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Brief Communication

Hepatitis E virus infection of transplanted kidneys

Jessica Schmitz [1], Julia Kracht [2], Katja Evert [3], Jürgen J. Wenzel [4], Mathias Schemmerer [4], Ulrich Lehmann [5], Marcus Panning [6], Lars Pape [7], Martin Pohl [2], Jan Hinrich Bräsen [1,*]

[1] *Nephropathology Unit, Institute of Pathology, Hannover Medical School, Hannover, Germany*
[2] *Department of General Pediatrics, Adolescent Medicine and Neonatology, Medical Center – University of Freiburg, Faculty of Medicine, University of Freiburg, Germany*
[3] *Institute of Pathology, University of Regensburg, Regensburg, Germany*
[4] *National Consultant Laboratory for HAV and HEV, Institute of Clinical Microbiology and Hygiene, University Medical Center Regensburg, Regensburg, Germany*
[5] *Molecular Pathology, Institute of Pathology, Hannover Medical School, Hannover, Germany*
[6] *Institute of Virology, University Clinics Freiburg, Freiburg, Germany*
[7] *Department of Pediatrics II, University Hospital of Essen, Essen, Germany*

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ABSTRACT

Immunocompromised patients are at risk of chronic hepatitis E (HEV) infection. Recurrent T cell and borderline rejections in a pediatric patient with high HEV copy numbers led us to study HEV infection within renal transplants. To investigate the frequency of renal HEV infection in transplanted patients, 15 samples from patients with contemporaneous diagnoses of HEV infection were identified at our center. Ten samples had sufficient residual paraffin tissue for immunofluorescence (IF) and RNA-fluorescence-in-situ-hybridization (RNA-FISH). The biopsy of the pediatric index patient was additionally sufficient for tissue polymerase chain reaction and electron microscopy. HEV RNA was detected in paraffin tissue of the index patient by tissue polymerase chain reaction. Subsequently, HEV infection was localized in tubular epithelial cells by IF, RNA-FISH, and electron microscopy. One additional biopsy from an adult was positive for HEV by RNA-FISH and IF. Focal IF positivity for HEV peptide was observed in 7 additional allografts. Ribavirin therapy was not successful in the pediatric index patient; after relapse, ribavirin is still administered. In the second patient, successful elimination of HEV was achieved after short-course ribavirin therapy. HEV infection is an important differential diagnosis for T cell rejection within transplanted kidneys. Immunostaining of HEV peptide does not necessarily prove acute infection. RNA-FISH seems to be a reliable method to localize HEV.

Abbreviations: FFPE, formalin-fixed paraffin-embedded; FISH, fluorescence in situ hybridization; HEV, hepatitis E virus; IF, immunofluorescence; PCR, polymerase chain reaction; RNA, ribonucleic acid; RT-qPCR, real-time reverse transcription quantitative polymerase chain reaction.

* Corresponding author. Nephropathology Unit, Institute of Pathology, Hannover Medical School, OE 5110, Carl-Neuberg-Str. 1, 30625 Hannover, Germany.
E-mail address: braesen.jan@mh-hannover.de (J.H. Bräsen).

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1. Introduction

Hepatitis E virus (HEV, *Paslahepevirus balayani*) is a single-stranded nonenveloped positive-sense RNA virus belonging to the family *Hepeviridae*.[1,2] Infection with HEV in immunocompetent humans follows a self-limiting course of illness sometimes including jaundice and nausea but is mostly asymptomatic. Immunocompromised patients have a risk of chronic infection (60%),[3,4] which can be treated off-label with ribavirin administration.[5,6] HEV genotypes 3 and 4 can cause severe (chronic) disease in solid-organ transplantation (SOT) patients.[7,8] HEV transmission via SOT has been reported since early 2010.[9]

