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Production of infectious ferret hepatitis E virus in a human hepatocarcinoma cell line PLC/PRF/5

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ABSTRACT

A strain of ferret hepatitis E virus (HEV), sF4370, isolated from an imported ferret was used to inoculate a human hepatocarcinoma cell line, PLC/PRF/5. The virus genome and capsid protein were detected in the cell culture supernatant. Immunofluorescence microscopy indicated that the capsid protein was located in the cytoplasm. The virus particles were purified from the culture supernatant by sucrose gradient ultracentrifugation. The capsid protein with molecular mass of ~72 kDa was detected in fractions with density of 1.150–1.162 g/cm^3, and particles of ferret HEV was associated with cell membrane. The virus recovered from the supernatant was serially passaged with PLC/PRF/5 cells and had the ability to infect ferrets by oral inoculation, indicating that the ferret HEV grown in PLC/PRF/5 was infectious. The establishment of ferret HEV cell culture system might be useful to understand the life cycle, mechanism of infection and replication of ferret HEV.

1. Introduction

Hepatitis E virus (HEV) is a single-stranded positive-sense RNA virus which primarily transmits by the fecal-oral route (Balayan et al., 1983; Chauhan et al., 1993; Reyes et al., 1993). HEV belongs to the genus *Hepevirus* in the family *Hepeviridae* (Meng et al., 2012), which infects a wide range of mammalian species, as well as chickens and trout (Batts et al., 2011; Meng, 2010; Smith et al., 2013). The family *Hepeviridae* was recently proposed to be divided into two genera, *Orthohepevirus* and *Piscihepevirus* (Smith et al., 2014) and this classification has already been included in the official ICTV taxonomy (http://ictvonline.org/virusTaxonomy.asp). The *Orthohepevirus* includes four species. *Orthohepevirus A* includes isolates from humans, pigs, wild boar, deer, mongooses, rabbits and camels; the genotype 1–4 (G1–G4) HEVs infect humans and cause a self-limiting acute hepatitis that may become chronic in immunosuppressed individuals (Emerson and Purcell, 2003; Kamar et al., 2013, 2008; Riezebos-Brilman et al., 2013). *Orthohepevirus B* includes isolates from chickens; *Orthohepevirus C* includes isolates from rats, greater bandicoots, Asian musk shrews, ferrets and mink; and *Orthohepevirus D* includes isolates from bats. The cutthroat trout virus belongs to the genus *Piscihepevirus*.

Ferret HEV was first detected in ferrets (*Mustela putorius*) in the Netherlands (Raj et al., 2012), and has since been detected in ferrets used as laboratory animals and pets in the US and Japan (Li et al., 2014, 2015c). The genome structure of ferret HEV is similar to that of other HEVs, and contains three open reading frames (ORFs 1–3). ORF1 encodes a nonstructural protein of 1589 or 1596 amino acids (aa), ORF2 encodes a capsid protein of 654 aa, and ORF3 encodes a functionally unknown phosphoprotein of 108 aa. In addition, a putative ORF4 encoding 183 aa was observed in the ferret HEV genome, although its function is unknown (Li et al., 2014; Raj et al., 2012). Ferret HEV-like particles (VLPs) have been produced by expression of a partial ferret HEV ORF2 gene by a recombinant baculovirus system, and an enzyme-linked immunosorbent assay (ELISA) for detection of anti-ferret HEV IgG and IgM antibodies has been established using VLPs as the antigen (Yang et al., 2013). The preliminary epidemiological studies indicated that the positivity rate for anti-ferret HEV IgG was above 20% in in the US and Japan (Li et al., 2015c; Yang et al., 2013). The ferret HEV RNAs detected in the US and Japan are genetically different from that isolated in the Netherlands (Li et al., 2014, 2015c). In addition, the anti-

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body against VLPs does not neutralize G3HEV, suggesting that the serotypes of these two HEVs are different (Yang et al., 2013). Ferret HEV is transmitted by an oral-fecal route and causes acute or persistent infection in ferrets (Li et al., 2015a, 2016). Our recent study indicated that rats and cynomolgus monkeys are not susceptible to ferret HEV, suggesting that the reservoirs of ferret HEV are limited (Li et al., 2015a).

Propagation of human, rabbit and rat HEV *in vitro* has been performed in human hepatocarcinoma cells, and the infectious virus was successfully produced (Jirintai et al., 2012, 2014; Tanaka et al., 2007). However, no cell culture system to grow ferret HEV is currently available, and the mechanism of the replication and infection of ferret HEV remain unclear. In the present study we inoculated the ferret HEV isolated from ferret stool specimens onto a human hepatocarcinoma cell line, PLC/PRF/5, and found that the ferret HEV grows in PLC/PRF/5 cells and the virus recovered from the cell culture is infectious.

2. Materials and methods

2.1. Ferret HEV

A ferret HEV strain, sF4370, was isolated from the fecal specimen of a ferret which was imported to Japan with other 62 ferrets from a ferret farm in the US. In this group 40 (63.5%) of these 63 ferrets were positive for ferret HEV RNA (Li et al., 2014). The stool specimens were diluted with 10 mM phosphate-buffered saline (PBS) to prepare a 10% suspension, shaken at 4 °C for 1 h, and clarified by centrifugation at 10,000 × *g* for 30 min. The supernatant was passed through a 0.45 μm membrane filter (Millipore, Bedford, MA) and stored at −80 °C until use. The titer of the ferret HEV was 4×10^6 copies/ml by a quantitative real-time RT-PCR.

