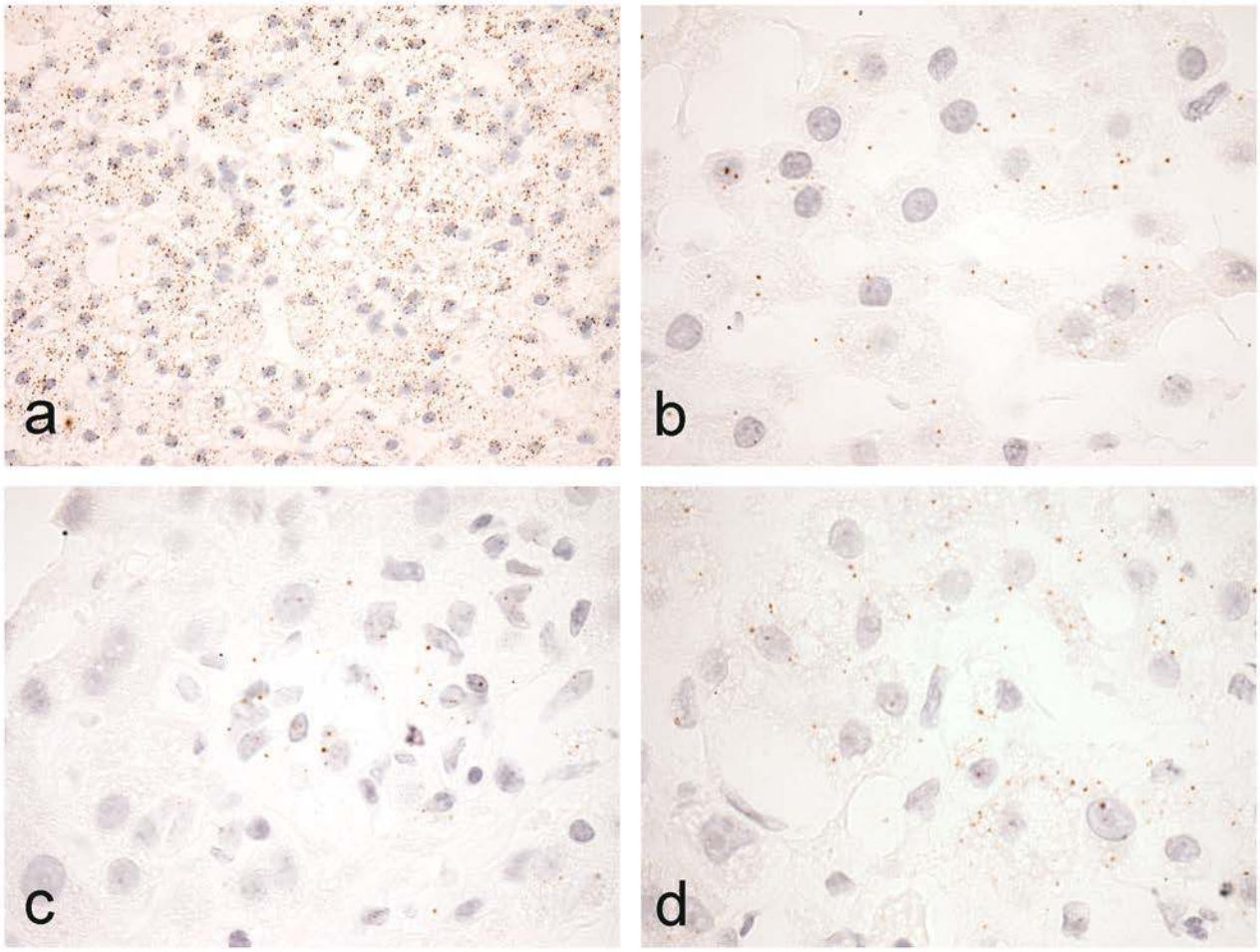
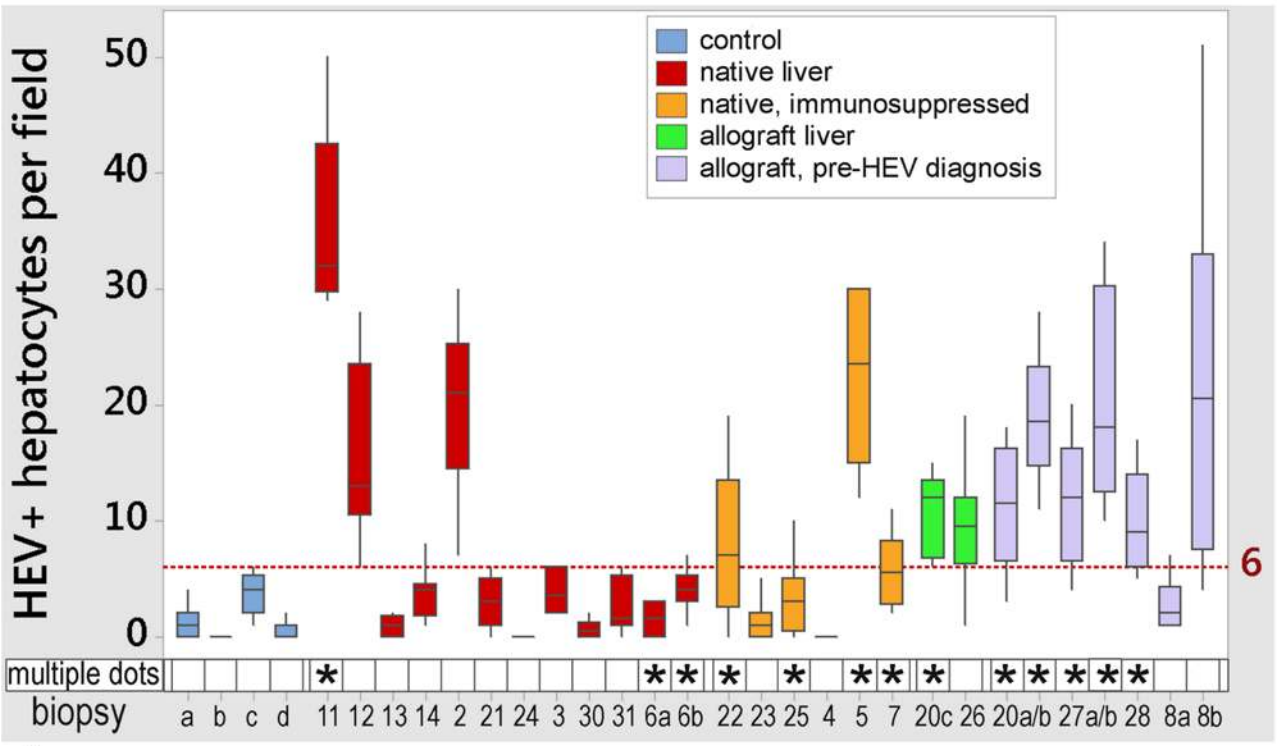
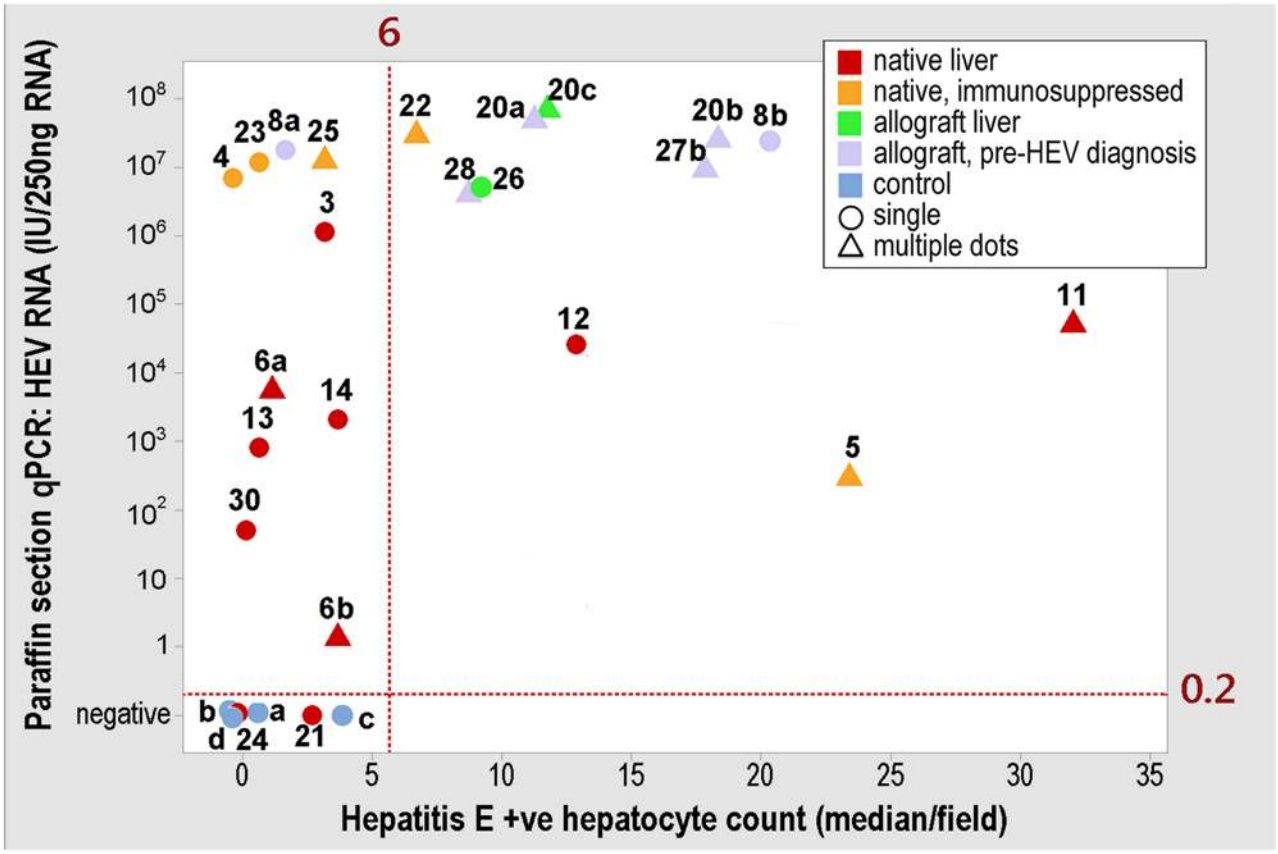