Our male index patient was 13 years old at presentation. He received a combined kidney and liver transplantation at 6 years of age, and a second living-donor renal transplantation was performed at 12 years of age. Initial best creatinine of the living allograft was 0.4 mg/dL. Repeated rejection episodes of this second renal allograft (3 times borderline, once Banff category 4 grade IB, later once again formally borderline) treated by prednisolone pulse therapies (for Banff 4 grade IB combined with antithymocyte globulin) in different centers, each time elevated creatinine (maximum 3.8 mg/dL) returned to normal (initial baseline 0.7 mg/dL), but retention parameters increased over time (to creatinine 1.8 mg/dL). A routine diagnostic workup after the fifth rejection therapy revealed a highly positive HEV polymerase chain reaction (PCR) (15 million IE/mL in serum) without elevated transaminases (aspartate transferase/alanine aminotransferase 47 [ref 10-50] U/L, gamma-glutamyltransferase 66 [ref 9-43] U/L). By retrospectively testing stored blood samples it was shown that HEV infection had occurred 4 months previously. Because of elevated creatinine level (2.73 mg/dL), the question arose whether HEV infection might have caused impaired function of the allograft, and the most recent biopsy was examined for direct viral infection of renal cells.

2. Methods

The renal biopsy closely prior to the timepoint of serum HEV detection by PCR (revealing borderline grade of tubulointerstitial T cell inflammation without any glomerular changes) was carefully re-evaluated: We detected HEV RNA in paraffin tissue by PCR. Subsequently, we localized HEV infection in tubular epithelial cells using immunofluorescence (IF) and fluorescent in situ hybridization (HEV oligo-FISH) within residual paraffin tissue as well as with electron microscopy.

Our study was conducted in accordance with the guidelines of the Hannover Medical School Ethics Committee (10183_BOK_2022).

2.1. Immunohistochemistry

IF staining for HEV peptide was carried out using mouse antibody (Millipore, clone 1E6), diluted 1:100, with 18 hours incubation time at 4 °C after EDTA pH 8 pretreatment. Detection was carried out using donkey anti-Mouse IgG (H+L) highly cross-adsorbed secondary antibody, Alexa Fluor™ 555 labeled, diluted 1:100, 30 minutes at room temperature (Thermo Fisher Scientific, Waltham, MA, USA). Negative controls omitting first antibody, tissues from HEV negatively tested patients, HEV infected and noninfected HuH-7-Lunet BLR and PLC/PRF/5 cells, a commercially available cell control array for viral infection (including HSV, CMV, EBV, SV40 except HEV; Zytomed Systems, Berlin, Germany) and liver tissue samples from patients with acute HEV infection were used in all staining procedures as positive and negative controls respectively.

2.2. FISH

Oligo-FISH-probe for HEV (conjugated with Atto488) was purchased from MetaSystems Probes (Altlussheim, Germany) and was applied according to the manufacturer's recommendations. Negative FISH stains omitting probes and the same control samples as listed for IF were included in all staining procedures.

2.3. Imaging

Whole slide images of all stains were digitized with the Metafer Scanning Platform (MetaSystems Hard & Software) at 40x. Additional images were acquired using a confocal imaging system (Leica-Inverted-3, Leica Microsystems, Wetzlar, Germany) at 63x objective.

2.4. Electron microscopy

The portion of the renal biopsy selected for electron microscopy during standard diagnostic workup was transferred from 4% neutral buffered formaldehyde into a 0.1M sodium-cacodylate buffer containing 1.25% glutaraldehyde overnight and rinsed in cacodylate buffer. Afterward, dehydration was performed using automated dehydration (Leica EM AMW) with osmium tetroxide contrasting step followed by graded ethanol, acetone, and resin series, final epon polymerization was performed in an oven at 60 °C for 16 hours. Semithin and ultrathin sections were cut on a Leica UC7 ultramicrotome (Leica) on coated copper grids (Science Services, Munich, Germany). Ultrathin sections were contrasted using uranyl acetate (Science Services) and lead citrate (Plano, Wetzlar, Germany) and analyzed with a LEO912 AB Omega electron microscope (Zeiss, Oberkochen, Germany) equipped with a digital electron microscope camera (Tröndle, Moorenweis, Germany).

