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Hepatitis E virus genotype 3 and capsid protein in the blood and urine of immunocompromised patients ☆,☆☆

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SUMMARY

Objectives: Hepatitis E virus genotype 3 (HEV3) is responsible for acute and chronic liver disease in solid organ transplant (SOT) recipients. HEV was recently found in the urine of some acutely and chronically genotype 4-infected patients.

Methods: We examined the urinary excretion of HEV3 by 24 consecutive SOT recipients at the acute phase of HEV hepatitis and characterized the excreted virus.

Results: Urinary HEV RNA was detected in 12 (50%) of the 24 transplanted patients diagnosed with HEV hepatitis. Urinary HEV antigen (Ag) was detected in all but one of the patients (96%). The density of RNA-containing HEV particles in urine was low (1.11-1.12 g/cm^3), corresponding to lipid-associated virions. The urinary HEV RNA/Ag detected was not associated with impaired kidney function or *de novo* proteinuria. Finally, there was more HEV Ag in the serum at the acute phase of HEV infection in SOT recipients whose infection became chronic.

Conclusions: HEV3 excreted via the urine of SOT recipients at the acute phase of HEV hepatitis has a lipid envelope. Renal function was not impaired. While urinary HEV Ag was a sensitive indicator of HEV infection, only acute phase serum HEV Ag indicated the development of a chronic infection.

Hepatitis E virus (HEV), a major cause of acute viral hepatitis worldwide, is enterically transmitted. Seven genotypes of the virus are currently identified.[1] Genotypes 1 and 2 infect only humans and are associated with epidemics in developing countries, mainly due to drinking contaminated water. Conversely, HEV genotypes 3 (HEV3), 4 and 7 are zoonotic and responsible for sporadic cases in developed countries.[2,3] HEV genotypes 5 and 6 are known to infect wild boar but no human infections have yet been reported. HEV infection results from direct contact with infected animals, eating infected pork or game products, or drinking contaminated water.[2] Cases of HEV infection via blood products and transplanted organs have been also reported.[4,5]

HEV is a single-stranded, positive-sense RNA virus that is a member of the *Hepeviridae*. The HEV genome is approximately 7.2 kb long and contains three open reading frames (ORFs).[2] ORF1 encodes nonstructural proteins, including methyltransferase, papain-like cysteine protease, helicase and RNA-dependent RNA polymerase.[6] ORF2 encodes the virus capsid protein whose sequence includes the immunogenic epitopes.[7] ORF3 encodes a 113 or 114 residue phosphoprotein involved in virion release; it interacts with the endosomal sorting complexes required for transport.[8] HEV virions are unenveloped in the environment and feces but are associated with a lipid membrane in cell culture supernatants and the serum of HEV-infected patients.[9,10] However, this HEV-membrane complex lacks the glycoprotein needed for recognizing the cell entry receptor for enveloped virus. Hence, HEV is presently considered to be a quasi-enveloped virus.[11]

While the liver is the main site of HEV replication, HEV RNA and antigen (Ag) have been detected in extra-hepatic sites like the gut, the central nervous system and the kidneys in animal

Abbreviations: Ag, antigen; ALT, alanine aminotransferase; AP, alkaline phosphatase; AST, aspartate aminotransferase; AUC, area under the curve; CI, confidence interval; eGFR, estimated glomerular filtration rate; GGT, gamma-glutamyl transpeptidase; HEV, hepatitis E virus; IQR, interquartile range; ORF, open reading frame; ROC, receiver operating characteristic; RT-PCR, real-time polymerase chain reaction; S/CO, signal-to-cutoff ratio; SOT, solid organ transplant.

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models.[12–15] Subjects acutely and chronically infected with genotype 4 excrete HEV in their urine,[16] but no data are currently available for genotype 3-infected patients and nothing is known about the characteristics of the urinary HEV3 virion or its association with lipid membrane.

Although most HEV infections are mild and self-limiting in immunocompetent subjects, immunocompromised patients, such as solid-organ transplant (SOT) recipients,[17] human immunodeficiency virus-infected patients with low CD4+ T lymphocyte counts,[18] hematological patients on chemotherapy[19] and those on immunotherapy,[20] can develop chronic hepatitis. The infections of more than 60% of SOT patients become chronic and about 10% of these develop HEV-related cirrhosis.[21] Reducing the immunosuppressive therapy of HEV-infected SOT recipients leads to HEV clearance in around one third of them. Any persistent HEV replication despite decreased immunosuppression 3 months after diagnosis can be treated with ribavirin, leading to a sustained virologic response in approximately 80% of patients.[22] It was suggested recently that measuring the HEV ORF2 Ag in sera could discriminate between acutely and chronically infected patients.[23] The discriminatory value of urinary HEV ORF2 Ag has not yet been studied.

This study investigates the urinary excretion of HEV by SOT recipients during the acute phase of HEV3 infection. It also assesses the values of urinary and serum HEV Ag concentrations as indicators of the development of a chronic infection.

Methods

Patients and Samples

We studied 24 consecutive SOT recipients followed in Toulouse University Hospital who developed an HEV infection between September 2010 and December 2016 and whose urine samples were available. The acute phase of HEV infection was defined as the first month following the diagnosis of HEV hepatitis, characterized by *de novo* elevated liver aminotransferase activity and the presence of HEV RNA in the blood. Other causes of hepatitis were excluded by serological and molecular tests for hepatitis A virus, hepatitis B virus, hepatitis C virus, Epstein-Barr virus and cytomegalovirus. The presence of biliary stones was excluded by abdominal ultrasound imaging in case of abdominal pain, fever or jaundice. All SOT recipients diagnosed with an HEV infection were placed on a minimal immunosuppressive regimen. A chronic HEV infection was defined as persistent HEV RNA in the blood 3 months after diagnosis, despite the reduced immunosuppressive therapy.[24]

Blood and urine samples tested in the study were collected the same day for a standard management, at the acute phase of infection for all patients and at the chronic phase for patients who did not eliminate the virus. Urine samples were cleared by centrifugation (3650 g/10 min). All serum and urine samples were stored at −80°C. This non-interventional study involved no additional sampling, hence requires no specific written informed consent (CSP Art L 1121-1.1). All data were analyzed using an anonymized database.