Figure 1. *In situ* hybridisation
a. Positive control probe (PPIB) for RNA, showing abundant cytoplasmic granular positive staining; occasional dots also overlie the nucleus.
b&c. HEV RNA probe, in a native liver biopsy from an immunosuppressed patient (relapsed lymphoma) with acute travel-acquired genotype 3 HEV who tested viraemic and IgM+ 11 days before the biopsy.
b: positive HEV RNA staining (brown dots) in most hepatocytes, with no associated inflammatory infiltrate. HEV RNA staining is predominantly cytoplasmic, although where hepatocytes have multiple dots some can overlie nucleus.
c: positive HEV RNA staining in some cells, topographically associated with a focus of lobulitis.
d: HEV RNA probe staining in many hepatocytes, without associated inflammation, in a native liver biopsy from a HEV-infected patient tested viraemic and seropositive (IgM, IgG) 16 days before this biopsy.



A



B

Figure 2: *in situ* HEV detection and correlation with qPCR in liver biopsy tissue

a. Boxplot of *in situ* HEV+ hepatocyte counts per biopsy, by clinical group. Boxes indicate interquartile range and are coloured by clinical grouping as specified. The dotted reference line bounds the negative control cases (HEV-naïve cases a-d) at the highest *single field* score. Other cases with a *median* score greater than this threshold were considered to show an RNA signal of HEV infection. Cases with multiple-dotted hepatocytes are indicated with an asterisk in the box on the x-axis, an alternative criterion for HEV-positivity (see text) Clinical group abbreviations: "control", HEV-naïve patient (patients labelled a-d); "native", acute HEV infection in immunocompetent patients; "native-immunosuppressed", HEV-infected native liver in immunosuppressed patients; "allograft", HEV-infected liver allograft; "allograft, pre-diagnosis", liver allograft biopsies preceding formal diagnosis of chronic HEV. The "biopsy" x-axis labels indicate the study patient number; any alphabetized suffix indicates one of multiple biopsies, in chronologic order.

b. Scatterplot of median HEV+ hepatocyte count and paraffin section qPCR yield for HEV, per biopsy. Triangular symbols indicate the cases with multiple-dotted hepatocytes. Symbol colours indicate the clinical grouping (as part a, above); datapoint labels indicate study patient number, an alphabetized suffix indicates one of multiple biopsies, in chronologic order. The 2 dotted reference lines bound the HEV-negative cases by PCR (y-axis) and median *in situ* hepatocyte count (x-axis).