2.5. Detection and sequencing of HEV RNA in formalin-fixed paraffin-embedded (FFPE) biopsy tissue

RNA was extracted with the Maxwell RSC RNA FFPE Kit (Promega, Madison, USA) on a Maxwell instrument (Promega) according to the manufacturer's recommendations in the Institute of Pathology at Hannover Medical School and sent to the Institute of Clinical Microbiology and Hygiene, University Medical Center Regensburg for further analysis. HEV RNA was detected by real-time reverse transcription quantitative PCR (RT-qPCR) according to a protocol by Jothikumar et al with a

modified probe.[10,11] The assay was calibrated against the WHO International Standard (code number 6329/10) and HEV RNA was quantified as International Units per mL (IU/mL). The lower limit of detection was 1400 copies/mL (1214 IU/mL), and the upper limit of the linear range was 1.0 × 10e8 copies/mL (8.7 × 10e7 IU/mL). The HEV RT-qPCR-positive sample was further characterized by amplicon sequencing. The initial amplification was performed by using specific primers for a fragment of HEV open reading frame (ORF) 1 (418 nt, FJ705359 pos. 54-471).[12,13] Moreover, an ORF2 fragment (626 nt, FJ705359 pos. 5934-6559) was amplified according to the unified European HEVnet protocol. The nested PCRs were performed with specific primers for HEV-ORF1 (286 nt, FJ705359 pos. 102-387) and ORF2 (566 nt, FJ705359 pos. 5973-6538) respectively. The PCR products were purified by using QIAquick columns (Qiagen, Hilden, Germany) and sequenced in both directions with the nested PCR primers (ORF1) or with specific sequencing primers (ORF2). Nucleotide sequences of amplicons were determined by using the BigDye Terminator cycle sequencing kit (Applied Biosystems) and separated on a model 3730xl genetic analyzer (Applied Biosystems). Nucleotide sequences of PCR products were analyzed by using CodonCode Aligner software (http://codon-code.com/).

2.6. Genotyping and phylogenetic analyses

A maximum likelihood phylogenetic consensus tree for the HEV ORF1 (242 nt) and ORF2 (466 nt) nucleotide sequence was inferred as described previously.[14] Sequence data from this article have been deposited with the International Nucleotide Sequence Database Collaboration Libraries (GenBank, DDBJ, and ENA) under the accession numbers MZ814655 and MZ814093.

2.7. Cell culture positive controls for IF, FISH, and electron microscopy

HuH-7-Lunet BLR and PLC/PRF/5 cells persistently infected with HEV subtype 3c strain 14-16753 (GenBank accession MK089849.1) and same cell lines without HEV infection served as positive and negative controls. The cells were prepared according to Schemmerer et al 2019.[15] Briefly, 2.5 × 10^6 viable cells were seeded in a Falcon T25 flask (Corning, Corning, NY, USA) and incubated at 37 °C and 5% CO_2 for 2 weeks refreshing the medium every 3 to 4 days. The 3-dimensional cell layer was then inoculated with 500 µL of HEV-3c positive cell culture supernatant for 75 minutes at room temperature. Medium was added and cells were incubated at 34.5 °C and 5% CO_2. The medium was completely refreshed 24 hours later and every 3 to 4 days afterward. Four weeks post inoculation, cells were detached and washed with 40 mL PBS without Ca^{2+} and Mg^{2+}. The cell pellet was then preserved in phosphate-buffered formaldehyde <5% and embedded in paraffin.

3. Results

Viral loads in RNA derived from FFPE tissue of the index patient were 3.9 × 10^4 copies/mL. Genotyping revealed an autochthonous HEV subtype 3c isolate, designated V19-14772.

