Research Article

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Viral Hepatitis

Detection and assessment of infectivity of hepatitis E virus in urine

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Background & Aims: Hepatitis E virus (HEV) is known to be excreted in the stool but there has been no report of its presence in urine. This study investigated the presence of HEV RNA and antigen (HEV-Ag) in urine and its possible transmission.
Methods: Serum and urine samples from patients with chronic or acute HEV infection and HEV infected monkeys were tested for viral and biochemical markers. Liver and kidney biopsies from the infected monkeys were analyzed by histopathology and immunohistochemistry. The infectivity of HEV from urine was assessed by inoculation into monkeys.
Results: HEV RNA and HEV-Ag were detected persistently in the urine of a patient with chronic HEV infection. Subsequently, HEV RNA was detected in the urine of three of the eight (37.5%) acute patients, all of whom had detectable HEV-Ag in their urine. HEV RNA and HEV-Ag were also detectable in the urine of HEV infected monkeys. The ratio of HEV-Ag to RNA in the urine of the infected monkeys was significantly higher than in their sera and feces. The parameters of routine urinalysis remained within the normal ranges in the hepatitis E patients and infected monkeys, however, pathological changes and HEV-Ag were observed in the kidneys of the infected monkeys. Furthermore, one of two monkeys became infected with HEV after inoculation with urine from another infected monkey.
Conclusions: HEV infection may result in kidney injury and the urine may pose a risk of transmission. HEV-Ag detection in urine may be valuable for diagnosis of ongoing HEV infection.

Keywords: Hepatitis E virus; Urine; Real-time RT-PCR; HEV ORF2 protein; HEV antigen; Enzyme immunoassay.
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Abbreviations: HEV, hepatitis E virus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ORF, open reading frame; GLU, Glucose; BUN, blood urea nitrogen; CRE, creatinine; CHO, cholesterol; TP, total protein; ALB, albumin; TBIL, total bilirubin; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; CK, creatine kinase; URO, urobilinogen; BIL, urobilin; KET, urine ketone; BLD, urine occult blood; AlbU, urine microalbuminuria; α1MU, urine α1-microglobulin; TrfU, urine transferrin; IgGU, urine Immunoglobulin IgG; EIA, enzyme immunoassay; DIP, days post inoculation.

Introduction

Hepatitis E virus (HEV) infection is known primarily to cause acute hepatitis, although the infection can become persistent in immunocompromised patients [1]. Several HEV infection-related extrahepatic manifestations, e.g., neurological symptoms, severe thrombocytopenia, myositis and kidney diseases, have been reported recently [2–5].

HEV is thought to replicate initially in the gut but the main site of replication is the liver, from which the virus is excreted into the intestines via the bile [6]. There is a period of viremia during the normal course of acute infection. Thus, the feces and blood of hepatitis E patients are infectious and HEV may be transmitted via the fecal-oral route or through transfusion [7,8]. Accordingly, detection of HEV RNA and antigen (HEV-Ag) in blood and fecal samples is the main tool for the diagnosis of ongoing HEV infection [8]. Recently, HEV RNA and HEV-Ag were detected in several different tissues during the viremic period [9] and replicative, negative-strand HEV RNA has been detected in the small intestines, lymph nodes, colon and kidneys in animal models [9,10].

To date, the mechanisms of HEV replication and the dynamics of HEV dissemination are not understood completely. There are few reports of the detection of HEV in body fluids and secretions and it is not clear whether HEV is excreted into the urine of hepatitis E patients. The objective of this study was to investigate the presence of HEV in the urine of infected individuals and to assess the infectivity of this urinary HEV.

Materials and methods

Patients and sample collection

The patient with chronic HEV infection, a 35-year-old female, was first diagnosed with hepatitis E in the Baoding Hospital of Infectious Diseases, China in July 2013. She was followed with collection of serum samples for detection of alanine aminotransferase (ALT), aspartate aminotransferase (AST), HEV RNA, anti-HEV IgM and IgG. From March 2014, urine samples were collected in parallel. In April 2013, she had been diagnosed as nephritic syndrome but the etiology of the disease was unknown. After one month of systemic treatment, she received immunosuppressive therapy with oral prednisone to control the disease. The dose of prednisone was reduced gradually and stopped completely in July 2014.