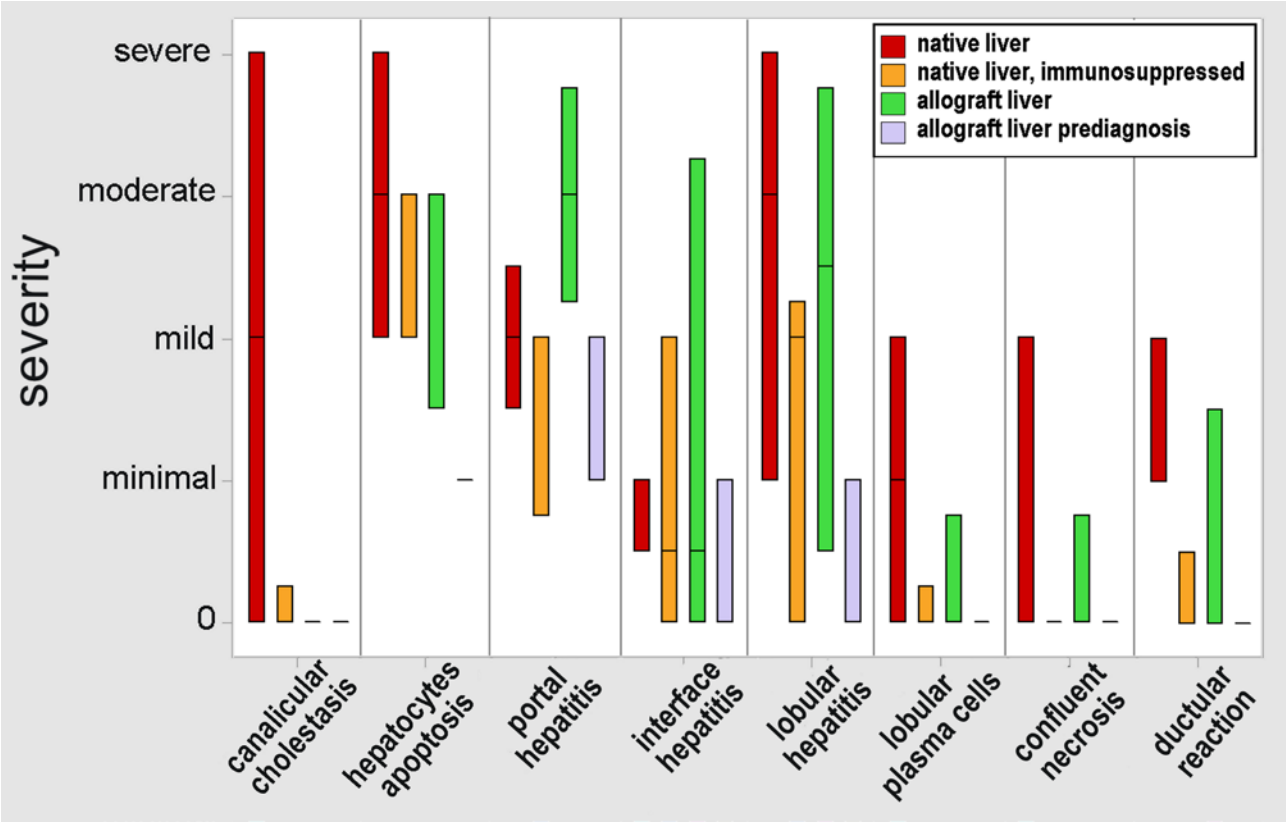
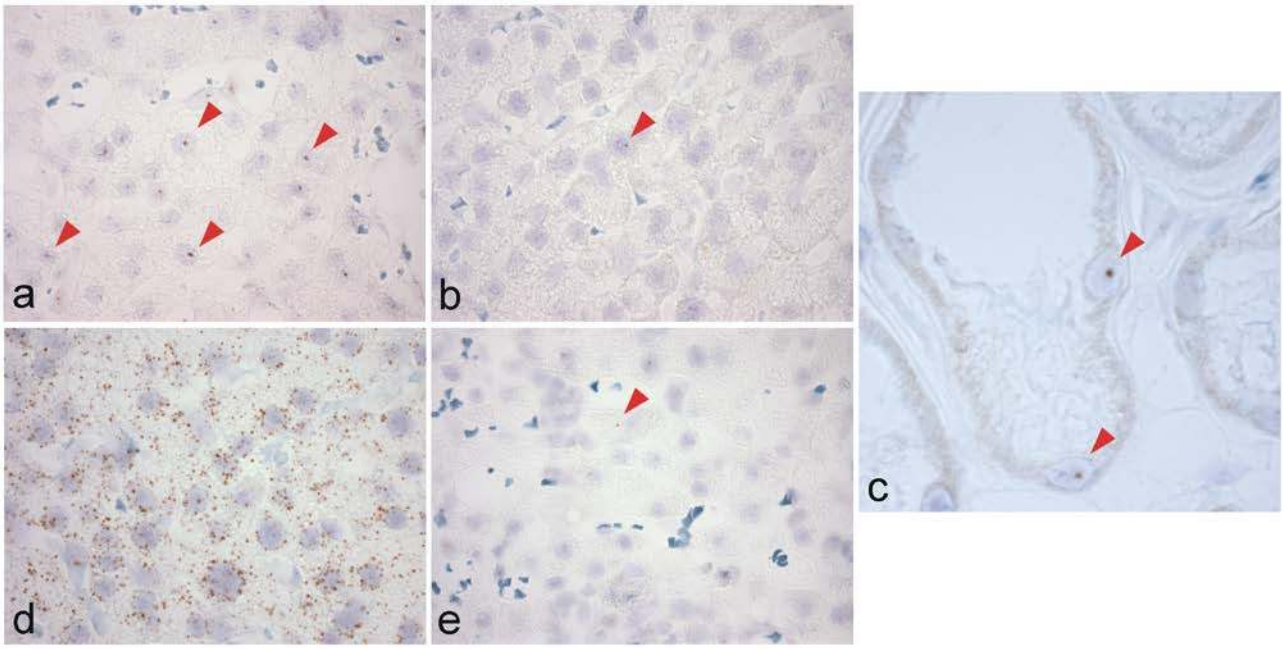
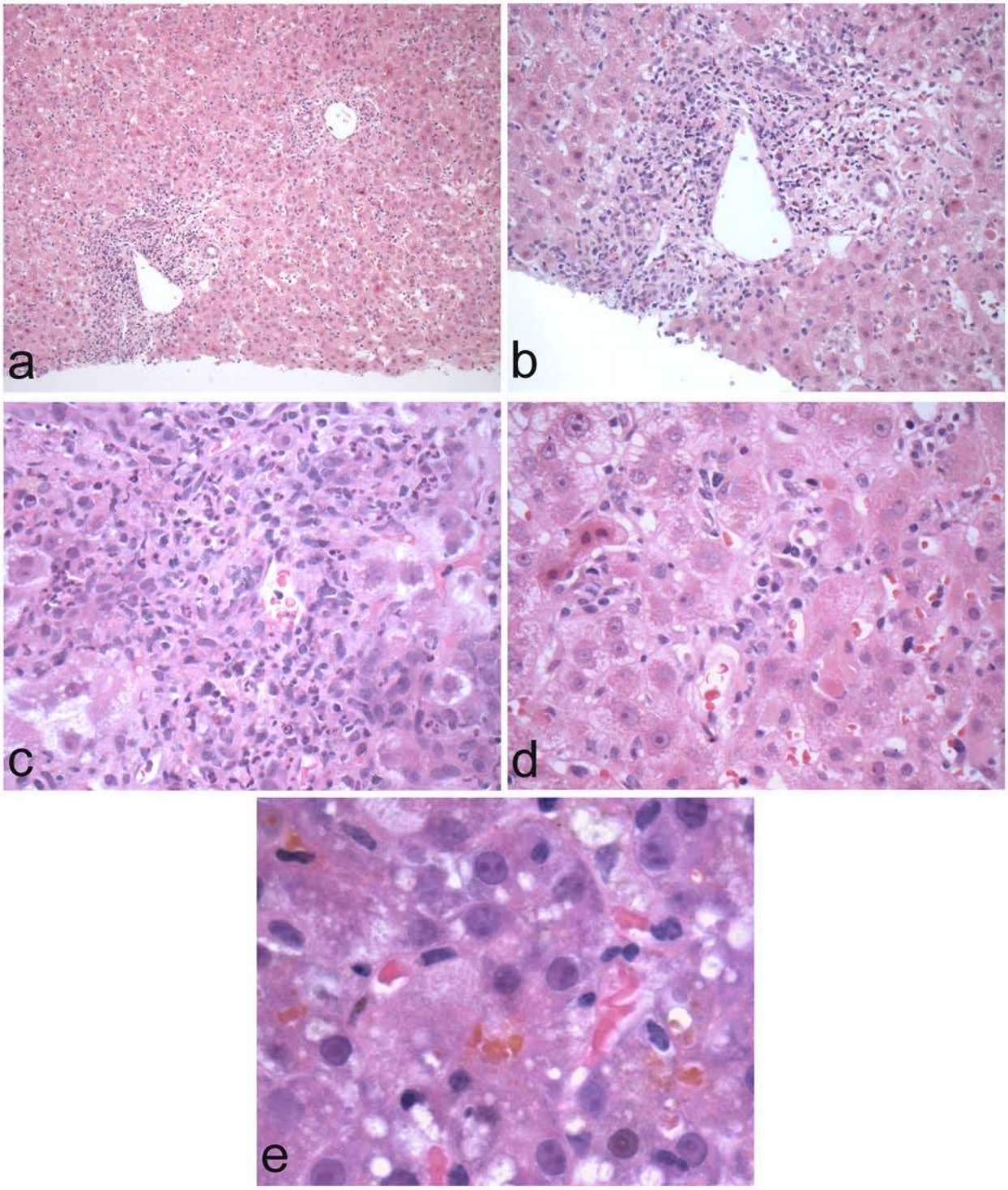


