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Viral RNA but no evidence of replication can be detected in the peripheral blood mononuclear cells of hepatitis E virus infected patients

Sirish Kumar Ippagunta[1], **Sita Naik**[2], **Shahid Jameel**[3], **Sudha Ramana KN**[4], and **Rakesh Aggarwal**[1]

Sirish Kumar Ippagunta: ippaguntask@gmail.com; Sita Naik: sitanaik@gmail.com; Shahid Jameel: jameelshahid@gmail.com; Rakesh Aggarwal: aggarwal.ra@gmail.com

[1]Department of Gastroenterology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow 226014, India

[2]Department of Immunology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow 226014, India

[3]Virology Group, International Centre for Genetic Engineering and Biotechnology, New Delhi 110067, India

[4]Sir Ronald Ross Institute of Tropical and Communicable Diseases, Government Fever Hospital, Hyderabad 530 044, India

SUMMARY

Hepatitis E virus (HEV) infection is an important cause of acute viral hepatitis in several developing countries, but has recently been shown to cause chronic hepatitis in immunosuppressed persons. Other hepatotropic viruses that cause chronic infection have been shown to infect peripheral blood mononuclear cells (PBMCs) and to persist in those cells. We therefore decided to look for evidence of replication of HEV in PBMCs obtained from patients with acute hepatitis E, using strand-specific assays for positive and negative HEV RNA. Of the 44 patients with acute hepatitis E during an outbreak in India, including 27 with detectable IgM anti-HEV and 19 with detectable serum HEV RNA, 11 had detectable HEV RNA in their PBMCs. However, of the six PBMC specimens with strong HEV RNA signal, none had detectable negative-strand-HEV RNA, a marker of viral replication. These findings indicate the presence of HEV RNA but the absence of its replication in PBMCs from patients with acute hepatitis E.

Keywords

Hepatitis E; peripheral blood mononuclear cells; replication; disease pathogenesis

INTRODUCTION

Hepatitis E virus (HEV) is a major cause of epidemic and sporadic acute hepatitis in several developing countries [1]. The virus is transmitted by the fecal-oral route, often through contaminated water. The infection is usually self-limited, but fulminant hepatic failure and death occur in about 0.1% of cases. The illness is particularly severe among pregnant women, with mortality rates of 15% to 25% [1]. Based on genomic sequence analysis, HEV

Correspondence to: Rakesh Aggarwal, Department of Gastroenterology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow 226014, India, Phone: +91 522 2494431, FAX: +91 522 2668017, aggarwal.ra@gmail.com.

isolates have been classified into four genotypes [2]; genotypes 1 and 2 are found in HEV endemic areas and affect humans, whereas genotypes 3 and 4 have been found in non-endemic and endemic areas and also infect several animal species.

It has been the belief that HEV infection is cleared rapidly and that chronic infection with HEV does not occur [1]. We have shown that HEV RNA disappears from the serum of patients with acute hepatitis E within 4 weeks of the onset of acute illness [3]. However, recently, Kamar et al. found evidence of chronic infection with genotype 3 HEV in French transplant recipients receiving immunosuppressive drugs [4]. Chronic HEV infection has since also been reported in persons with HIV infection. These chronic HEV infections could arise from newly acquired infection. However, the frequency of transmission of HEV in the areas where chronic HEV infection has been reported is quite low. Another possibility is that previously dormant HEV in an as yet unidentified compartment, such as peripheral blood mononuclear cells (PBMCs), might start replicating during immunosuppression. PBMCs have been shown to serve as a reservoir for other hepatotropic viruses that cause chronic infection, such as hepatitis B virus (HBV) and hepatitis C virus (HCV) [5,6]. No data are available on the presence and replication of HEV in PBMCs. We therefore decided to investigate this.

MATERIAL AND METHODS

Specimens

Blood specimens were collected from 44 patients (age = 13–57 [mean ± SD 26 ± 10] years; 32 male) with acute hepatitis during an outbreak of hepatitis E in Hyderabad, Andhra Pradesh, India. The duration of illness varied from 2 to 10 (7.2 ± 2.3) days. The patients included five pregnant women. PBMCs were isolated from EDTA blood (10 ml) by density gradient centrifugation using Histopaque-1077 (Sigma, St. Louis, USA) as per standard protocol, washed four times with phosphate buffer saline (pH 7.4) and stored in Trizol (Invitrogen, Carlsbad, CA, USA) at −80°C. In addition, serum was stored at −80°C.