Urine samples were collected from eight patients with acute HEV infection from April to June, 2014, one or two days after the collection of serum. HEV markers were detected immediately or the samples were stored at −80 °C. Ten urine

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samples were collected as controls, five from hepatitis B patients and five from healthy volunteer donors. None of these individuals had any known renal disease.

The study was approved by the Institutional Review Committee of the Health Science Center, Hebei University. Each patient gave written informed consent for the use of their samples and clinical records.

Monkeys, inoculation and sample collection

Seven, 1–2 year old, healthy male cynomolgus monkeys (*Macaca fascicularis*) were assigned to the study. The monkeys were numbered randomly and housed in individual cages. Prior to inoculation, all monkeys were negative for HEV markers.

Feces and urine collected from an acute patient in the viremic period (genotype 4 HEV, GenBank accession number, KM517193), and stored feces collected from a genotype 1 HEV (GenBank accession number, JQ655734) infected patient, were used as inocula. The fecal inocula were prepared using the procedures described previously [11]. Urine was filtered through 0.45 μm and 0.22 μm filters. The final RNA concentrations of the two fecal and one urine inocula, determined by real-time RT-PCR, were 2.45×10^6 IU/ml for the genotype 4, and 7.51×10^5 IU/ml for the genotype 1, fecal suspensions and 2.12×10^4 IU/ml for the urine inoculum.

Each monkey was inoculated intravenously twice on consecutive days with 2 ml of fecal suspension or urine. Monkeys 001 and 002 were inoculated with fecal suspensions of genotype 4 and genotype 1 HEV, respectively, monkeys 003 and 004 with urine from the patient and monkey 005 with PBS as a negative control. After inoculation, serum, urine and fecal samples were collected from each monkey twice per week. All samples were tested immediately or and stored at −80 °C until use.

Another two monkeys (006, 007) were inoculated with urine collected from monkey 001 during the period of viremia at 4 weeks p.i. The inoculum preparation, inoculation and sample collection were as described above; the final HEV RNA concentration in this inoculum was 1.06×10^5 IU/ml.

The animal protocol was approved by the Committee of Laboratory Animal Welfare and Ethics of the National Institutes for Food and Drug Control, China.

Biochemical analysis of blood and urine

The clinical chemistry parameters of the monkey sera were measured, including glucose (GLU), blood urea nitrogen (BUN), creatinine (CRE), cholesterol (CHO), triglyceride (TG), calcium (Ca^{2+}), potassium (K^+), sodium (Na^+), chloride (Cl^-), carbon dioxide (CO_2), ALT, AST, total protein (TP), albumin (ALB), total bilirubin (TBIL), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and creatine kinase (CK) using Hitachi 7060 (Hitachi Ltd., Tokyo, Japan) and Bayer Rapidchem™ 744 (Bayer Corp., Tarrytown, NY, USA). The monkey urine samples also were analyzed, including urobilinogen (URO), urobilin (BIL), urine ketone (KET), urine occult blood (BLD), urine protein, urine microalbuminuria (AlbU), urine α1-microglobulin (α1MU,), urine transferrin (TrfU), urine Immunoglobulin IgG (IgGU) using a Roche Modular auto-biochemical Analyzer P800 (Roche Corp., NY, USA) and red blood cells (RBC) by light microscopy. In order to determine the normal ranges of the above parameters, blood and urine samples isolated from 10 random healthy monkeys were analyzed.

Detection of anti-HEV IgM, anti-HEV IgG and HEV-Ag

Anti-HEV IgM, anti-HEV IgG and HEV-Ag were detected in the serum and urine samples by enzyme immunoassays (EIA), using commercial kits (Wantai, Beijing, China) according to the manufacturer's instructions. In the HEV-Ag system, goat polyclonal anti-ORF2 antibodies are used for antigen capture and enzyme linked monoclonal antibodies detected the ORF2 protein [12].