Figure 3. Severity of histologic features in the HEV-infected study groups
The boxplot shows the median and interquartile severity scores for selected histologic variables, according to the clinical grouping of the case (as described for figure 2).

Supplementary Information Description
Supplementary Figure 1. *in situ* RNA staining
Supplementary Figure 2. Histological features of acute HEV infection in native liver without immunosuppression.
Supplementary Figure 3 . Histological features of HEV-infected native liver under immunosuppression.
Supplementary Figure 4. Histological features of HEV-infected allograft liver.
Supplementary table 1. Case details
Supplementary table 2. Histological feature scores
Supplementary table 3. Methods for histological scoring
Supplementary Data 1. RNAscope probe design and genotyping



Supplementary Figure 1. *in situ* RNA staining
The figure compares non-specific nuclear hybridisation in different settings (a,b,c) with strong positive staining (d).
a. Shows extensive non-specific hepatocyte nuclear hybridisation (a dot in multiple nuclei, some indicated with arrowheads) due to severe overdigestion (dapB negative control probe). No cytoplasmic dot.
b. Rare non-specific nuclear hybridisation signal (arrowhead) in a properly digested section (dapB, negative control probe). No cytoplasmic dot.
c. Two non-specific nuclear hybridisation dots in Kidney tubular cells (HEV RNA probe on a kidney needle biopsy, negative tissue control). No cytoplasmic dot.
d. Positive staining in hepatocytes: numerous cytoplasmic dot signals, with occasional dots overlying nucleus in strongly positive cells (PPIB positive RNA control probe).
e. HEV probe. Single hepatocyte cytoplasmic dot in a negative control case



Supplementary Figure 2. Histological features of acute HEV infection in native liver without immunosuppression.