All patients underwent an exhaustive clinical and biological examination during their acute infection phase. We measured liver enzyme activities (AST, ALT, GGT and AP), total bilirubin, kidney parameters (serum creatinine, estimated glomerular filtration rate (eGFR), proteinuria (mg/g urine creatinine) and cytology), hematological parameters (hemoglobin, platelet count, lymphocyte count) and immunosuppressive regimen.

Laboratory Methods

HEV Ag was measured in all serum and urine samples using the Wantaï HEV Antigen ELISA Plus kit (Beijing Wantaï Biological Pharmacy Enterprise Co., China) according to the manufacturer's instructions.[25] Briefly, samples (50 µL) were incubated in microwell strips coated with anti-ORF2 antibody. The wells were then washed with buffer and incubated with peroxidase-conjugated anti-ORF2 antibody followed by chromogen solution. The optical densities were read and results expressed as signal-to-cutoff ratios (S/CO). A ratio of >1.1 was taken as positive for HEV Ag, 0.9–1.1 as borderline and <0.9 as negative. Linear ranges of HEV Ag ELISA were determined for both serum and urine samples by serial dilutions. Out-of-range samples (S/CO >15) were diluted and retested. Because the urinary excretion of creatinine can be considered to be constant throughout the day in the presence of stable renal function,[26] the urine S/COs were normalized to urinary creatinine to compensate for concentration bias.[27]

The concentrations of HEV RNA in the plasma and urine were measured by real-time polymerase chain reaction (RT-PCR) targeting the ORF3 gene (detection limit: 60 IU/mL; 1.78 $\log_{10}$IU/mL).[28,29] Samples were genotyped by sequencing a 345-nucleotide fragment within the ORF2 gene.[30] The sequences were compared to reference HEV strains.[31]

We used the Wantaï HEV IgM enzyme immunoassay kit and the Wantaï HEV IgG enzyme immunoassay kit to detect anti-HEV IgM and IgG, according to the manufacturer's instructions (Beijing Wantaï Biological Pharmacy Enterprise Co., China). The concentrations of anti-HEV IgG were determined using the WHO anti-HEV IgG reference material (95/284), as previously described.[32]

Iodixanol gradients (7.5 to 40%) were prepared manually with solutions of OptiPrep Density Gradient Medium (Sigma-Aldrich). One milliliter of a positive urine sample, with or without 1% NP-40 detergent solution (Thermo Scientific) treatment, was layered on top of the gradient and centrifuged in a SW41Ti® rotor (Beckman Coulter) at 125,000 g and 10°C for 14 h (Optima L80-XP ultracentrifuge, Beckman Coulter). Fractions (500 µL) were collected starting from the top and the density of each fraction was measured by refractometry. The HEV RNA and Ag in each fraction were quantified by one-step RT-PCR and Wantaï® HEV Antigen test, respectively.

The tissue culture infectious dose 50 (TCID50) was determined by an endpoint dilution method and calculation of the Spearman-Karber formula.[33–35] Briefly, HepG2/C3A cells (CRL-10741, ATCC) that had been seeded in 96-well plates (10^5 cells per well) a day earlier were infected with tenfold dilutions of viral suspensions in Dulbecco's modified Eagle's medium (DMEM). Six replicates of each dilution were tested. The cells were incubated with virus at 35.5°C for 6 h. The inoculum was then removed and the cells were washed five times with phosphate-buffered saline (PBS). HEV-infected HepG2/C3A were incubated at 35.5 °C in a 5% CO_2 humidified atmosphere in DMEM supplemented with 10% heat inactivated fetal bovine serum, 2% dimethyl sulfoxide (DMSO), 10,000 U/mL penicillin G/streptomycin and 25 µg/mL amphotericin B. Half of each culture medium was changed every two to three days for a total of 12 days. Cells were lysed by freezing/thawing the plates and HEV RNA in the lysates was detected by RT-PCR. Wells with detectable HEV RNA were considered positive and wells with undetectable HEV RNA as negative (limit of detection: 60 IU/mL).

Urine inoculum was obtained from the urine of an acutely HEV3-infected patients (subgenotype 3chi), selected for its high HEV RNA content (~4.0 $\log_{10}$IU/mL). The initial urine inoculum (~6.0 $\log_{10}$IU/mL) was obtained by passing the urine sample through an Amicon® filter (Amicon® Ultra 15 mL Centrifugal Filters Millipore; molecular weight cutoff 100 kDa) and centrifuging it at 4000 g and 25°C for 40 min. Unenveloped HEV virions of the same strain were obtained directly from the patient's stools. Briefly, stools were diluted in PBS, centrifuged at 3560 g for 10 min and filtered through a 0.22 µm filter unit. Quasi-enveloped HEV virions of the same strain were obtained from the patient's plasma

and a culture supernatant of HepG2/C3A infected with the patient's stools.

Statistical analysis

Data were analyzed using GraphPad Prism v 7.04 (GraphPad Software, Inc., La Jolla, USA). Discrete variables are shown as counts and continuous variables as medians (interquartile range, IQR[25-75]). Correlation was assessed by Spearman analysis. Fisher's exact test was used to compare proportions. The Mann-Whitney *U*-test was used to compare continuous variables, the Wilcoxon signed rank test was used to compare paired data, while a Bonferroni correction was applied for multiple comparisons. A p value <0.05 was considered significant in all analyses.

Results

Patient characteristics

The median age of the 24 HEV-infected SOT patients (18 men; sex ratio: 3.0) was 53 [43–63] years and the average time between transplantation and HEV infection was 77 [32-154] months (Table 1). The immunosuppressive regimens consisted of tacrolimus ($n=16/24$, 67%), mTOR inhibitors ($n=5/24$, 21%), tacrolimus plus mTOR inhibitors ($n=2/24$, 8%) and belatacept ($n=1/24$, 4%). Seventeen patients were given mycophenolic acid ($n=17/24$, 71%) and 14 were given steroids ($n=14/24$, 58%). A total of 9 patients spontaneously cleared the virus ($n=9/24$, 37.5%), the others ($n=15/24$, 62.5%) developed chronic infections.

HEV infection was associated with cytolytic hepatitis and cholestasis but the renal (creatinine, eGFR or proteinuria) and hematological (hemoglobin, lymphocyte, neutrophil and platelet counts) parameters remained unchanged (Supplementary Table 1).