All study subjects provided an informed consent. An institutional ethics committee approved the study protocol.

IgM anti-HEV testing

Sera were tested for IgM anti-HEV antibodies using a commercial enzyme immunoassay (Genelabs, Singapore), which detects antibodies against recombinant proteins corresponding to open reading frames (ORF) 2 and ORF 3 of HEV. A cut-off value was calculated as recommended by the manufacturer.

Detection of HEV RNA in serum

RNA was extracted from serum (100 µl) using the QIAamp viral RNA kit (Qiagen, Valencia, CA, USA) in 50 µl of water, as described previously [7]. In brief, a reverse transcription was done using superscript II reverse transcriptase (SS II RT; Invitrogen, Carlsbad, CA, USA) and a reverse primer (PR3, antisense: 5’-TAA TAA CGG CCA TAT TCC AGA CAG TAT TCC-3’), followed by addition of the forward primer (PF2, sense: 5’-TGT TTG AGA ATG ACT TTT CTG AGT TTG ACT-3’) and 35 cycles of PCR amplification [7]. A 1:1000 dilution of 10% suspension (in 0.01M PBS, pH 6.8) of feces from a patient with acute epidemic hepatitis E and RNase free water were used as positive and negative controls, respectively. The PCR products were subjected to 1% agarose gel electrophoresis and Southern hybridization using a digoxigenin-labelled oligonucleotide probe (5’-CTA GAG TGT GCT ATT ATG GAG GAG TGT GGG ATG CCG CAG TGG C-3’) to detect a 237-bp band [7].

Detection of HEV RNA in PBMCs

RNA was extracted from PBMCs [~ 3 million] from each patient using Trizol reagent (Invitrogen). The extracted RNA was initially tested for HEV RNA, as described above. For specimens testing positive, another aliquot was tested using strand-specific RT-PCR separately for positive and negative strand HEV RNA, as described below.

Development of strand-specific assays for positive and negative strand HEV RNA

A recombinant plasmid generated by insertion of a 744 bp (nt 5131 to 4388) ORF1 fragment of human HEV genotype I into pGBKT7 vector was digested with EcoRI (New England Biolabs, Beverly, MA, USA) to produce DNA templates for run-off transcription. Transcription reactions (20 μl) were set up to generate RNA corresponding to HEV negative strand using a MEGAscript kit (Ambion, Austin, TX, USA). Transcribed RNA was digested with 5 U/μg DNA of DNase I (Ambion) for 60 min at 37°C and followed by two rounds of purification using Trizol. After verifying the absence of residual plasmid DNA by PCR without reverse transcription, this RNA was used as a control in assays for detection of negative strand HEV RNA.

Initially, we tried a modification of the RT-PCR assay described above to detect negative-strand HEV RNA, by replacing the primer PR3 with PF2 during reverse transcription. However, since this assay using SS II RT showed only limited discrimination between the amplification of positive and negative HEV RNA strands, we resorted to a RT-PCR assay that used the rTth polymerase. This enzyme being thermostable, carries out reverse transcription at 70°C and minimizes false priming. Using synthetic RNAs, this method has previously been shown to provide about 10,000-fold discrimination in sensitivity for detection of opposing strands of HCV RNA [8].

In brief, for rTth-based assays, cDNA synthesis was carried out in 20 μl reactions containing 5.4 pmol of the appropriate primer (PR3 for positive strand RNA, and PF2 for negative strand RNA) from the ORF1 region of the HEV genome, 1X RT buffer (Applied Biosystems, Foster City, CA, USA), 1 mM $MnCl_2$, 200 μM of each deoxynucleotide triphosphate, and 5 U of rTth DNA polymerase (Applied Biosystems). After 20 min at 70°C, Mn^{+2} was chelated out with 8 μl of the supplied 10X chelating buffer (Applied Biosystems). The opposite primer (5.4 pmol) was then added, followed by adjustment of the reaction volume to 100 μl and of the $MgCl_2$ concentration to 2.2 mM. Amplification conditions were similar to those for the non-strand-specific PCR. The products of RT-PCR (10 μl) were subjected to a semi-nested second round PCR using a forward primer (5'- CTT TTC TTT GGG TCT AGA GTG TGC- 3'), and PR3 for 40 cycles. PCR products (192 bp) were visualized using 1% agarose gel electrophoresis and Southern hybridization with a digoxigenin-labelled HP1 probe, as described above [7]. For standardization, the strand-specific rTth assays were performed on 10-fold serial dilutions (1:10 to $1:10^9$) of HEV RNA extracted from a stool specimen containing positive-strand RNA and on *in vitro* generated negative-strand RNA.