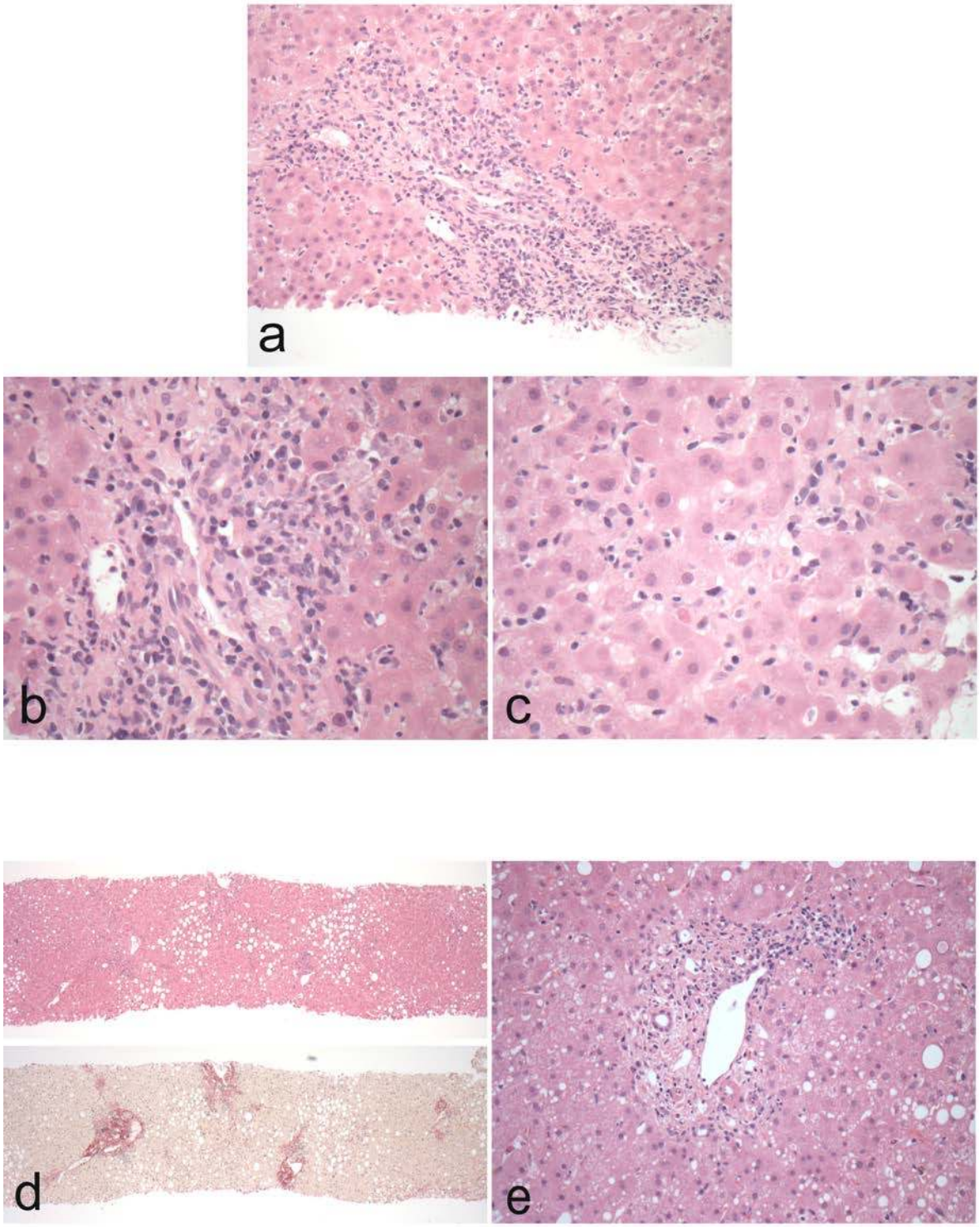
a. Mild expansion of portal tracts with a predominantly mononuclear cell infiltrate, with increased mononuclear inflammatory cells in sinusoids and scattered hepatocyte cell death (H&E)

b. Same case as (a) showing more detail of inflamed portal tract, including oedema, interface inflammation but only mild ductular reaction (H&E).

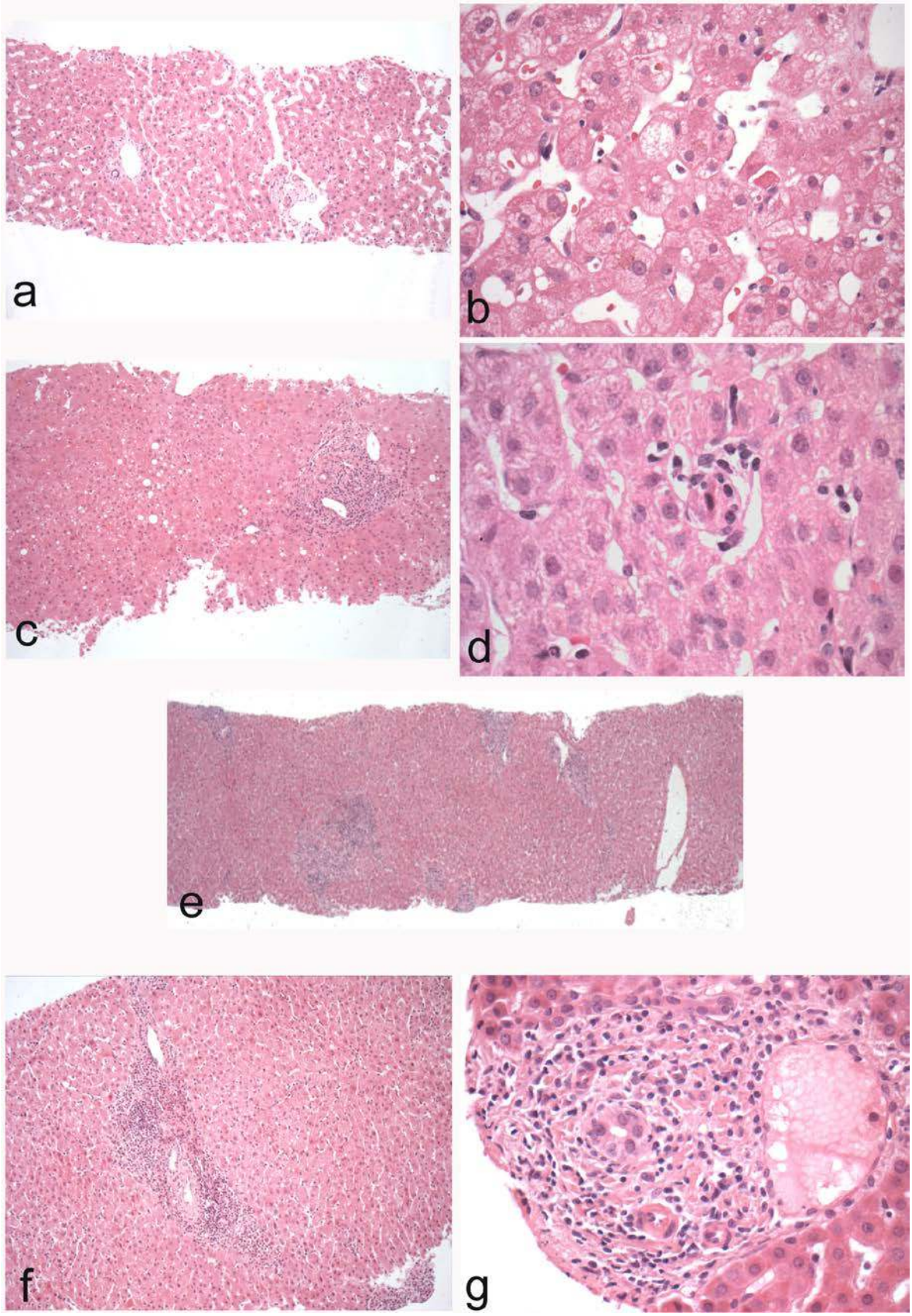
c. Different case, showing more pronounced ductular reaction with numerous neutrophils (H&E).