The median serum HEV RNA concentration at diagnosis was 5.52 [5.31–6.84] $\log_{10}$IU/mL. The sera of all but one patients ($n=23/24$; 96%) tested positive for HEV Ag, with a median value of 3.56 [2.14–3.84] $\log_{10}$S/CO. The patient whose serum HEV Ag was undetectable had a low HEV RNA concentration (2.19 $\log_{10}$IU/mL). The serum HEV Ag and HEV RNA concentrations were correlated ($r=0.674$; $p<0.001$; $n=24$) (Fig. 1.A). No correlation was found between HEV Ag values and anti-HEV IgG concentrations ($r=-0.046$; $p=0.83$).

The HEV3 subgenotype was determined for 22/24 strains: genotype 3e ($n=2$), 3f ($n=10$), 3c ($n=6$), 3i ($n=1$) and 3chi ($n=3$).

HEV RNA and antigen in urine and characterization of HEV particles

The urine of 12 patients ($n=12/24$, 50%) tested positive for HEV RNA at the acute phase of HEV infection. The median urinary HEV RNA concentration was 2.28 [1.83–3.42] $\log_{10}$IU/mL. The urine of 23 patients ($n=23/24$, 96%) tested positive for HEV Ag; thus the same samples from 11 of the 12 patients with no detectable urinary HEV RNA tested positive for HEV Ag. The patient whose urine HEV Ag was undetectable was the same patient who had a negative HEV Ag and a low HEV RNA concentration in serum. Finally, the urine median HEV Ag concentration (3.86 [2.87–4.19] $\log_{10}$S/CO/g) was higher than that of the serum (3.56 [2.14–3.84], $p=0.04$).

The urinary HEV Ag and HEV RNA concentrations were not correlated ($r=0.346$; $p=0.098$; $n=24$), unlike those of the serum (Fig. 1.B). However, the HEV Ag in the urine was correlated with that of the circulating serum HEV Ag ($r=0.617$; $p=0.001$; $n=24$) (Fig. 1.C).

We also compared the ratios of HEV Ag ($\log_{10}$S/CO) to HEV RNA ($\log_{10}$IU/mL) in the urine and serum at the acute phase of HEV infection (Fig. 1.D). The urinary HEV Ag to RNA ratios (1.76 [0.94–2.28], $n=12$) were significantly higher than those for the blood (0.56 [0.39–0.66], $n=24$; $p<0.001$).

The densities of the HEV particles in the urine (Fig. 2.A) were compared to those of enveloped HEV particles obtained from the plasma of patients infected with HEV3 and unenveloped HEV particles isolated from the feces of a chronically HEV3-infected patient (Fig. 2.B). The urine HEV particles formed a major peak at a density of 1.11–1.12 g/cm^3, close to the 1.08–1.09 g/cm^3 peak density observed for enveloped HEV particles from the plasma. Treating the urine with detergent (1% NP40) shifted the HEV-RNA-containing particles to a higher density (1.18–1.20 g/cm^3) corresponding to that of fecal HEV particles. These data indicate that urinary HEV particles were associated with a lipid membrane, as were those in the serum and culture supernatants. Measurements of the HEV Ag in each fraction of the urine gradient (Fig. 2.A) indicated that HEV Ag was present only in very low-density fractions, with no detectable HEV RNA (1.00–1.08 g/cm^3). HEV Ag was not detected in any HEV RNA-positive fractions, regardless of density. The results for NP40-treated urine were similar, indicating that removing the lipid membrane did not enable the Wantaï® Ag test to recognize the capsid-associated ORF2 protein.

The infectivity of urinary HEV particles was assessed by an endpoint dilution method on HepG2/C3A cells culture system.[34] We also studied viral particles from stools (unenveloped virions), plasma (quasi-enveloped virions) and culture supernatant (quasi-enveloped virions) of the same HEV strain (from the same patient). HEV RNA and HEV Ag concentrations for each inoculum were measured. Both urine and plasma-derived virions were similarly infective *in vitro* (respectively 148 ±2 and 135 ±3 infectious particles/10^6 IU HEV RNA) (Fig. 3). Conversely, the HEV Ag concentration in the urine was 30-times higher than that of the plasma inoculum and did not seem to influence infectivity *in vitro*. Unenveloped HEV virions were more infectious than quasi-enveloped virions from both clinical samples (plasma and urine) and HEV-infected HepG2/C3A cells (supernatant), while the HEV Ag concentrations associated with enveloped-virions were broadly higher in urine and plasma samples than in culture supernatants (Fig. 3).

Factors associated with the detection of HEV RNA in urine

Patients with detectable HEV RNA in their urine had greater serum virus RNA loads and higher urine and serum Ag concentrations than did those with no urinary HEV RNA (Table 2). And patients whose urine was HEV RNA-positive at diagnosis had a deeper tacrolimus trough (9.1 [7.4–10.2] ng/mL) than did patients whose urine was HEV RNA-negative (5.9 [4.8–6.5] ng/mL, $p<0.001$). The serum creatinine, eGFR and proteinuria data for the two groups were similar, as were those for liver and hematological parameters. HEV excretion in the urine was not sub-genotype-specific. There was no association between the presence of HEV RNA or HEV Ag in the urine and the organ transplanted (kidney *versus* liver transplants).

HEV markers associated to the development of a Chronic HEV infection

The 24 SOT recipients included fifteen (62.5%) who developed a chronic infection; the other nine spontaneously cleared the virus. Chronic phase serum and urine HEV Ag concentrations were also available for 12 patients who developed chronic HEV infections.

The acute phase serum HEV RNA concentration of patients who developed a chronic HEV infection was higher (6.41 [5.48–6.86] $\log_{10}$IU/mL) than that of patients who eliminated the virus (5.30 [3.64–5.55] $\log_{10}$IU/mL; $p=0.018$). Moreover, patients who developed a chronic infection had a significantly higher acute phase

Table 1
Patient characteristics at diagnostic of HEV infection.