PBMC specimens that showed strong amplification signals by non-strand-specific RT-PCR were tested using the strand-specific rTth RT-PCR assay for positive and negative strand HEV RNA in separate tubes, simultaneously. The assays were done on undiluted RNA as well as on serial 10-fold dilutions of each RNA specimen.

RESULTS

Of the 44 sera, 27 (61%) tested positive for IgM anti-HEV and 19 (43%) had detectable HEV RNA. Overall, 35 (80%) of the 44 sera were positive for one or both of these markers.

Of the 44 PMBC specimens, 11 (25%) tested positive for HEV RNA in the SS II RT based assay (Fig. 1). Of these, 10 were positive for serum IgM anti-HEV and/or HEV RNA, but one was negative for both these markers in the serum.

Using HEV RNA derived from serial dilutions of a stool suspension as template, a signal could be detected up to dilutions of $1:10^6$ and $1:10^4$ using the SS II RT assays for positive-strand and negative-strand HEV RNA, respectively. This 100-fold strand-specific discrimination was considered inadequate. In addition, in SS II RT based assays, an amplification signal was seen even when no primer was added during the reverse transcription step (Fig. 2a).

In comparison, the rTth-based assays showed a better strand specificity. Using the stool suspension as HEV RNA template, a positive signal was detected up to a dilution of $1:10^4$ in the rTth-based assay for positive-strand HEV RNA and up to 10^0 using the assay for negative-strand HEV RNA (Fig. 2b). Similarly, using the *in vitro* generated negative-strand HEV RNA as a template, a signal could be detected up to a dilution of $1:10^9$ using the assay specific for negative strand HEV RNA (Fig. 3a), and up to $1:10^5$ using the positive-strand detection assay (Fig. 3b). Thus, the rTth assays for the positive and the negative strand HEV RNA showed a strand-specific discrimination of 10,000-fold. In addition, the rTth-based assay did not show amplification in the control reaction in which the primer had been omitted during the reverse transcription step.

RNA extracted from PBMCs from 6 patients were tested for HEV RNA using strand-specific rTth RT-PCR assays for positive and negative strand HEV RNA. Of these, one tested positive in rTth PCR assay for positive-strand HEV RNA up to a dilution of 10^3 (Fig. 4a) but was negative in the rTth PCR assay for negative-strand HEV RNA (Fig. 4b). The other five specimens tested positive in assay for positive-strand HEV RNA up to a dilution of 10^2, but were negative in the assay for negative-strand HEV RNA, even when tested undiluted.

DISCUSSION

In the present study, HEV RNA was detected in repeatedly-washed PBMCs from nearly one-fourth of patients with acute hepatitis E. However, these PBMC specimens tested negative in a strand-specific assay for negative-strand HEV RNA, indicating that viral replication in PBMCs was unlikely.

Other hepatotropic viruses that cause chronic infection, namely HBV and HCV, have been shown to replicate in PBMCs, in addition to liver, the main site of infection and viral replication. Laskus et al. reported integration of HBV DNA in PBMCs obtained from patients with chronic hepatitis B [5]. Similarly, presence of HCV RNA has been shown in PBMCs from patients with chronic HCV infection [9–12]. This has prompted several studies to look for evidence of HCV replication, i.e. presence of negative-strand HCV RNA, in these cells. Using sensitive and highly strand-specific assays, several workers have shown the presence of negative-strand HCV RNA in PBMCs [13–19] though, admittedly, some studies have failed to detect such RNA [8,20]. Thus, though the issue remains somewhat controversial, it appears that replication of HBV or HCV in extrahepatic sites, in particular PBMCs, may play a role in the pathogenesis of these infections and in reactivation of these infections in persons receiving immunosuppressive drugs, as also for infection of naïve allograft organs following liver transplantation [21,22].