Using FISH and electron microscopy, HEV was located supranuclear within tubular cells (Fig. 1) in the index patient. Glomeruli were unremarkable, the biopsy was negative for immune complexes or other changes like hypercellularity (excluding glomerulonephritis due to HEV infection). One month later, virus levels increased again rapidly (up to 1000 IU/mL) and ribavirin was repeatedly initiated. There were often changing findings with once negative PCR and then again renewed virus

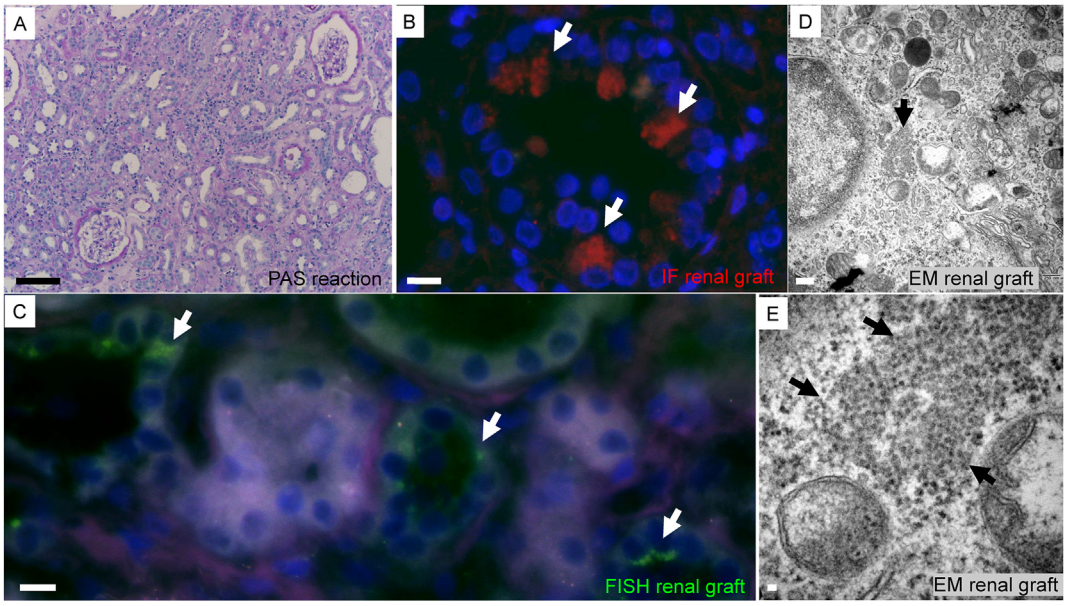

Figure 1. Index case for hepatitis E virus (HEV) in renal graft. (A) Periodic acid–Schiff reaction showing the representative region of renal transplant biopsy with T cell-rich inflammation of borderline type (bar: 100 µm). (B) Immunofluorescence showing positive staining for HEV ORF2 antigen (red, white arrows) in tubular epithelia (bar: 10 µm). (C) FISH showing HEV RNA (green fluorescence) within tubular epithelia (white arrows, bar: 10 µm). (D, E) Transmission electron microscopy identifies HEV particles (black arrows, bar: 250 nm in D and 25 nm in E). FISH, fluorescence in situ hybridization.

detection. Concomitantly, the serum creatinine level remained high. In consultation with the virology department, the virus detection in the PCR was considered "positive" even at low levels, and ribavirin therapy was continued in the presence of clinically persisting elevated serum creatinine at a dose of 200 mg/day (46 kg body weight, eGFR 29 mL/min). After 10 months, there was then at least a short-lasting negative PCR detection, in combination with markedly improved serum creatinine <2.0 mg/dL. During ribavirin therapy, hemoglobin tended to be lower—starting values were at 11 g/dL, minimally at 8 g/dL during ribavirin administration, mostly between 9 to 10 g/dL. The patient did not require erythrocyte concentrates because of ribavirin therapy; so the long administration was possible, and was then restarted after a short interruption. There was no evidence of hemolysis; Lactate dehydrogenase was stable, sometimes minimally elevated to values around 300 U/L, and haptoglobin was normal (but determined only once). No thrombocytopenia and always normal platelets (mostly > 200 000/μL) were observed. Allograft function stabilized during follow-up (creatinine level 1.7-2.0 mg/dL, last follow-up 1.64 mg/dL).