Patient infection evolution	Gender M/F	Age years	Organ transplanted	Duration of transplantation, months	Immunosuppressive regimen	AST IU/L	ALT IU/L	Bilirubin μmol/L	Anti-HEV IgM	Anti-HEV IgG (titer[a])	S/CO[b] serum	S/CO/g[b] urine
Clearance												
1	F	68	Kidney	67	Tacrolimus, MPA, steroids	104	195	8.1	+	+ (>400.0)	2.99	3.89
2	M	62	Kidney	107	Tacrolimus, MPA	43	92	14.3	+	+ (0.3)	3.51	3.94
3	F	52	Liver	160	Tacrolimus, MPA	97	157	23.0	+	-	0.48	2.74
4	M	63	Kidney	70	Tacrolimus, MPA, steroids	16	16	6.0	-	-	0.00	0.00
5	M	62	Kidney	29	Tacrolimus, MPA, steroids	216	461	5.6	+	-	1.26	2.31
6	M	43	Liver	83	Tacrolimus, MPA	768	1172	16.9	+	+ (1.6)	3.02	4.21
7	F	50	Kidney	335	Tacrolimus, MPA	338	677	5.9	+	+ (0.3)	2.32	3.33
8	M	51	Kidney	24	Belatacept, MPA, steroids	177	378	6.4	+	+ (20.1)	3.29	3.59
9	M	36	Liver	159	mTORi	543	1232	8.1	+	+ (4.5)	0.70	2.27
Persistence												
10	M	75	Kidney	236	mTORi, MPA, steroids	63	60	12.0	+	+ (5.4)	4.30	3.50
11	M	32	Kidney	35	Tacrolimus, MPA, steroids	64	209	14.0	-	-	3.80	4.12
12	F	74	Kidney-Liver	138	Tacrolimus, mTORi	388	502	8.0	+	+ (394.2)	2.05	3.13
13	F	49	Kidney	163	Tacrolimus, MPA, steroids	83	217	9.4	+	-	4.58	4.25
14	M	55	Liver	3	Tacrolimus, steroids	90	240	71.6	-	-	4.70	5.01
15	M	59	Kidney	175	Tacrolimus, MPA, steroids	43	66	8.8	+	+ (22.4)	4.25	2.71
16	M	43	Kidney-Liver	24	Tacrolimus, MPA, steroids	60	71	17.0	+	+ (>400.0)	3.69	3.10
17	M	39	Liver	34	mTORi, steroids	140	164	11.0	+	-	3.64	4.41
18	M	70	Liver	133	mTORi, steroids	70	105	11.1	+	-	3.76	4.06
19	M	44	Kidney	13	Tacrolimus, MPA, steroids	61	106	9.3	+	+ (0.4)	3.78	4.22
20	M	54	Kidney	132	mTORi, MPA	254	564	12.7	+	+ (5.0)	2.59	3.94
21	F	53	Kidney-Pancreas	107	Tacrolimus, azathioprine	91	57	6.0	+	-	3.60	4.24
22	M	50	Liver	42	Tacrolimus, MPA, steroids	105	140	23.5	+	+ (41.9)	3.90	3.84
23	M	34	Kidney	31	Tacrolimus, mTORi, steroids	65	172	6.7	+	+ (42.6)	1.97	2.75
24	M	75	Liver	44	Tacrolimus, MPA, steroids	33	54	6.9	+	-	3.85	4.09

Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; S/CO, signal-to-cutoff ratio; S/CO/g, signal-to-cutoff ratio normalized to urine creatinine; mTORi, mammalian target of rapamycin inhibitors; MPA, mycophenolic acid.

[a] anti-HEV IgG titers are expressed in U/mL.[32]

[b] S/CO values are expressed in $\log_{10}$.