Replication of HEV in host cells is poorly understood due to the lack of an efficient cell-culture system. However, since HEV has a positive-stranded RNA genome that codes for a putative RNA-dependent RNA polymerase, its replication is likely to involve a negative-

stranded RNA intermediate [23]. Tam et al. showed the presence of both positive and negative strand HEV RNA in hepatocytes infected *in vitro* with HEV [24]. In a swine HEV model, negative-strand HEV RNA was detected in the small intestine, lymph node, colon and liver [25]. In a recent study, Billam et al. identified replicative viral RNA in gastrointestinal tissues, including the colorectal, cecal, jejunal, ileal, duodenal, and cecal tonsil tissues, in addition to the liver in chickens experimentally infected with avian HEV [26]. Negative-strand HEV RNA has also been shown in HepG2 cells transfected *in vitro* with an infectious full-length HEV cDNA clone [27]. Thus, the presence of negative strand RNA appears to indicate ongoing viral replication in a tissue.

Following acute HEV infection, viral RNA can be detected in serum and stool for only a limited period [3]. Chronic HEV infection has not been shown to occur in regions where hepatitis E is endemic. In recent years, however, chronic HEV has been detected in developed countries among persons receiving immunosuppressive drugs [4]. Further, HEV RNA has been detected in a small proportion of healthy blood donor sera. These findings raise the possibility of ongoing low-level viral replication in a compartment such as PBMCs, which serves as a source for infection of the liver once immunosuppression sets in. Thus, whether HEV persists and multiplies in PBMCs appears to be an important issue.

To address this question, we developed a strand-specific rTth assay, with 10,000-fold discrimination between detection of HEV RNA of the desired strand and the alternative strand. In our initial assay, we could detect the presence of HEV RNA in repeatedly-washed PBMCs from a proportion of subjects with acute hepatitis E, including one who tested negative for HEV RNA in the serum. However, using the strand-specific assays, the HEV RNA detected in PBMCs was shown to be exclusively positive-stranded, indicating absence of HEV replication in PBMCs. This finding suggests that HEV RNA does not replicate in PBMCs in patients with uncomplicated acute hepatitis. However, our findings may not exclude persistence of HEV RNA in PBMCs and its replication in the presence of immune suppression.

Our data may be limited by the relatively lower sensitivity of the strand-specific rTth RT-PCR assay. Our rTth assay for positive-strand HEV RNA could detect positive-strand RNA in a stool suspension at a dilution of $1:10^4$ compared to detection at a dilution of $1:10^6$ by a SS II RT based assay; however, the latter assay was not strand-specific and hence could not be used to look for negative-strand HEV RNA. Development of more sensitive assays specific for negative-strand HEV RNA will be useful for confirming our findings.

In conclusion, our data indicate that PBMCs from patients with acute hepatitis E do not contain negative-strand HEV RNA. Hence, these cells are unlikely to be a site for replication of HEV in immunocompetent persons with HEV infection. Further studies are needed to determine whether PBMCs can serve as a reservoir site for reactivation of HEV infection in immunosuppressed persons.

Acknowledgments

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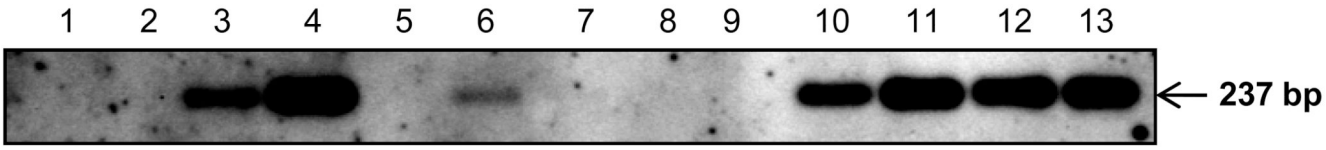

Fig. 1.
HEV-RNA positive-strand detection in PBMCs by RT-PCR using SS II RT. Lane 1: negative control; lanes 2–12: PBMC lane 13: positive control.