To investigate the frequency of HEV infection in renal transplants, we identified a total of 14 additional kidney samples (13 transplant kidneys [1 nephrectomy], 1 native kidney biopsy) from transplanted patients with a contemporaneous diagnosis of HEV infection (stool or serum PCR) from our archives at Hannover Medical School (Table). All patients had undergone an indication renal biopsy due to deterioration of renal (graft) function (independent of diagnosed HEV infection) and biopsies were routinely analyzed for rejection, polyomavirus infection, or other changes including recurrent or de novo renal disease (including electron microscopy). Diagnosis of HEV infection by PCR was performed in clinical routine diagnostic workup. Only some of the patients were tested for HEV infection because of elevated liver enzymes (Table). We used HEV oligo-FISH technology and IF on the remaining paraffin tissues which were very scarce in some cases. Only 10 samples (including the index patient) had sufficient residual paraffin tissue for IF and RNA-FISH; no conclusive result was obtainable in 5 biopsies (only minimal material left in paraffin blocks). One biopsy showed diffuse, 7 focal tubular IF positivity for HEV-ORF2-antigen, and 1 biopsy was negative. Using HEV oligo-FISH probe, 1 of the additional biopsies from a transplanted kidney showed HEV-positive signals (corresponding to IF, patient number 5 in Table, Fig. 2A, B) but had only a very weak positive signal in RT-qPCR (~7 copies/PCR; detected below the lower limit of quantification) which could not be verified in nested PCR (probably due to very small amount of tissue available and thus very low amount of RNA) nor used for sequencing. No viral particles could be detected in the ultrastructure either. The biopsy was taken from a 31-year-old male patient, who underwent transplantation 3 years before. His serum creatinine was 171 μmol/L, proteinuria 0.47g/L. Two weeks prior, the kidney biopsy HEV was positive in serum (anti-HEV IgG by ELISA and RNA-PCR in blood, no copy numbers determined) with normal liver enzymes (aspartate transferase 36 U/L [ref. < 35], alanine aminotransferase 37 U/L, [ref. < 45] gamma-glutamyltransferase 28 U/L [ref. < 55]). Two weeks after the kidney biopsy, ribavirin therapy was initiated and resulted in rapid clearance of HEV RNA from serum (1 month later and further PCR tests were negative). Afterward, the patient developed nephrotic syndrome and repeated biopsy revealed a recurrent Henoch-Schoenlein purpura/IgA glomerulonephritis (mesangiocapillary proliferation with crescents); he received a cyclophosphamide and steroid pulse therapy with good response of the recurrent IgA nephropathy.

All other biopsies did not include enough material for successful RNA isolation, except for the nephrectomy, which was negative for HEV PCR. Control experiments in infected human liver, HEV-infected and noninfected HuH-7-Lunet BLR (Fig. 2C-F), and PLC/PRF/5 cells and a commercially available cell control array for viruses (Fig. 3) proved the specificity of our methods (IF, FISH, and electron microscopy) and confirmed our results in renal grafts.

4. Discussion

To the best of our knowledge, we herewith describe a direct HEV infection of renal tubular epithelia in a renal allograft for the first time. As HEV infection of tubular cells has been shown in cell culture[16,17] and pigs,[18,19] it may occur in immunosuppressed transplant recipients causing tubulointerstitial damage with inflammation that cannot be distinguished from T cell rejection using conventional diagnostic methods. Immunosuppression targeting T cells might lead to failure of T cell function including exhaustion,[20] preventing elimination of viruses. In our index patient, ribavirin medication failed to persistently eradicate HEV infection; the patient is still under antiviral therapy with impaired but stable renal transplant function (creatinine 1.83 mg/dL), as well as normal liver transplant function which was impaired meanwhile. The patient did not develop severe anemia which is described in the literature in similar pediatric cases receiving immunosuppression and ribavirin.[21] Infection was most probably acquired after and independent from transplantation since the earliest positive HEV RNA in historic serum samples occurred 4 months before the reported positive biopsy was taken. However, HEV transmission via transplantation cannot be ruled out completely as the viral status of the donor is not known.

HEV clearance using ribavirin therapy was achieved rapidly in our second case, but the patient developed recurrent IgA glomerulonephritis (which might have been triggered by HEV infection).