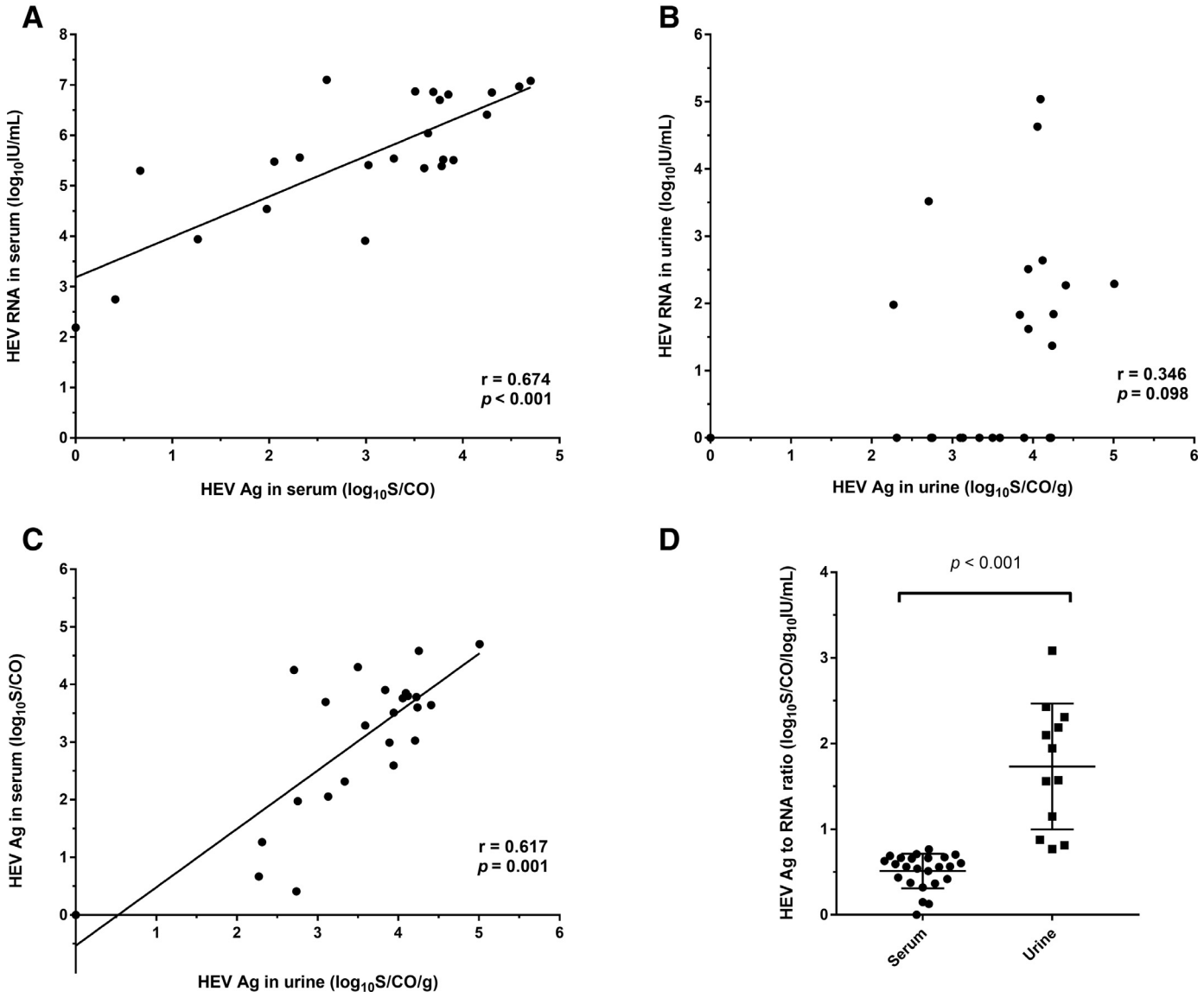

Fig. 1. HEV Ag and HEV RNA in serum and urine. A, HEV RNA (log_{10}IU/mL) and HEV Ag (log_{10}S/CO) in the serum ($r = 0.674$; $p < 0.001$; $n = 24$); B, HEV RNA (log_{10}IU/mL) and HEV Ag (log_{10}S/CO/g) in the urine ($r = 0.346$; $p = 0.098$; $n = 24$); C, HEV Ag (log_{10}S/CO) in the serum and HEV Ag (log_{10}S/CO/g) in the urine ($r = 0.617$; $p = 0.001$; $n = 24$); D, HEV Ag:RNA ratios (log_{10}S/CO/log_{10}IU/mL) in serum and urine.
Abbreviations: HEV, hepatitis E virus; Ag, antigen; S/CO, signal-to-cutoff ratio; S/CO/g, signal-to-cutoff ratio normalized to urine creatinine.

serum Ag level (log_{10}S/CO: 3.78 [3.60–4.25]) than those who spontaneously cleared the virus (2.32 [0.56–3.18]; $p = 0.002$) (Fig. 4.A). ROC analysis showed that the AUC used to predict the evolution toward chronicity was lower when based on serum HEV RNA concentrations (AUC: 0.793, 95%CI [0.595–0.990]; $p = 0.019$) than the AUC obtained using the serum HEV antigen values (AUC: 0.896, 95%CI [0.769–1.023]; $p = 0.001$). We identified the HEV Ag threshold for distinguishing between patients whose infection became chronic and those who recovered using an ROC curve (Fig. 4.B). An acute phase log_{10}S/CO threshold of 3.56 differentiated SOT recipients whose infection would become chronic from those who recovered; its sensitivity was 80% (95%CI [52–96]) and specificity was 100% (95%CI [66–100]). For this threshold, the positive and negative predictive values of the HEV Ag assay were 100% and 75%.

Finally, the acute (median log_{10}S/CO: 3.56 [2.25–3.81]; $n = 15$) and chronic phase (median log_{10}S/CO: 3.65 [3.17–4.07]; $n = 12$) serum HEV Ag concentrations of patients whose infections evolved toward chronicity were not significantly different ($p = 0.648$).

The urinary acute phase HEV Ag concentrations in patients whose infections became chronic (log_{10}S/CO/g: 4.06 [3.13–4.24]) and those who cleared the virus (3.34 [2.29–3.92]) were not significantly different ($p = 0.123$) (Fig. 4.C). We detected similar concentrations of HEV Ag in the urine of chronic phase patients (3.24 [2.72–4.48]). Urinary HEV RNA tended to be more frequent in acute phase patients who developed a chronic infection ($n = 10/15$, 67%) than in those who spontaneously cleared the virus ($n = 2/9$, 22%), but the difference was not statistically significant ($p = 0.089$). The urine of 8 patients ($n = 8/12$, 67%) tested positive for HEV RNA at the chronic phase (median RNA concentration: 2.64 [2.16–2.73] log_{10}IU/mL). The urine HEV RNA concentrations were similar whatever the phase of infection or the disease evolution.

Discussion

The results of this study in immunocompromised subjects indicate that HEV RNA and Ag are frequently present in the urine of both acutely and chronically HEV3-infected patients.

