Hepatitis E Virus Outbreak in Monkey Facility, Japan

Hiroshi Yamamoto, Juri Suzuki, Atsushi Matsuda, Takafumi Ishida, Yasushi Ami, Yuriko Suzaki, Isao Adachi, Takaji Wakita, Naokazu Takeda, and Tian-Cheng Li

An outbreak of hepatitis E virus occurred in an outdoor monkey breeding facility in Japan during 2004–2006. Phylogenetic analysis indicated that this virus was genotype 3. This virus was experimentally transmitted to a cynomolgus monkey. Precautions should be taken by facility personnel who work with monkeys to prevent infection.

Wild or reared monkeys have been used as disease models in animal facilities worldwide. Because disease caused by hepatitis E virus (HEV) is a zoonosis (*1–4*), monkeys might be infected. We examined the prevalence of antibodies against HEV in serum and fecal samples collected from monkeys in animal facilities at the Primate Research Institute of Kyoto University in Japan for 6 years (2004–2009). We found that spontaneous infection and transmission of HEV occurred in a monkey facility.

The Study

There are 9 monkey colonies (A–I) at the Primate Research Institute of Kyoto University. Colonies A–G contained Japanese monkeys (*Macaca fuscata*), and colonies H and I contained rhesus monkeys (*Macaca mulatta*). Each colony was bred in a separate outdoor breeding facility. A total of 588 monkey serum samples were collected during September–November 2004–2009 and tested for IgG and IgM against HEV and for HEV RNA by ELISA or reverse transcription PCR (RT-PCR) as described (*5–7*). Samples from colonies G and F were collected during 2004–2006, whereas in 2009 samples were collected from colonies A, C, D, and I.

The prevalence of IgG against HEV was 0% in 2004, 20.0% in 2005, and 78.5% in 2006, followed by a gradual decrease to 35.9% in 2009 (Table 1). The prevalence of IgM against HEV increased from 0% in 2004 to 2.5% in 2005 and to 6.6% in 2006, and then decreased to 1.1% in 2007 and 0% in 2008 and 2009.

Table 1. Prevalence of IgG and IgM against hepatitis E virus in monkeys at monkey facility, Japan, 2004–2009

Year	No. positive/no. tested (%)	
	IgG	IgM
2004	0/110	0/110
2005	24/120 (20.0)	3/120 (2.5)
2006	96/121 (78.5)	8/121 (6.6)
2007	73/96 (76.0)	1/96 (1.1)
2008	47/90 (52.2)	0/90
2009	18/51 (35.3)	0/51

IgG against HEV was not detected in any of the 9 colonies in 2004, indicating that HEV infection did not occur before October 2004. However, in 2005, the prevalence of IgG reached 100% in colony D and 20% in colony G (Figure 1). ELISA titers were high, ranging from 0.293 to 1.641 in colony D and from 0.230 to 0.845 in colony G. These results suggested that HEV infection occurred after October 2004 in the monkey facility. The prevalence of IgG was higher in colony D than in colony G, and IgM was not detected in colony D, suggesting that HEV infection occurred earlier in colony D than in colony G. These colonies adjoined each other, indicating that the first HEV infection occurred in colony D and was then transmitted to colony G. Colonies A, C, D, E, and H each had an IgG prevalence of 90%–100%, and colonies B and G had an IgG prevalence >80% in 2006 (Figure 1). These results demonstrated that infection spread over a large area, except for colony F, during 2005 and 2006.

To compare the kinetics of IgG formation during 2004–2009, serum samples from 25 monkeys awhose peak ELISA optical density (OD) values for IgG against HEV were each higher than 1.0 were selected. In most monkeys, OD values for IgG increased rapidly and then decreased gradually year by year. The kinetic pattern of monkey M1543 was different from those of other monkeys that had high OD values (2.568–2.738). IgM was detected exclusively in this monkey in 2006 (OD value 0.620).

Serum samples from the 25 monkeys were used to detect HEV RNA by RT-PCR. Four serum samples were positive for HEV RNA; all were from the same monkey (M1543) from which samples were collected in 2006, 2007, 2008, and 2009. Nucleotide sequences of 348 bp coding the partial open reading frame 2 showed 100% identity. This result indicated that monkey M1543 was infected persistently with HEV and produced virus continuously.

To examine whether HEV was present in feces, 2 fecal samples were collected from monkey M1543 in September and November 2009 for detection of HEV RNA. Both samples were positive for HEV RNA. Nucleotide sequences of these samples were identical to those detected from serum samples.

Author affiliations: University of Toyama, Toyama, Japan (H. Yamamoto, I. Adachi); Kyoto University, Aichi, Japan (J. Suzuki); University of Tokyo, Tokyo, Japan (A. Matsuda, T. Ishida); National Institute of Infectious Diseases, Tokyo (Y. Ami, Y. Suzaki, T. Wakita, T.-C. Li); and Osaka University, Osaka, Japan (N. Takeda)

DOI: http://dx.doi.org/eid1812.120884

Primers were designed on the basis of sequences of swine HEV (GenBank accession no. AB248522), and RT-PCR was performed to amplify the viral genome except for the N terminus noncoding region. This strain was designated the monkey HEV Inuyama strain (JQ026407). Phylogenetic analysis of its genome indicated that this strain belongs to HEV genotype 3 (Figure 2). Infectivity of the monkey HEV strain was examined ex vivo with a human hepatocarcinoma cell line (PLC/PRF/5), and in vivo with 2 HEV-negative cynomolgus monkeys. Both experiments showed that the virus was infectious (online Technical Appendix Figures 1 and 2, wwwnc.cdc.gov/EID/pdfs/12-0884-Techapp.pdf).

