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Rat hepatitis E virus derived from wild rats (*Rattus rattus*) propagates efficiently in human hepatoma cell lines☆

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ABSTRACT

Although rat hepatitis E virus (HEV) has been identified in wild rats, no cell culture systems for this virus have been established. A recent report suggesting the presence of antibodies against rat HEV in human sera encouraged us to cultivate rat HEV in human cells. When liver homogenates obtained from wild rats (*Rattus rattus*) in Indonesia were inoculated onto human hepatocarcinoma cells, the rat HEV replicated efficiently in PLC/PRF/5, HuH-7 and HepG2 cells, irrespective of its genetic group (G1–G3). The rat HEV particles released from cultured cells harbored lipid-associated membranes on their surface that were depleted by treatment with detergent and protease, with the buoyant density in sucrose shifting from 1.15–1.16 g/ml to 1.27–1.28 g/ml. A Northern blotting analysis revealed genomic RNA of 7.0 kb and subgenomic RNA of 2.0 kb in the infected cells. The subgenomic RNA of G1–G3 each possessed the extreme 5′-end sequence of GUAGC (nt 4933–4937), downstream of the highly conserved sequence of GAAUAACA (nt 4916–4923). The establishment of culture systems for rat HEV would allow for extended studies of the mechanisms of viral replication and functional roles of HEV proteins. Further investigation is required to clarify the zoonotic potential of rat HEV.

1. Introduction

Hepatitis E virus (HEV) is the causative agent of acute and fulminant hepatitis in many developing countries in Asia and Africa, where the poor sanitary conditions are associated with fecal–oral transmission (Emerson and Purcell, 2013). However, hepatitis E has been found to be endemic in industrialized countries, including Japan, the United States and Europe, where the zoonotic food-borne transmission of HEV from domestic pigs, wild boars and wild deer to humans plays an important role (Colson et al., 2010; Meng, 2013; Takahashi and Okamoto, 2013; Tei et al., 2003; Yazaki et al., 2003). Hepatitis E is typically a self-limiting disease with variable severity, presenting as acute icteric hepatitis with clinical symptoms. However, chronic HEV infection has recently been documented in immunocompromised patients, such as solid-organ transplant recipients and human immunodeficiency virus-infected patients (Dalton et al., 2009; Gerolami et al., 2008; Haagsma et al., 2009; Kamar et al., 2013; Zhou et al., 2013). HEV is classified as the sole member of the genus *Hepevirus* of the family *Hepeviridae* (Meng et al., 2012). Its genome is a single strand, positive-sense RNA of approximately 7.2 kilobases (kb) in size and contains a short 5′-untranslated region (5′-UTR), three open reading frames (ORFs: ORF1, ORF2 and ORF3) and a short 3′-UTR terminated by a poly(A) tract (Tam et al., 1991). ORF1 at the 5′ end of the genome encodes several non-structural proteins involved in replication, while ORF2 codes for a capsid protein of 660 amino acids (aa). ORF3 encodes a small protein of only 113–114 aa that is required for viral infectivity in animals (Graff et al., 2005; Huang et al., 2007) and virion egress (Emerson et al., 2010; Yamada et al., 2009a). ORF2 and ORF3 overlap, and the ORF2 and ORF3 proteins are translated from a single bicistronic subgenomic RNA measuring 2.2 kb in length (Graff et al., 2006; Ichiyama et al., 2009).

☆ The nucleotide sequences of rat HEV isolates reported herein have been assigned DDBJ/EMBL/GenBank accession nos. AB897758–AB897782.

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Four genotypes (genotypes 1–4) of HEV have been identified in humans (Okamoto, 2007). Genotype 1 and 2 HEVs are restricted to humans and often associated with large outbreaks and epidemics in developing countries in Asia and Africa. Genotype 3 and 4 HEVs have a wider host range with several animal species as hosts, including humans as well as pigs, wild boars, deer, mongooses and rabbits (Meng et al., 1997; Nakamura et al., 2006; Sonoda et al., 2004; Takahashi et al., 2004; Zhao et al., 2009): the molecular epidemiological evidence on transmission from reservoirs to humans indicates that genotype 3 and 4 HEVs are zoonotic (Colson et al., 2010; Li et al., 2005; Tei et al., 2003; Yazaki et al., 2003). Recently, additional new putative genotypes of HEV have been identified in wild boars in Japan (Takahashi et al., 2010a, 2011), ferrets in the Netherlands (Raj et al., 2012) and wild rats (*Rattus norvegicus*) in Germany (Johne et al., 2010). Rat HEV strains have been identified in other countries including the United States, Vietnam, Indonesia and China (Li et al., 2011, 2013b; Mulyanto et al., 2013; Purcell et al., 2011). The phylogenetic analysis revealed that rat HEV strains are segregated into three distinct genetic groups [a German type (G1), Vietnamese type (G2) and Indonesian type (G3)] that differ from each other by 19.5–23.5 (22.0 ± 1.7)% over the entire genome (Mulyanto et al., 2014). Of interest, various rat HEV strains classifiable into three genetic groups have been identified in Indonesia, suggesting the global distribution of heterogeneous rat HEV strains in this country (Mulyanto et al., 2014). However, whether rat HEV can be transmitted to humans remains unclear, and no cell culture system for rat HEV has yet been established (Johne et al., 2010).

Recently, Dremsek et al. (2012) reported the detection of anti-rat HEV antibodies in human serum samples, which encouraged us to cultivate rat HEV in human cell lines. In the present study, homogenates of liver specimens obtained from wild rats (*Rattus rattus*) with high viral load in Lombok and Java, Indonesia (Mulyanto et al., 2014), were inoculated onto human hepatocarcinoma cells in order to propagate rat HEV. The rat HEV replicated efficiently in cultured human cells, and the progeny viruses in the culture supernatant and genomic RNAs in the cell lysates were characterized to support the successful propagation of rat HEV in human cells.