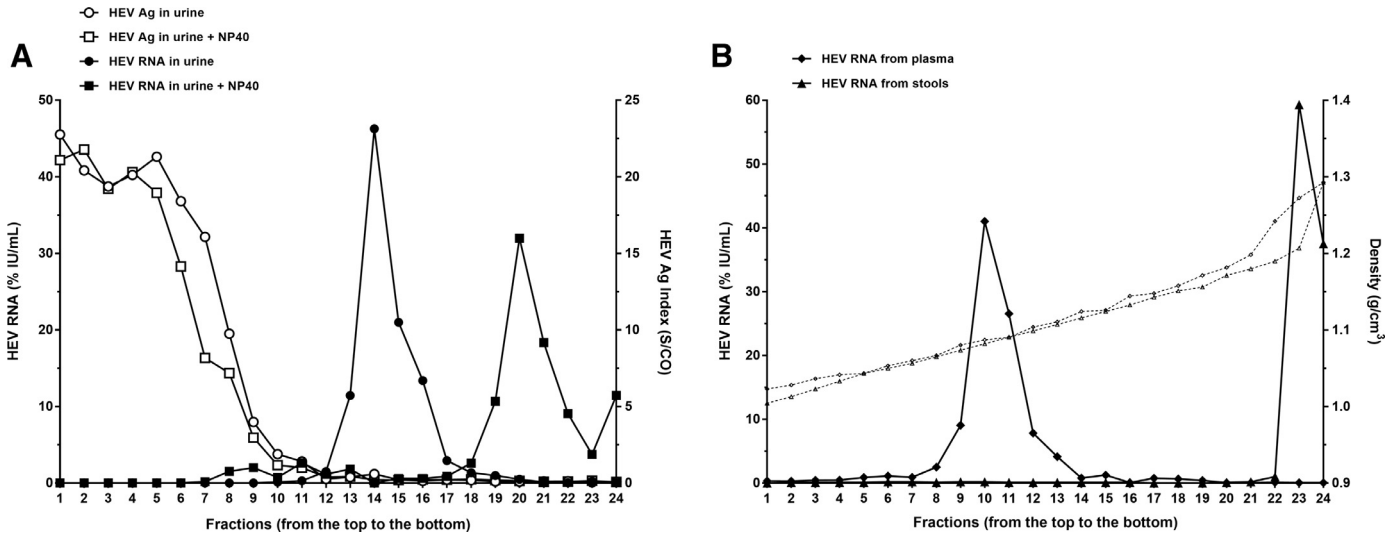

Fig. 2. Iodixanol density gradient centrifugation of HEV particles from the urine, plasma and stools.
A. HEV-RNA-containing particles from urine (●) migrated at a major peak density of 1.11–1.12 g/cm^3; Greater density (1.18–1.20 g/cm^3) after treatment with the nonionic detergent NP40 (■), corresponding to unenveloped particles. HEV Ag was detected in detergent-treated (□) and untreated (○) fractions of urine, after iodixanol density gradient centrifugation. B. Enveloped HEV particles (♦) from the plasma of a patient infected with HEV3. Their migration density of 1.08–1.09 g/cm^3 reflects their association with a lipid membrane. The unenveloped HEV particles (▲) from the feces of a patient chronically infected with HEV3 migrated at a higher density (1.20–1.22 g/cm^3). Abbreviations: HEV, hepatitis E virus; Ag, antigen; S/CO, signal-to-cutoff ratio.

Table 2
Patients with and without HEV RNA in their urine during the acute phase of infection.

Variable	HEV RNA positive urine ($n=12$)	HEV RNA negative urine ($n=12$)	p-value
Age (years)	54 [42–61]	52 [43–67]	0.810
Gender (male/female)	10/2	8/4	0.640
Serum creatinine (µmol/L)	108.5 [72.3–145.3]	100.0 [65.0–137.5]	0.561
eGFR CKD-EPI (mL/min/1.73m^2)	59.5 [41.0–100.0]	65.5 [50.0–91.8]	1
Proteinuria (mg/g)	40.0 [13.0–127.0]	14.5 [8.3–77.5]	0.230
Hematuria (> 10 red cells/mm^3, n)	0	0	–
AST activity (IU/L)	87 [48–131]	101 [62–308]	0.541
ALT activity (IU/L)	152 [73–234]	184 [80–492]	0.590
GGT activity (IU/L)	203 [130–413]	170 [62–342]	0.319
AP activity (IU/L)	123 [84–277]	158 [94–467]	0.551
Bilirubin (µmol/L)	11.1 [8.3–14.2]	8.1 [6.1–15.7]	0.173
Hemoglobin (g/dL)	13.4 [11.3–15.4]	13.7 [13.1–15.3]	0.486
Platelet count (G/L)	177 [139–243]	216 [173–259]	0.340
Lymphocyte count (/mm^3)	1 026 [596.5–2421]	1750 [1417–2450]	0.101
Neutrophil count (/mm^3)	3900 [2450–5250]	5150 [3850–6050]	0.155
Tacrolimus trough (ng/mL)	($n=8$)	($n=10$)	
	9.1 [7.4–10.2]	**5.9 [4.8–6.5]**	**<0.001**
mTOR inhibitors trough (ng/mL)	($n=3$)	($n=3$)	0.100
	14.2 [12.4–18.8]	7.1 [5.9–8.7]	
Subgenotypes (3: e/f/c/chi/i/na)	1/7/2/2/–/–	1/3/4/1/1/2	0.537
HEV RNA in serum ($\log_{10}$IU/mL)	**6.58 [5.51–6.95]**	**5.40 [3.92–5.56]**	**0.015**
HEV Ag in serum ($\log_{10}$S/CO)	**3.78 [3.53–4.19]**	**2.77 [1.57–3.62]**	**0.017**
HEV Ag in urine ($\log_{10}$S/CO/g)	**4.08 [3.87–4.25]**	**3.24 [2.74–3.83]**	**0.028**

Abbreviations: HEV, hepatitis E virus; eGFR, estimated glomerular filtration rate; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; AP, alkaline phosphatase; mTOR, mammalian target of rapamycin; na, not available; Ag, antigen; S/CO, signal-to-cutoff ratio; S/CO/g, signal-to-cutoff ratio normalized to urine creatinine.

This study of a homogeneous cohort of HEV3-infected SOT recipients revealed that 12/24 patients (50%) had detectable HEV RNA in their urine during the acute phase of HEV infection. We detected HEV Ag in the urine and blood of all but one of them (96%). Other hepatotropic viruses - hepatitis A virus, hepatitis B and hepatitis C virus - have also been detected in the urine of infected patients.[36–38] Thus, 7–57% of patients having an HCV infection had detectable HCV RNA in their urine.[37,39] Geng et al. also found that the urine of all 8 of their immunocompetent patients with acute HEV genotype 4 infections tested positive for HEV Ag and they detected HEV RNA in 3/8 (38%) of them.[16] The sensitivity of the ORF2 Ag test kit is reported to be 60–91% for the serum of immunocompetent subjects and 94% for that of immunocompromised patients.[25,40] Although detection of HEV RNA in blood remains the gold standard for diagnosing HEV infections, screening urine samples for HEV Ag could be a practical alternative in resource-limited regions, as it needs no molecular diagnostic devices or venipuncture. If its utility is confirmed in immunocompetent patients, it could be particularly appropriate for monitoring epidemics in resource-limited countries.