d. Marked lobular inflammation with hepatocyte cell death, singly and in a small cluster (H&E).

e. Pronounced canalicular and cytoplasmic cholestasis (H&E).



Supplementary Figure 3 . Histological features of HEV-infected native liver under immunosuppression. a-c. Acute HEV infection. (a) shows portal expansion with mononuclear cell infiltrate, (b) higher power of (a), illustrating the lymphoplasmacytic infiltrate flooding periportal inlet venules and sinusoids. (c) illustrates increased sinusoidal mononuclear cells, Kupffer cell hypertrophy, occasional hepatocyte single cell death. d-e. Chronic HEV infection, after stem cell transplantation. (d) shows H&E and picrosirius red stains of the same area of the biopsy core, illustrating periportal fibrous expansion with fibrous spurring and macrosteatosis. (e) same case as (d), showing a sparse portal mononuclear cell infiltrate toward one end of the tract spilling into adjacent parenchyma (H&E).



Supplementary Figure 4. Histological features of HEV-infected allograft liver.
a-b. Pre-diagnosis biopsy (qPCR and in situ positive), (a) low magnification view, showing no apparent inflammation or obvious architectural change (H&E). (b) higher magnification to show occasional hepatocyte apoptosis without parenchymal inflammation (H&E).
c-d. (same patient, 1 year later) (c) shows focal portal hepatitis and sparse macrosteatosis without appreciable lobular inflammation. (d) illustrates a focus of lymphocytic lobulitis.
e-g. (different patient), (e) illustrates widespread inflammatory portal expansion, seen at higher magnification in (f), within a tract having fibrous expansion, and in (g), associated with focal lymphocytic cholangitis but no venulitis.

Supplementary table 1. Case details

clinical group	case	age	sex	biopsy indication	comorbidities	liver transplant indication	months after liver transplant	biopsy type	biopsy length mm (section for in situ RNA)	portal tracts (section for in situ RNA)
native	1	68	f	acute hepatitis, alcohol, diabetes, foreign travel	Diabetes, alcohol	*	*	bx	14	11
native	2	26	m	acute hepatitis after foreign travel		*	*	TJ	24	20
native	3	56	f	acute hepatitis/jaundice		*	*	bx	15	13
native	11	43	f	acute hepatitis		*	*	bx	12	10
native	12	48	f	acute hepatitis/jaundice, alcohol	Alcohol	*	*	bx	15	10
native	13	63	m	acute hepatitis/jaundice		*	*	bx	10	5
native	14	81	m	acute hepatitis/jaundice, fatty liver on US, possible biliary dilation		*	*	bx	8	3
native	21	43	f	acute hepatitis after foreign travel		*	*	bx	24	21
native	24	53	f	acute hepatitis, autoimmune hepatitis	Autoimmune hepatitis	*	*	bx	31	33
native	30	32	f	acute hepatitis, Graves disease	Graves disease	*	*	bx	14	16
native	31	64	m	acute hepatitis, high ferritin, alcohol, possible biliary dilation	Alcohol	*	*	bx	25	28
native	6a	67	f	rising LFT not responding to steroids, autoimmune hepatitis, cirrhosis	Autoimmune hepatitis cirrhosis	*	*	TJ	7	6
native	6b	67	f	hepatectomy	Autoimmune hepatitis cirrhosis	HEV/AIH cirrhosis	*	hepatectomy	*	*
native - is	4	61	m	screening abnormal LFT pre-bone marrow transplant	Non-Hodgkin lymphoma	*	*	bx	29	27
native - is	5	72	m	acute hepatitis/jaundice, chronic lymphocytic leukaemia, alcohol	Chronic lymphocytic leukaemia, alcohol	*	*	TJ	19	12
native - is	7	61	m	abnormal LFT, Hodgkin disease	Hodgkin disease	*	*	bx	17	14
native - is	15	62	f	Acute hepatitis, renal allograft	Renal allograft	*	*	bx	14	13