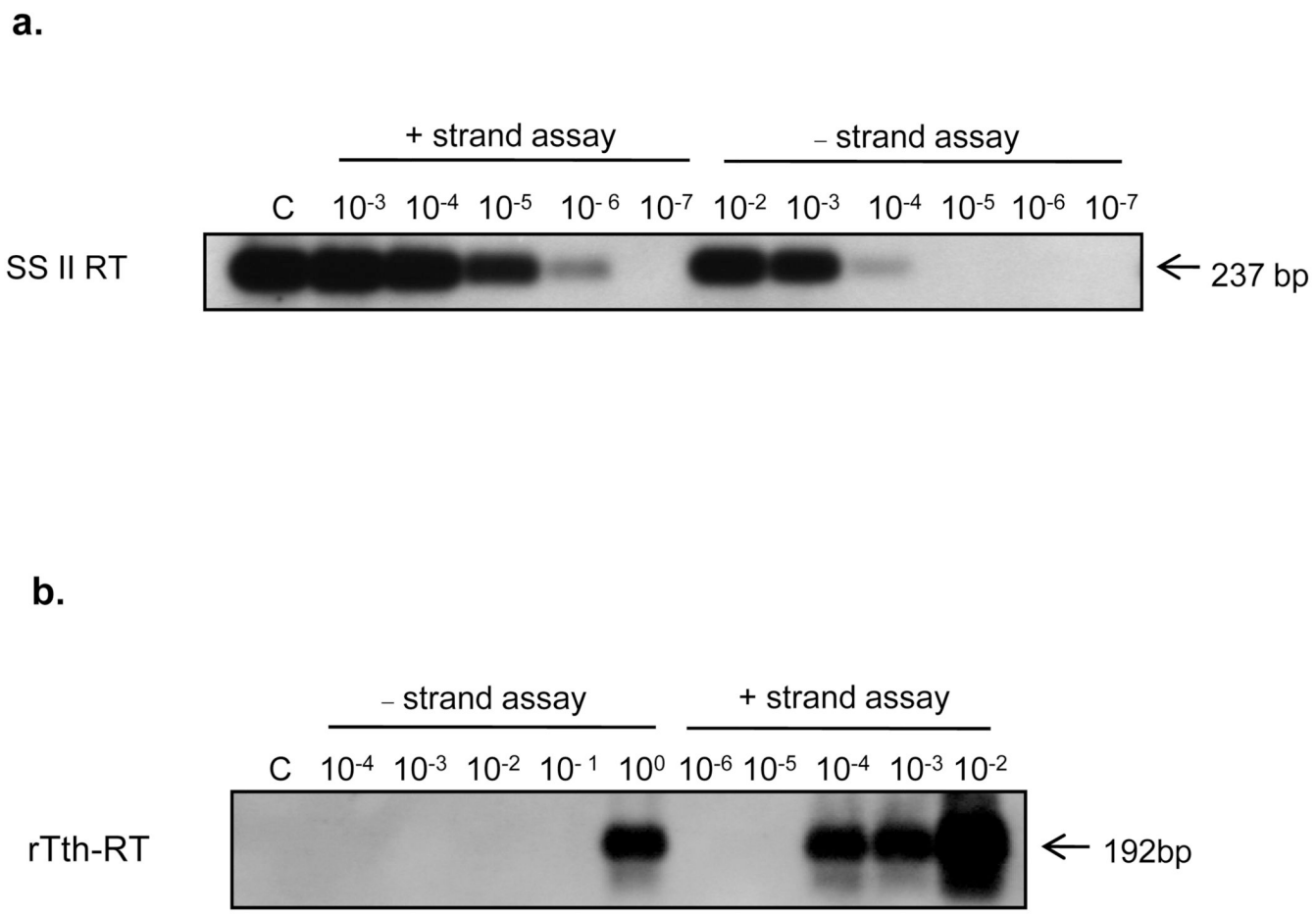

Fig. 2.

Sensitivity and specificity of RT-PCR using SS II RT and rTth (a). Strand-specific RT-PCR assays on 10-fold serial dilutions of positive-strand HEV RNA using SS II RT. Lane C, control-reaction where primer for cDNA synthesis is omitted (b). Strand-specific RT-PCR assays on 10-fold serial dilutions of positive-strand HEV RNA using rTth. Lane C, control-reaction where primer for cDNA synthesis is omitted