The HEV virions in urine have never been characterized. We showed that urinary RNA-containing HEV particles form a low density (1.11–1.12 g/cm^3) band after fractionation on an iodixanol gradient, at a slightly higher density than that of HEV particles

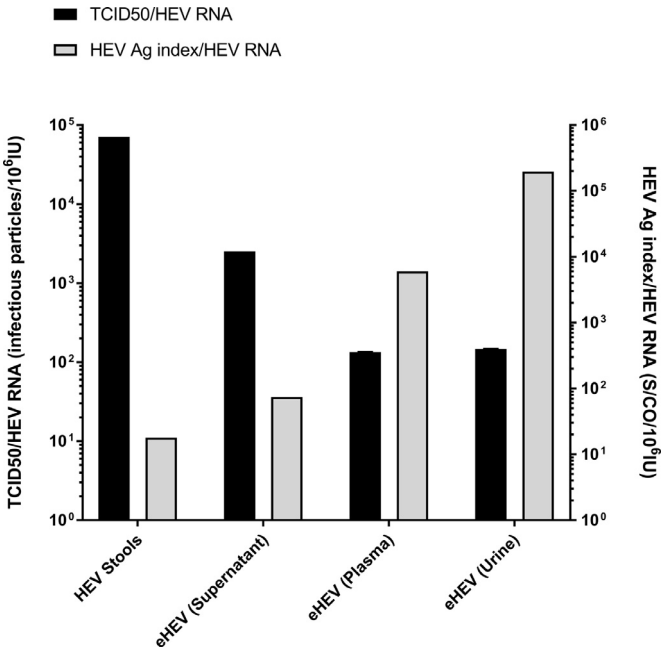

Fig. 3. Infectivity of HEV virions derived from stools (unenveloped virions) and plasma, urine or cell cultures supernatant (quasi-enveloped virions).
Abbreviations: HEV, hepatitis E virus; eHEV, enveloped HEV; Ag, antigen; S/CO, signal-to-cutoff ratio.

from the plasma of infected patients (1.08–1.09 g/cm^3). The difference in the densities of the plasma and urine HEV particles could be due to small differences in lipid membrane compositions. Particles treated with NP40 detergent to remove the lipid membrane had a higher density, similar to that of unenveloped particles (1.18–1.20 g/cm^3). Thus, urinary HEV virions seem to be quasi-enveloped like the particles in the plasma and culture supernatants of HEV infected cells.[9,10]

Geng et al. showed that intravenous inoculation of genotype 4 urine-derived HEV virions could infect cynomolgus monkeys.[16] One of our patients had a urinary HEV RNA load high enough to assess urine HEV3 virions infectivity *in vitro*. The urine-derived HEV particles from this patient were as infectious as his plasma HEV particles.[34] Unenveloped HEV particles were more infectious than quasi-enveloped virions, as previously reported.[10,34,41] Thus, although the infectivity of urinary HEV seemed to be low (~1 infectious particle/10^4 IU HEV RNA), it may well contribute to the spread of virus in the environment.

We did not detect HEV RNA in the urine of all our patients. We have previously failed to detect HEV RNA in the urine of acutely and chronically infected SOT recipients.[42] The populations that were the subjects of these two studies were distinct. We could have failed to amplify some HEV genomes because most of the urinary HEV virus loads were low. But we have also improved our in-house RT-PCR assay for quantifying HEV RNA by using the WHO reference standard.[28,29] Furthermore, only those patients with deep tacrolimus troughs and greater HEV markers concentrations, with high virus loads in the serum and elevated HEV Ag index in both serum and urine, were likely to have HEV RNA-positive urines. Calcineurin inhibitors enhance HEV replication *in vitro*[43] and promote the development of chronic hepatitis in SOT recipients.[21] Thus detecting virus in the urine may indicate over-immunosuppression. Just how tacrolimus favors HEV infection and the persistence of virus in the urine requires further study.

Montpellier et al. identified three forms of the ORF2 capsid protein,[44] and showed that glycosylated by-products (ORF2g, approximately 90 kDa, and ORF2c, approximately 75 kDa) are secreted by infected cells, independently of any infectious particles. This