Cryoglobulins[22,23] and glomerulonephritis[24,25] both were linked to HEV infection in some former studies but for glomerulonephritis, contrary results were published.[26] In our cohort, neither HEV-related glomerulonephritis nor cryoglobulins within the biopsies were found during histopathological diagnostic workup. Also, as far as accessible, none of the patients had clinical signs of cryoglobulins or glomerulonephritis (no active sediment).

Immunostaining of HEV peptide does not necessarily prove acute infection of renal tissues, since FISH for HEV RNA was positive only in 2 of 10 cases, 9 of which showed positive tubular epithelial staining for HEV peptide (including the index patient). Further, in our routine biopsies, we observed a number of positive immunostainings prompting HEV-testing which excluded active

Table

Characteristics of all identified patients with a transplant and HEV (hepatitis E virus) infection.

No	Sex	Age	TX since	Type of TX	IS	Banff19 (cat/grade)	Ther	HEV viral load	Time HEV/BX	Ribav	HEV IF	HEV FISH	Crea (μMol/L)	Crea (Δ μMol/L)	ALT (U/L)	AST (U/L)	PU g/L	Cryo	HEV dur	Rep BX
1	f	12 y	13 mo	k	Tac, MMF, Sir, Pred	2, III +4, III	none	n.d.[a]	n.d.[a]	n.t. [a]	TE foc +	neg	112	+6	82	173	neg	n.t.	11 mo	n
2[a]	M	13 y	7 y	k & li	Tac, Ever, Pred	3	Ster	1.5 × 10e6	–4 mo	Y	TE foc +	foc +	218	+118	17	33	neg	n.t.	2 y	n
3	F	18 y	4.5 y	k	MMF, Sir, Pred	4, IA	Ster	"pos"	+5 d	Y	TE foc +	neg	281	+56	594	224	0.3	n.t.	n.a.	n
4	F	25 y	17 d	k	MMF, Tac, Pred	1, ATI	none	3 × 10e5	+24 d	Y	TE foc +	neg	119	+27	16	22	0.3	n.t.	n.a.	n
5	M	31 y	3 y	k	MMF, Tac, Pred	1, ATI	none	"pos"	–14 d	Y	TE foc +	foc +	207	+36	51	45	0.47	neg	3 mo	y[b]
6	M	32 y	26 mo	k	MMF. Tac, Pred	2, II	Ster, PE, ivIG	2.3 × 10e5	+2 d	Y	TE foc +	neg	204	+39	94	45	neg	neg	5 mo	y[c]
7	M	33 y	16 y	k	MMF, Tac, Pred	1, ATI	none	2 × 10e5	+12 d	Y	TE foc +	neg	299	+64	44	31	0.28	n.t.	1.5 y	
8	F	38 y	12 mo	k	Tac, Ever, Pred	5, PVN class 3[d]	IS red	"pos"	– 8 d	Y	TE foc +	neg	290	+70	15	43	neg	neg	2 y	y[d]
9	M	46 y	7.5 y	k	MMF, Pred, Eculizumab	3	Ster	"pos"	–25 d	Y	TE foc +	neg	276	+46	15	13	0.49	neg	3 mo	n
10	M	56 y	12 y	h	MMF, Tac, Pred	n.a.	none	"pos"	+ 2 d	Y	neg	neg	151	+7	47	31	0.1	neg	2 m	n

ALT, alanine transaminase; AST, aspartate transferase; Banff19 cat, category according to Banff19 consensus (1, no rejection, 2, antibody mediated rejection, 3, borderline, 4, acute T cell rejection, 5, polyomavirus nephropathy [PVN], [d]preexisting PVN since 6 months, repeated biopsy again with PVN, ATI, acute tubular injury); BX, biopsy; Crea, creatinine (Δ indicates change); Cryo, cryoglobulins; Ever, everolimus; F, female; FISH, fluorescent in situ hybridization; h, heart; HEV dur, duration of HEV positivity; IF, immunofluorescence; IS, immunosuppression; ivIG, intravenous immune globulins; k, kidney, li, liver; M, male; MMF, mycophenolate mofetil; mo, month(s); n, no; n.t., not tested (transplant nephrectomy case was not tested, but 13 y later HEV infection identified and eradicated); PE, plasma exchange; pos, positive; Pred, prednisolone; PU, proteinuria; RepBX, repeated biopsy following HEV diagnosis; Ribav, ribavirin; Sir, sirolimus; ster, steroid pulse; Tac, tacrolimus; TE, tubular epithelia; Ther, rejection therapy following biopsy diagnosis; time HEV/BX, time between biopsy and HEV diagnosis (–, before; +, after biopsy); TX, transplant; Y, yes.