RNA extraction, RT-nested-PCR, DNA sequencing and phylogenetic analysis

RNA extraction and quantitative NAT procedures were essentially the same as described previously [11–13]. Briefly, total nucleic acids were extracted from 140 μl of samples using the QIAamp Viral RNA Mini kit and procedures (QIAGEN GmbH, Hilden, Germany) and were recovered with 50 μl of the elution buffer. A 282 nt amplicon from HEV ORF1 was amplified by RT-nested PCR and sequenced as described previously [14]. The 282 nt sequences were aligned with the corresponding regions of reference sequences available in GenBank. Phylogenetic analysis was performed with a neighbor-joining algorithm (Molecular Evolutionary Genetics Analysis, MEGA5.2).

Real-time RT-PCR

The real-time RT-PCR was performed using a One Step Real Time kit for HEV RNA (Jinhao, Beijing, China) as described previously [13].

The ratio of HEV-Ag to RNA in urine, serum and fecal samples of monkeys

Serial dilutions of recombinant ORF2 protein and the WHO International HEV Standard for NAT (WHO/BS/2011.2175) [15] were used for validation of the EIA Wantai) and real-time RT-PCR (Jinhao), respectively. Standard curves were drawn for both assays. The concentrations of HEV RNA and HEV-Ag in the urine, serum and fecal samples of HEV infected monkeys were calculated based on the standard curve.

Histopathology and immunohistochemical analysis of liver and kidney biopsies

Liver puncture and renopuncture were performed before inoculation and after the peak ALT level in each monkey. Tissue biopsies were fixed in 10% neutral buffered formalin and embedded in paraffin. Specimens were cut into 3–4 μm serial sections. Slides were stained with hematoxylin-eosin and subjected to histopathological microscopic examination. HEV-Ag were detected immunohistochemically in formalin-fixed, paraffin-embedded needle aspirates of liver and kidney using a mouse-anti-HEV ORF2 monoclonal antibody.

Results

HEV was detected persistently in a patient with chronic HEV infection

From March 2014, urine samples were collected in parallel with sera from the patient who was diagnosed as hepatitis E in July 2013. HEV-Ag was detected in the first urine sample with a higher S/CO (signal to cut-off) than that in serum, 22.4 *vs.* 14.7. HEV RNA concentrations were 1.6×10^5 GE/ml in the urine and 5×10^4 GE/ml in the serum. Anti-HEV IgM and IgG were positive in serum but negative in urine (Table 1). At the same time, her serum tests were as follows: ALT 178 U/L, AST 92 U/L, TBIL 41.1 μmol/L, ALP 65 U/L, GGT 48 U/L. The results of routine urinalysis and kidney function tests were within the normal ranges. HEV RNA and HEV-Ag were also present in the subsequent urine samples and, when the dose of prednisone was gradually reduced, the concentrations of HEV RNA decreased in both urine and serum. Three weeks after stopping the treatment, HEV RNA was undetectable in the serum and urine but HEV-Ag remained positive in the urine for two further weeks, after which it became undetectable (Table 1).

The 282 bp PCR amplicons from the HEV ORF1 region were sequenced and deposited in GenBank (Accession No: KM517191). The sequences from urine and serum were identical and phylogenetic analysis showed that the HEV isolate belongs to genotype 4.

HEV was detected in the urine of patients with acute HEV infection

Three of the eight urine samples collected from patients with acute HEV infection were positive for both HEV RNA and HEV-Ag and the remaining five were positive only for HEV-Ag (Table 2). The HEV-Ag S/CO varied from 9.4–25.5 and 7.2–22.6 in the urine and serum samples, respectively. The HEV-Ag S/CO appeared higher in the urine than in the serum of each patient. Ten control urine samples were negative for HEV RNA, HEV-Ag, anti-HEV IgM and IgG antibodies.