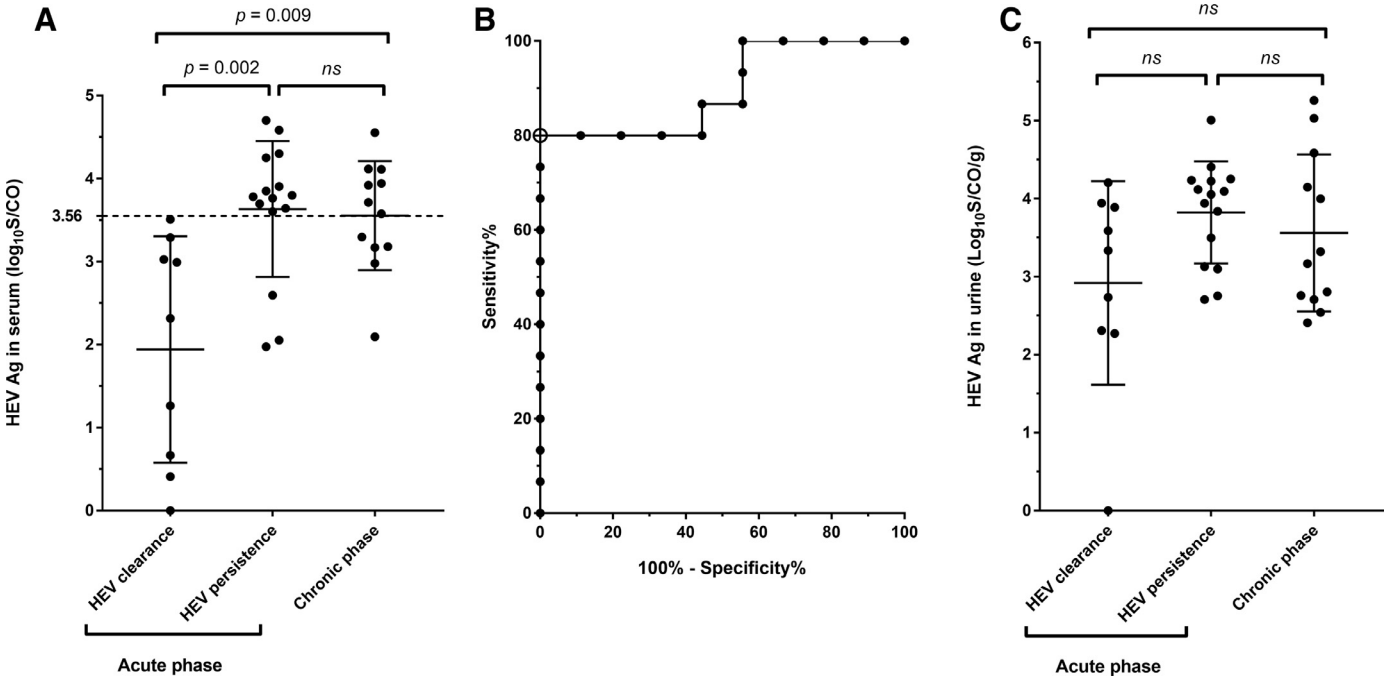

Fig. 4. HEV Ag in the urine and serum during an HEV infection. The HEV Ag concentrations in the sera (A) and urines (C) of SOT patients during the acute and chronic phases of HEV infection were assayed. The dashed line in panel A indicates a putative $\log_{10}$S/CO threshold (3.56) for distinguishing patients with chronic evolution from those who cleared their HEV, as calculated in panel B. B, receiver operating characteristic curve showing the true positive rate plotted against the false positive rate at different serum HEV Ag threshold values to identify patients according to the evolution of their HEV infection. The open circle represents 80% sensitivity (95%CI [52–96]) and 100% specificity (95%CI [66–100]) at an HEV Ag value of 3.56 (area under the curve: 0.896, 95%CI [0.769–1.023]; $p = 0.001$). Differences were tested with a Bonferroni corrected Mann-Whitney *U*-test; ns: $p > 0.05$.
Abbreviations: HEV, hepatitis E virus; Ag, antigen; SOT, solid organ transplant; S/CO, signal-to-cutoff ratio; S/CO/g, signal-to-cutoff ratio normalized to urine creatinine.

observation is in line with the recent study by Yin et al.[45] The secreted form of ORF2 proteins could be the main antigens recognized by the Wantaï® test.[44] The HEV Ag and the virus RNA in the urine formed bands at different densities after iodixanol density gradient centrifugation, as they do in the serum.[9,45] Moreover, we found that HEV Ag concentrated in the urine with a higher RNA to Ag ratio than that in the serum, similarly to the situation in immunocompetent patients.[16] This indicates that there was a high concentration of free HEV Ag in the urine, independently of the number of RNA-containing HEV particles. With its elevated molecular weight, ORF2 protein, like lipid-associated HEV virions, should not freely cross the glomerular filtration barrier.[44,46] However, it is possible that some lower molecular weight HEV antigen byproducts could be excreted in the urine and detected by the Wantaï test. Otherwise, HEV antigen could be secreted into the urine by kidney epithelial cells, resulting in a higher HEV antigen concentration in urine than in plasma. Therefore, the kidney or the urinary tract could be HEV reservoirs.

None of our subjects showed any sign of kidney injury (increased serum creatinine or proteinuria), whatever the HEV status of their urine. The hepatitis A virus also continues to replicate in renal tubules and particles are shed into the urine without causing any clinical renal dysfunction.[47] HEV positive and negative-strand RNA and ORF2 protein have also been detected in the kidneys of infected animals.[12,13,16,48] The main damage shown by histopathological analysis of kidney biopsies was tubule-interstitial with interstitial inflammatory cell infiltrates.[13,16,48] Peptidome analysis of samples from patients with acute hepatitis E showed increased urine alpha-1-microglobulin, a marker of tubular dysfunction in several nephropathies.[49] *De novo* membranous nephropathy and some cases of cryoglobulinemic membrano-proliferative glomerulonephritis have been reported in both immunocompetent[50] and immunocompromised HEV-infected patients.[51,52] Our study of 51 SOT patients also showed that the glomerular filtration rate tended to be decreased during an acute hepatitis E infection (mean eGFR decrease: 5 mL/min).[42] However, the pathogenicity of HEV for the human kidney is essentially unknown.

As a chronic HEV infection can lead to liver fibrosis and extrahepatic manifestations,[53] distinguishing patients who will develop chronic infections from those who will spontaneously eliminate the virus at diagnosis could justify the early initiation of antiviral therapy. Although we reduced the dose of immunosuppressant, 15 of our 24 acutely-infected SOT recipients (62.5%) developed chronic hepatitis, in agreement with previous studies.[17,21] Finding HEV Ag and RNA in the urine of patients during the acute phase of infection did not discriminate between SOT recipients who recovered from those who developed a chronic infection. Conversely, the acute phase serum HEV Ag and RNA concentrations of patients whose infection became chronic were higher than those of SOT recipients with resolving hepatitis E. ROC analysis indicated that serum HEV Ag discriminated between the two groups and a $\log_{10}$S/CO HEV Ag threshold > 3.56 was associated with 80% sensitivity and 100% specificity. Behrendt et al. recently found that HEV Ag was significantly higher in chronically infected patients than in acutely infected subjects.[23] The threshold proposed for discriminating between acutely and chronically infected patients was lower ($\log_{10}$S/CO >1.20) but most of the acutely infected patients were immunocompetent and we have reported that the serum HEV Ag level in immunocompetent patients is lower than in immunocompromised individuals.[25] In our study, the acute and chronic phase serum HEV Ag concentrations of patients whose infections evolved toward chronicity were not significantly different. Thus, measuring the serum HEV Ag at diagnosis provides a useful predictive marker of the development of a chronic infection in SOT patients.

Our study has some limitations. Although our cohort was homogeneous and all patients were on similar T-cells targeting immunosuppressive regimens, the group was small. Studies on larger cohorts are needed to confirm that serum HEV Ag can be used to assess the risk of developing a chronic hepatitis E infection. Also, we did not examine kidney tissue for HEV infection and replication. Lastly, our work was restricted to immunocompromised patients. Studies in immunocompetent patients need to be done.

To conclude, we have shown that HEV3 RNA and Ag are often present in the urine of SOT recipients at both the acute and chronic phases of an HEV infection, without any apparent adverse renal effect. Urinary RNA-containing HEV particles were quasi-enveloped and most of the HEV Ag detected in the urine was not associated with infectious virions. The utility of urinary HEV Ag screening should now be studied in immunocompetent patients. Finally, the value of the serum HEV Ag concentration as a marker of developing a chronic infection should be defined by studies on a larger cohort.

Conflict of interest

None of the authors has anything to declare.

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Authors' contributions Virological work-up: OM, NC, SL, MD, MP and FA; data collection and statistical analyses: OM; paper preparation and review: OM, SL, FA, NK, JI; study design: JI.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2019.01.004.

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