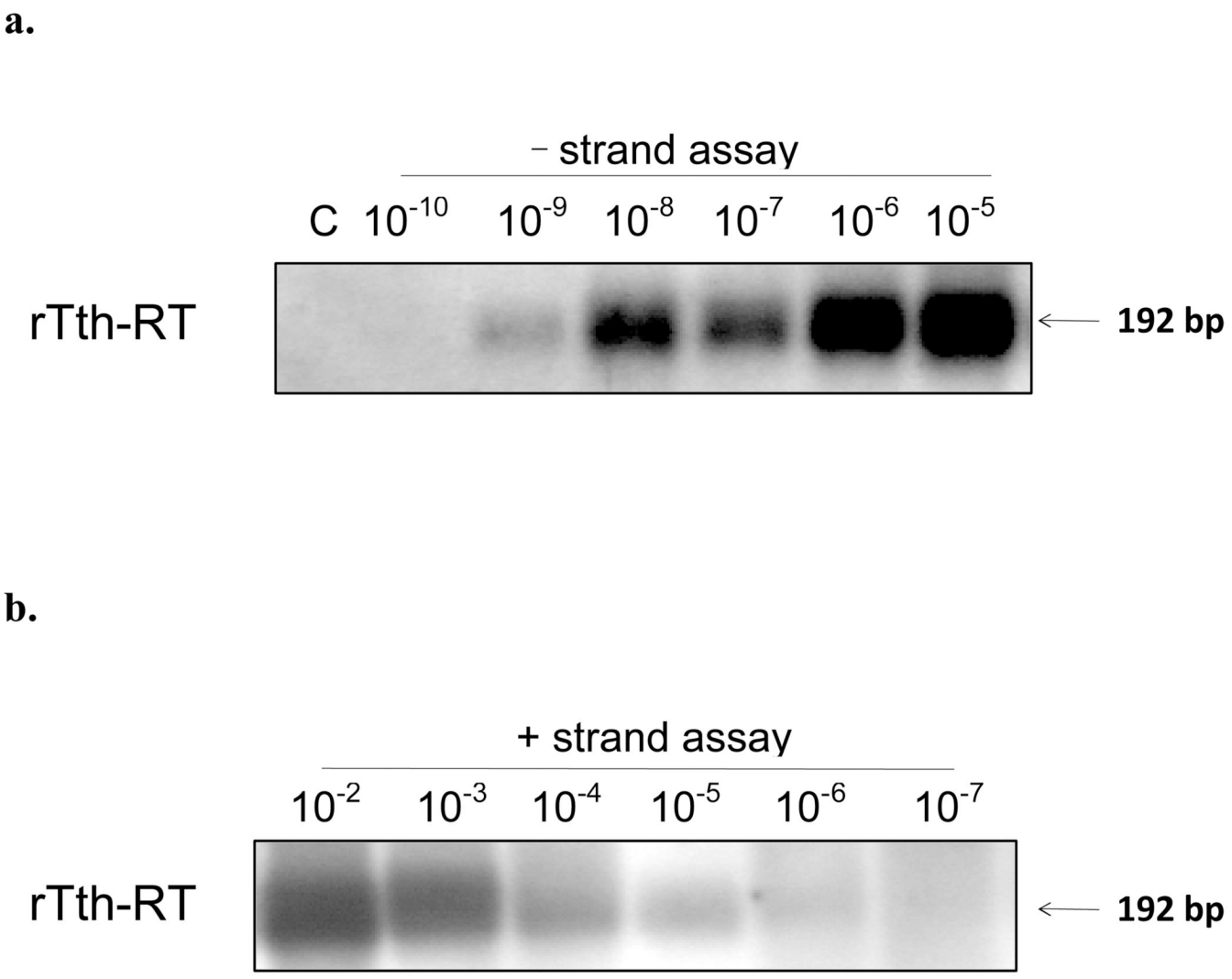

Fig. 3.

Sensitivity and specificity of RT-PCR using rTth (a) Negative-strand-specific assay on 10-fold serial dilutions of negative-strand HEV RNA using rTth. Lane C, control-reaction where primer for cDNA synthesis is omitted (b). Positive-strand-specific assay on 10-fold serial dilutions of negative-strand HEV RNA using rTth.

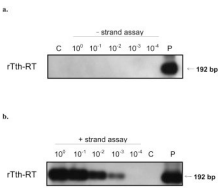

Fig. 4.

HEV-RNA detection in PBMC by strand-specific rTth RT- PCR. (a) Negative-strand-specific assay on 10 fold serial dilutions of total RNA. Lane C, control-reaction where primer for cDNA synthesis is omitted; lane P, positive control. (b) Positive-strand-specific assay on 10 fold serial dilutions of total RNA. Lane C, control-reaction where primer for cDNA synthesis is omitted; lane P, positive control.