[a] Index patient.

[b] Recurrence of IgA glomerulonephritis.

[c] ATI.

[d] Pre-existing PVN since 6 months, repeated biopsy again with PVN; persistent PVN.

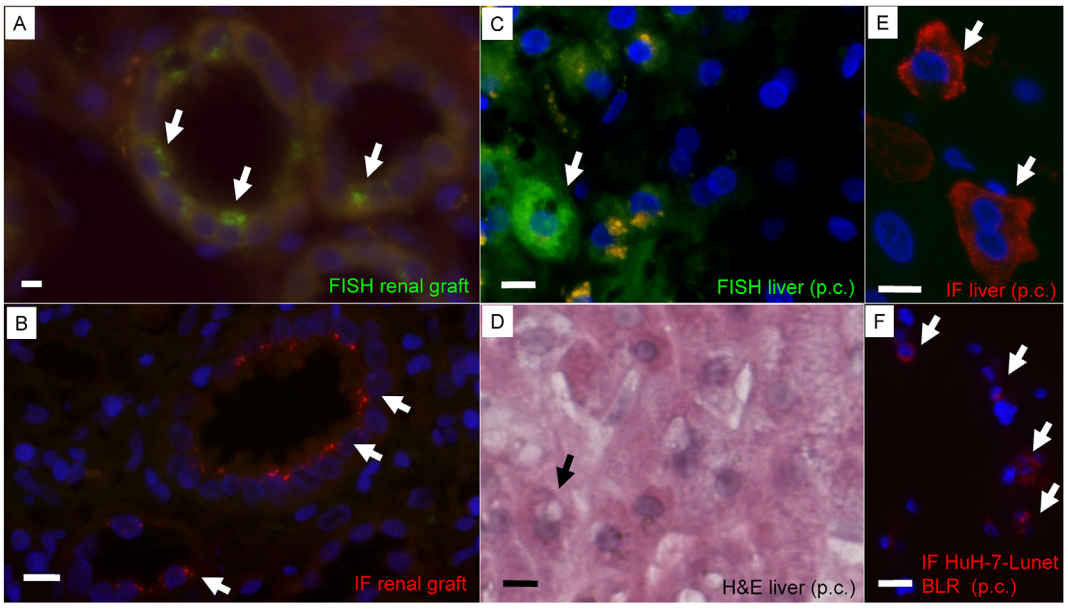

Figure 2. FISH for hepatitis E virus (HEV) RNA (green, arrows, A) and immunofluorescence for HEV peptide (red, arrows, B) of second kidney transplant case with HEV infection. Positive controls (C-F): HEV-infected native liver showing positive hepatocytes in FISH (green, arrow, C, H&E stain of identical section in D). Immunofluorescence shows positive signal of ORF2 antigen in the same liver (red, arrows, E) as well as in HuH-7-Lunet BLR cells persistently infected with HEV-3c strain 14-16753 (red, arrows, F,). Bars represent 5 μm in A, 10 μm in B-E, and 20 μm in F. FISH, fluorescence in situ hybridization.

infection (PCR for HEV RNA negative, data not shown). Thus, peptide positivity in renal biopsies can possibly be an epiphenomenon based on viral fragments, which might be filtered and reabsorbed within the kidney.[27] On the other hand, unspecific antibody binding to other epitopes cannot be ruled out completely, although we proved antibody specificity in infected liver biopsy and in cell lines infected with HEV (Figs. 2 and 3) and included technical and PCR-negative controls in all staining procedures.