native - is	16	60	m	Acute hepatitis, renal allograft	Renal allograft	*	*	bx	27	22
native - is	17	54	f	Acute hepatitis, renal allograft	Renal allograft	*	*	bx	12	11
native - is	22	56	f	persistent abnormal LFT, stem cell transplant	Stem cell transplant	*	*	bx	16	19
native - is	23	60	m	abnormal LFT, stem cell transplant	Stem cell transplant	*	*	bx	19	14
native - is	25	50	m	abnormal LFT, right heart failure, cardiac allograft	Cardiac allograft	*	*	bx	20	13
native - is	33	39	m	persistent abnormal LFT, renal allograft	Renal allograft	*	*	bx	25	39
allograft	18	67	m	Acute hepatitis, liver allograft	Liver allograft	PSC	95	bx	26	18
allograft	26	53	f	abnormal LFT, liver allograft	Liver allograft	PSC, Crohn	45	bx	25	31
allograft	20c	56	m	abnormal LFT, liver allograft	Liver allograft	ALD	18	bx	18	25
allograft	32c	64	m	persistent abnormal LFT, liver allograft	Liver allograft, diabetes	ALD/NAFLD	27	bx	23	23
allograft-pre	28	54	f	rising ALT, liver allograft	Liver allograft	HCV	10	bx	17	13
allograft-pre	20a	55	m	abnormal LFT, itch, liver allograft	Liver allograft	ALD	5	bx	14	19
allograft-pre	20b	56	m	worsening LFT, liver allograft	Liver allograft	ALD	9	bx	15	11
allograft-pre	27a	32	m	rising ALT, liver allograft	Liver allograft	ALD/NAFLD	6	bx	29	21
allograft-pre	27b	33	m	abnormal LFT, alcohol, liver allograft	Liver allograft	ALD/NAFLD	15	bx	21	24
allograft-pre	32a	62	m	rising ALT, diabetes, liver allograft	Liver allograft, diabetes	ALD/NAFLD	4.5	bx	28	23
allograft-pre	32b	62	m	persistent abnormal LFT after steroid trial, liver allograft	Liver allograft, diabetes	ALD/NAFLD	8	bx	41	41
allograft-pre	8a	66	f	abnormal LFT and creatinine, liver & renal allograft	Liver/Kidney allografts	ADPKD	8	bx	28	16
allograft-pre	8b	68	f	rising ALT, liver & renal allograft	Liver/Kidney allografts	ADPKD	28	bx	18	17

Supplementary table 2. Histological feature scores

		hepatitis metrics					fibrosis (1-6)	fat %	steato-hepatitis (y/n)	portal changes						cholestatic reaction					lobular inflammatory cells			
Clinical group	case	portal	interface	lobular	confluent necrosis	dropout				plasma cells	eos.	neutro	oedema	biliary atrophy	duct inflam-mation	ductular reaction	cholestasis				sinusoidal mononu-cleosis (0-4)	plasma cells	eos	neutro
																	ductular	canalicular	chronic y/n	cytoplasmic y/n				
native	1	1	1	0	0	2	5	20	1	0	0	2	2	0	0	2	1	4	0	1	0	0	0	3
native	2	3	1	4	2	4	2	0	0	1	2	3	1	0	1	3	0	3	0	1	2	1	0	1
native	3	2	1	4	0	4	0	0	0	2	1	2	1	1	1	2	0	1	0	1	2	2	0	0
native	11	2	1	4	2	4	0	1	0	2	0	0	0	0	0	2	0	4	0	1	3	2	0	1
native	12	2	1	4	0	3	0	0	0	2	0	2	2	2	2	2	0	4	0	1	2	2	0	1
native	13	1	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	0	0	0
native	14	2	2	3	0	2	0	2	0	1	0	0	0	1	0	1	0	0	0	1	3	1	0	0
native	21	0	0	0	0	2	0	0	0	0	0	1	1	0	1	0	0	2	0	1	0	0	0	0
native	24	2	1	4	6	4	0	0	0	1	3	0	0	0	2	4	0	0	0	1	4	1	3	2
native	30	2	0	2	0	1	0	0	0	0	0	0	0	0	1	0	0	2	0	1	2	0	0	0
native	31	3	1	4	2	3	3	0	0	2	1	2	1	1	1	2	1	3	0	1	2	2	0	1
native	6a	2	0	3	0	3	6	0	0	1	0	0	0	0	0	2	0	0	0	0	2	0	0	0
native	6b	3	1	0	3	2	6	0	0	0	0	0	0	2	0	2	3	4	1	1	0	0	0	0
native - is	4	2	1	3	0	3	0	60	0	1	0	1	2	2	2	2	0	0	0	0	2	1	1	1
native - is	5	0	0	0	0	2	4	10	1	0	0	2	1	1	0	3	0	4	0	1	0	0	0	3
native - is	7	2	2	4	0	3	0	10	0	2	2	1	1	2	1	1	0	1	0	0	3	1	0	0
native - is	15	2	0	2	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
native - is	16	2	0	2	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
native - is	17	2	2	2	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0
native - is	22	3	3	2	0	3	2	0	0	2	1	0	0	1	2	2	0	0	0	0	2	0	0	0
native - is	23	2	2	2	0	2	0	20	0	0	0	0	0	2	0	0	0	0	0	0	2	0	0	0
native - is	25	0	0	0	0	2	0	20	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1
native - is	33	1	0	0	0	2	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
allograft	18	2	0	0	0	1	0	0	0	1	0	0	0	2	1	2	0	0	0	0	2	0	0	0
allograft	26	3	0	3	1	3	0	0	0	0	2	2	1	1	2	1	0	0	0	0	3	0	1	0
allograft	20c	4	4	4	0	3	1	20	0	1	2	1	1	1	0	0	0	0	0	0	1	0	1	0
allograft	32c	3	1	2	0	3	1	0	0	1	1	2	1	0	1	1	0	0	0	0	3	1	0	0
allograft-pre	28	1	0	0	0	1	0	30	0	0	0	0	0	0	0	1	0	0	0	0	2	0	0	0
allograft-pre	20a	1	0	0	0	1	0	0	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0
allograft-pre	20b	2	2	1	0	1	0	20	0	0	1	2	1	0	1	2	0	0	0	0	3	0	0	0
allograft-pre	27a	2	0	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0
allograft-pre	27b	2	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
allograft-pre	32a	1	0	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0
allograft-pre	32b	2	0	1	0	2	0	0	0	0	0	0	0	0	1	0	0	0	0	0	2	0	0	1
allograft-pre	8a	0	0	0	0	1	0	2	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
allograft-pre	8b	2	1	1	0	1	1	5	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0

Supplementary table 3: methods for histological scoring.

Hepatitis feature scoring was adapted from Ishak et al[43], specifying 'minimal' to take account of anticipated slight changes in immunosuppressed patients. Confluent necrosis was scored exactly as per Ishak et al[43]. These and other features scored are enumerated below.