2. Materials and methods

2.1. Liver specimens from wild rats with high viral load

In our previous study, paired serum and liver specimens were obtained from 136 black wild rats (*R. rattus*) in Solo (also called Surakarta), a city in Central Java, between September 24 and October 5, 2012 and from 233 wild rats on Lombok Island between October 2 and 22, 2012, and the serum samples were used to investigate the prevalence of rat HEV infection and analyze rat HEV genomes (Mulyanto et al., 2014). In the present study, the rat HEV RNA in the liver specimens of 99 viremic rats was quantitated, and liver homogenate samples with a high load of rat HEV belonging to each of three genetic groups (G1, G2 or G3) were used as inocula in cell cultures, as described below (see Table 1). All paired serum and liver samples were stored at −20 °C in Indonesia and −80 °C after being sent to Japan and preserved until testing.

2.2. Qualitative and quantitative detection of rat HEV RNA

Total RNA was extracted from 20 to 100 μl of each liver homogenate or cell lysate using the TRIzol reagent (Life Technologies, Carlsbad, CA) or 10–100 μl of each culture medium using the TRIzol LS reagent (Life Technologies) according to the manufacturer's instructions. In order to detect rat HEV RNA in the liver homogenate or culture medium, a portion of the ORF1 and ORF2 junction region (primarily the ORF1 region) was amplified *via* nested reverse transcription (RT)-PCR using the primer sets HE607 (sense) and HE604 (antisense) in the first round and HE608 (sense) and HE606 (antisense) in the second round (ORF1-PCR), as previously described (Mulyanto et al., 2013). These sets generated amplification products of 899 base pairs (bp) (nt 4098–4996) and 880 bp (nt 4103–4982), respectively; the nucleotides are numbered in accordance with the prototype rat HEV strain [rat/R63/DEU/2009 (GU345042)], unless otherwise stated. The RT-PCR assay was performed in duplicate, and its reproducibility was confirmed. The specificity of the RT-PCR assay was verified using a sequence analysis, as described below.

The RNA of rat HEV was quantitated in various specimens using real-time RT-PCR according to the previously described method (Mulyanto et al., 2014), employing an *in vitro*-transcribed rat HEV RNA as a standard. Briefly, using the pT7Blue T-Vector (Merck Millipore, Tokyo, Japan) containing a 341-nt fragment of ratIDE079 cDNA (nt 35–375) as a template and the AmpliScribe™ T7-*Flash*™ Transcription Kit (Epicentre Biotechnologies, Madison, WI), rat HEV RNA was transcribed *in vitro*, purified and used as a standard of rat HEV RNA. The RNA preparations obtained from the liver homogenates, culture media, cell lysates and fractions *via* ultracentrifugation (see below) were subjected to real-time RT-PCR with a QuantiTect Probe RT-PCR Kit (QIAGEN, Tokyo, Japan) using the sense primer HE655 [5′-CCA CGG GGG TTA ATA CTG C-3′ (nt 36–54)], the antisense primer HE656 [5′-CGG ATG CGA CCA AGA AAC AG-3′ (nt 189–208)] and a probe (HE657-P) consisting of an oligonucleotide with a 5′-reporter dye (6-carboxyfluorescein, FAM) and a 3′-quencher dye (6-carboxytetramethyl-rhodamine, TAMRA) [5′-FAM-CGG CTA CCG CCT TTG CTA ATG C-TAMRA-3′ (nt 81–102)] on an Applied Biosystems 7900HT Fast Real-Time PCR System (Life Technologies). The thermal cycler conditions were 50 °C for 30 min, 95 °C for 15 min and 50 cycles of 94 °C for 15 s followed by 56 °C for 30 s. From standard dilutions analysis, the sensitivity of this real-time RT-PCR assay was estimated to be 2–5 copies/test or 20–50 copies/ml (when 100 μl of samples were used). It is expected that the sensitivity of this assay is the same for the genetic groups 1, 2 and 3, since the primers and a probe used were derived from well-conserved genomic area among the three genetic groups. The reproducibility of the quantitative assay was assessed by testing each sample in duplicate, and the mean value was adopted.

2.3. Sequence analysis of the PCR products

The amplification products were sequenced directly on both strands using a BigDye Terminator v3.1 Cycle Sequencing kit (Life Technologies) on an Applied Biosystems 3130xl Genetic Analyzer (Life Technologies). The sequence analysis was performed using the Genetyx software program (version 11.1.2; Genetyx Corp., Tokyo, Japan), and multiple alignments were generated by the CLUSTAL Omega software program (version 1.2.0) (Goujon et al., 2010). Phylogenetic trees were constructed according to the neighbor-joining method (Saitou and Nei, 1987) with the Kimura two-parameter model and 1000 replicates of bootstrap resampling, as implemented in the MEGA5 software program (version 5.2.1) (Tamura et al., 2011).

2.4. Preparation of inocula for cell culture

A piece of liver tissue (200 mg) was minced with a razor blade and homogenized with a BioMasher II (Nippi Incorporated, Tokyo, Japan) in the presence of 1.8 ml of phosphate buffered saline (pH 7.5) without Mg^{2+} and Ca^{2+} [PBS(−)] and clarified *via* centrifugation in a high-speed refrigerated microcentrifuge (Tomy Seiko, Tokyo, Japan) at 7900 × *g* at 4 °C for 10 min, and a clear supernatant was obtained. The 10% (w/v) homogenates were used as the inoculum. Aliquots of culture supernatant containing the rat HEV progeny