2.2. Cell culture and virus inoculation

A human hepatocarcinoma cell line, PLC/PRF/5 (JCRB0406), and a human lung carcinoma cell line, A549 (IF050153), were obtained from the Health Science Research Resources Bank, Japan. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Nichirei, Biosciences. Inc. Tokyo, Japan) and 100 U penicillin and 100 mg streptomycin (Gibco, Grand Island, NY) at 37 °C in a humidified 5% CO_2 atmosphere. For virus inoculation, confluent cells were trypsinized, diluted 1:3 and cultured in a 25 cm^2 tissue culture flask. The next day, the medium was removed and the cells were washed two times with PBS. A total of 1 ml of the 10% stool suspension sample was inoculated onto PLC/PRF/5 cells. After adsorption at 37 °C for 1 h, the cells were washed two times with PBS, and then replaced with 10 ml maintenance medium consisting of medium 199 (Invitrogen, Carlsbad, CA) containing 2% (v/v) heat-inactivated FBS and 10 mM $MgCl_2$. Further incubation was done at 36 °C. The culture medium was replaced with new medium every 4 days and used for the detection of ferret HEV capsid protein and RNA. The cells were observed daily by light microscopy for the cytopathic effect.

2.3. Quantitative real-time RT-PCR (RT-qPCR) for detection of ferret HEV

The RNA was extracted using a MagNA Pure LC system with MagNA Pure LC Total Nucleic Acid isolation (Roche Applied Science, Mannheim, Germany) according to the manufacturer's recommendations. To determine the ferret HEV RNA copy numbers, a TaqMan assay was performed by using a 7500 FAST Real-Time PCR System (Applied Biosystems, Foster City, CA) with TaqMan Fast Virus 1-step Master Mix (Applied Biosystems, Foster City, CA). The primers consisted of 900 nmol/l forward primer (5′-TGAGTGGTGTTTTTATGGCGA-3′, nt 4006–4026) and 900 nmol/l reverse primer (5′-CAAACTCAGAAAAATCATTCTCAAAGAC-3′, nt 4082–4109), and 250 nmol/l probe (5′-6FAM-GCCATGCCGCGGTTTGAGGCGGCTGT-TAMRA-3′, nt 4034–4059). The condition for the one-step RT-qPCR was 15 min at 48 °C, a 10-min incubation at 95 °C, and 50 cycles of 15 s at 95 °C and 1 min at 60 °C. The capped *in vitro*-transcribed RNA of ferret HEV was used as the standard to calculate the copy number of the RNA molecule. A 10-fold serial dilution of the RNA standards (10^7–10^1 copies) was used for the quantitation of viral genome copy numbers. Amplification data were collected and analyzed with Sequence Detector software version 1.3 (Applied Biosystems). This RT-qPCR system, with a sensitivity of 10 copies, was used exclusively for ferret HEV.

2.4. Western blot analysis

The protein samples were separated by 5–20% SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane. The membrane was then blocked with 5% skim milk in 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, and incubated with rabbit anti-ferret HEV-LPs polyclonal antibody (Yang et al., 2013). Detection of the rabbit IgG antibody was achieved using alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (1:1000 dilution) (Chemicon International, Temecula, CA). Nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate *P*-toluidine were used as coloring agents (Bio-Rad Laboratories, Hercules, CA).

2.5. Detection of the ferret HEV capsid protein

An antigen capture ELISA was used to detect ferret HEV capsid protein. Briefly, duplicate wells of flat-bottom 96-well polystyrene microplates (Dynex Technologies Inc., Chantilly, VA) were coated with 100 μl of a coating buffer (0.1 M carbonate–bicarbonate buffer, pH 9.6) containing 1:2000 diluted rabbit anti-ferret HEV-LPs serum. The coating was performed at 4 °C overnight. Unbound antibodies were removed by washed wells twice with 10 mM PBS containing 0.05% Tween 20 (PBS-T). The blocking was carried out at 37 °C for 1 h with 150 μl of 5% skim milk (Difco Laboratories, Detroit, MI) in PBS-T. 100 μl of the cell culture supernatant was added to the wells after well washing and incubated for 1 h at 37 °C. Then 100 μl of guinea pig anti-VLPs hyperimmune serum (1:10,000 dilution with PBS-T containing 1% skim milk) was added to the wells and the plate was incubated for 1 h at 37 °C. The plate was washed 3 times with PBS-T, and then horseradish peroxidase-conjugated goat anti-guinea pig IgG antibody (1: 2000 in PBS-T containing 1% skim milk; Cappel, Durham, NC) was added to each well. After incubation for 1 h at 37 °C, the plate was washed 3 times with PBS-T and 100 μl of substrate o-phenylenediamine was added. The plate was left for 30 min at room temperature, and then the reaction was stopped with 50 μl of 4 N H_2SO_4. The absorbance at 492 nm was measured with a microplate reader (Molecular Devices Corp., Tokyo, Japan). In one plate three wells were added with the normal cell culture supernatants as negative control. When the ratio of OD values between the sample and negative control was higher than 3.0, the sample was judged to be positive.

2.6. Anti-ferret IgG and IgM antibodies

Anti-ferret HEV IgG and IgM antibodies were detected by enzyme-linked immunosorbent assays (ELISAs) by using ferret HEV-LPs as antigens according to the previously described methods (Yang et al., 2013).