	Portal hepatitis
Minimal	marginally increased mononuclear cell infiltrate in a few portal areas
Mild	easily appreciable mononuclear cell infiltrate, either expansile in a few portal areas or non-expansile in most portal areas
Moderate	expansile/dense portal mononuclear cell infiltrate in most portal areas
Severe	dense, expansile portal mononuclear cell infiltrate in all portal areas

	Interface hepatitis
Minimal	scanty interface hepatitis in only 1-2 portal areas
Mild	focal in < 50% portal areas
Moderate	at least focal in most portal areas (or affecting most of the circumference in some)
Severe	affecting most of the circumference of most portal areas

	Lobular hepatitis; also for Hepatocyte dropout (apoptosis, single cell necrosis)
Minimal	~1 focus per lobule
Mild	2-4 foci per lobule
Moderate	5-10 foci per lobule
Severe	>10 foci per lobule

	Specific inflammatory cell types (in lobule or portal area), Canalicular cholestasis
Minimal	sparse in some (lobules/portal tracts)
Mild	Sparse-occasional in most (lobules/portal tracts)
Moderate	prominent in some (lobules/portal tracts), otherwise occasional
Severe	prominent in most (lobules/portal tracts)

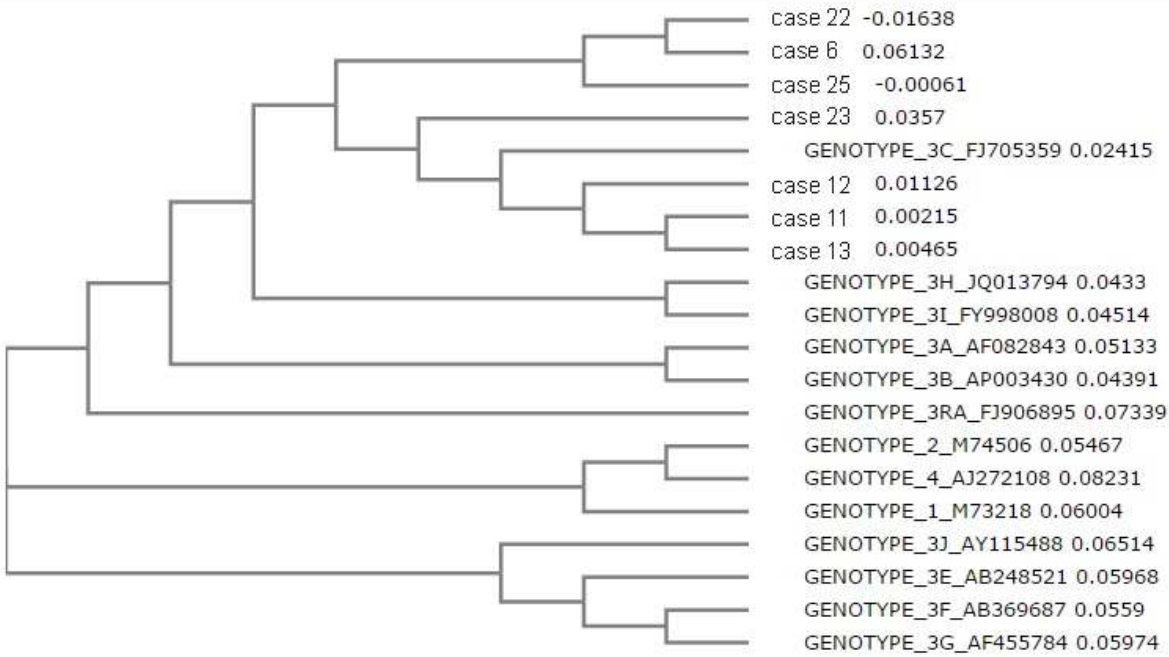
	Bile duct inflammation
Minimal	1-2 ducts infiltrated by occasional inflammatory cells
Mild	inflammatory cells infiltrating upto 50% ducts, with signs of duct epithelial reactivity
Moderate	most ducts infiltrated by inflammatory cells and showing epithelial reactivity
Severe	as moderate, with severe epithelial injury, duct damage (basement membrane breaks)

	Ductular reaction
Minimal	some ductules around a few tracts
Mild	at least occasional ductules around most portal areas or prominent around some
Moderate	prominent ductular aggregation around most portal areas
Severe	expansile prominent ductular reaction around/between portal areas

Supplementary Data 1: RNAscope probe design and genotyping

RNAscope probe design : Oligonucleotide probes specific for Hepatitis E genotypes 1 to 4 were prepared using the comprehensive sequence library of published and unpublished hepatitis E sequences of all genotypes, maintained and kindly made available by Prof Peter Simmonds, Edinburgh virus evolution group .
A consensus sequence for each genotype was generated and a pool of 4 probes (V-HEV-pool 420621) targeting genotypes 1 to 4 was created by ACDBio, each probe containing 40 Z oligo probe pairs. The name of subtype consensus method and accession number of the public sequence with highest similarity is listed as follows.
V-HEV-GT1-C1: Hepatitis E virus genotype 1 consensus sequence, 98% identical to gb|M73218.1|HPESVP. Gene length 7207; LE pairs 40. Start position 2678; end position 5090.
V-HEV-GT2-C1: gi|330017|gb|M74506.1|HPENSSP Hepatitis E virus (Mexican strain) structural proteins and a nonstructural polyprotein genes complete, genotype 2. Gene length 7180; LE pairs 40; start position 1828; end position 3756.
V-HEV-GT3-C1: Hepatitis E virus genotype 3 consensus sequence, 94% identical to dbj|AB089824.1|. Gene length 7269; LE pairs40; start position 3105; end position 5097.
V-HEV-GT4-C1: Hepatitis E virus 4 consensus sequence, 94% identical to dbj|AB097812.1|. Gene length7259; LE pairs 40; start position 628; end position 2787.

Genotyping : Phylogentic analysis of partial ORF2 sequences from 7 patient HEV isolates and genotype reference sequences from GenBank. Amplicons were generated using the Erker et al ORF2 PCR, TA cloned and sequenced by GATC Biotech. Sequences were aligned using Clustal Omega, branch distances are indicated.



Erker JC, Desai SM and Mushahwar IK, 1999. Rapid detection of Hepatitis E virus RNA by reverse transcriptase-polymerase chain reaction using universal oligonucleotide primers. Journal of Virological Methods, 81, pp109-113