Based on our findings, HEV infection of kidney tissue in immunocompromised patients is an important differential diagnosis of T cell rejection but data on incidence are missing. HEV

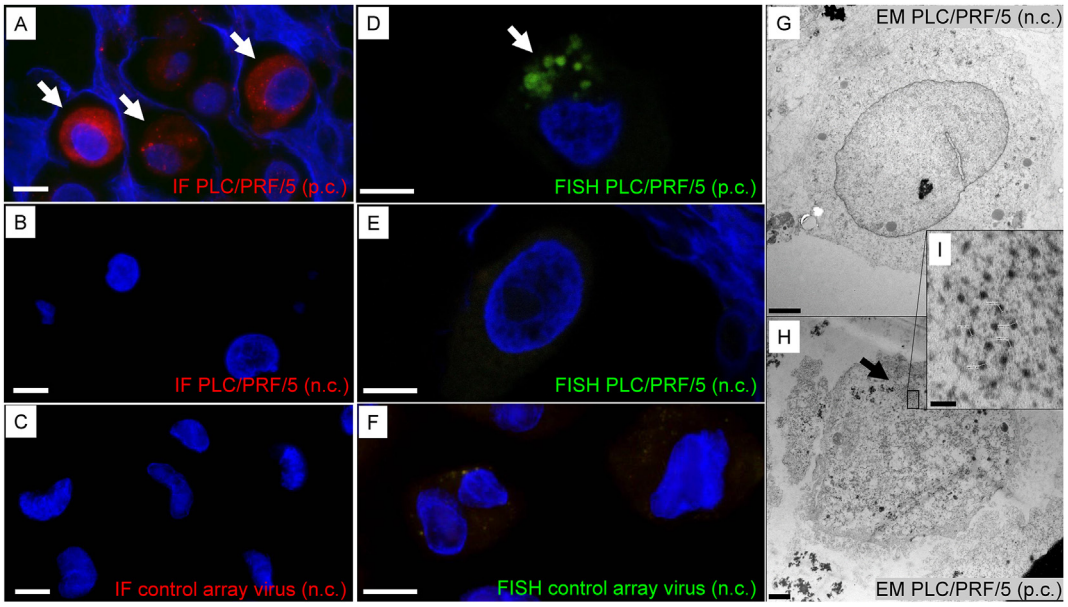

Figure 3. Fluorescence in situ hybridization (FISH) for HEV RNA (green) and immunofluorescence (IF) for HEV peptide (red) of PLC/PRF/5 cells infected versus noninfected with HEV-3c strain 14-16753 and a commercially available cell control array containing different viruses other than HEV served as additional control experiments for staining specificity. (A) IF for HEV (red) in infected cells, (B) negative staining in noninfected cells of the same cell line, and (C) negative staining in the commercial virus control block. (D) RNA-FISH staining for HEV (green) in infected cells, I shows negative FISH in noninfected cells of the same cell line, and in (F) negative FISH in the commercial virus control block. (G) shows the electron microscopy image of noninfected cells, (H) virus particles in HEV-infected cells, (I) shows higher resolution of perinuclear virus particles measuring 32 to 34 nanometers. Bars represent 10 μm in A to F, 2.5 nm in G, 1 nm in H, and 100 nm in I.

infection can be verified using FISH on a single FFPE section if PCR cannot be performed due to poor tissue availability

Declaration of competing interest

The authors of this manuscript have no conflicts of interest to disclose as described by the American Journal of Transplantation.

Data availability

Data will be shared upon reasonable request.

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ORCiD

Jessica Schmitz https://orcid.org/0000-0001-9843-7339
Katja Evert https://orcid.org/0000-0003-0984-5563
Jürgen J. Wenzel https://orcid.org/0000-0001-8422-6581
Mathias Schemmerer https://orcid.org/0000-0003-3782-8226
Ulrich Lehmann https://orcid.org/0000-0003-2350-6584
Marcus Panning https://orcid.org/0000-0003-3596-5964
Lars Pape https://orcid.org/0000-0002-3635-6418
Jan Hinrich Bräsen https://orcid.org/0000-0002-2863-3067

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