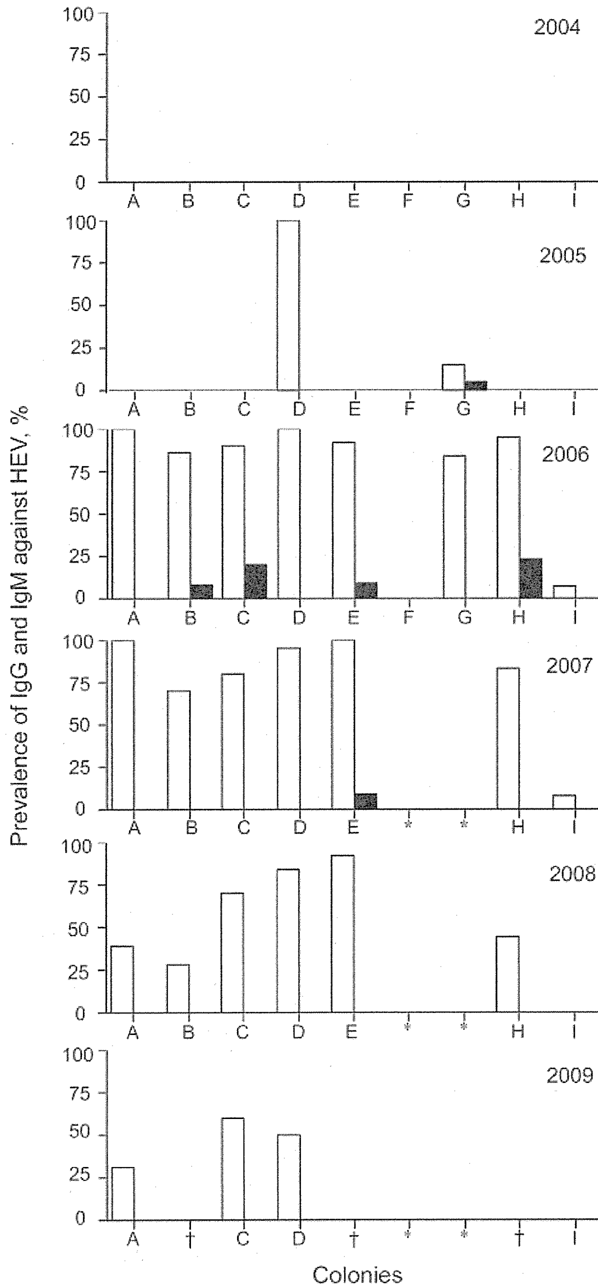


Figure 1. Prevalence of IgG (white bars) and IgM (black bars) against hepatitis E virus (HEV) in monkey facility, Japan, 2004–2009. *Monkeys were moved to another animal facility; †specimen not available.

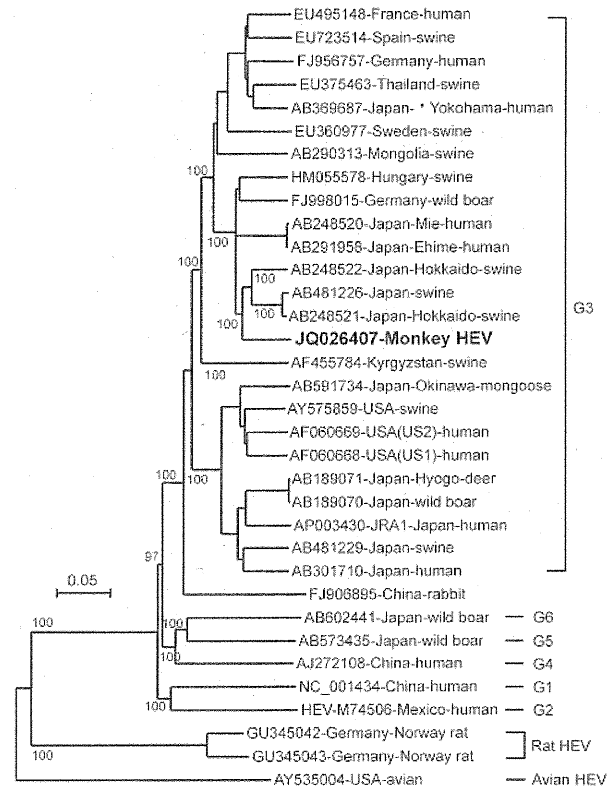


Figure 2. Phylogenetic analysis of monkey hepatitis E virus (HEV) Inuyama strain on the basis of nucleotide sequence of the HEV genome except for a 5′ noncoding region (7,206 nt) by using avian HEV as an outgroup. Values along the branches are bootstrap values determined on the basis of 1,000 resamplings of datasets. **Boldface** indicates strain isolated in this study. Genotypes are indicated on the right. Scale bar indicates nucleotide substitutions per site.

A total of 94 human serum samples were collected from staff of the Primate Research Institute and subjected to ELISA for detection of IgG and IgM against HEV. All serum samples were negative for IgM against HEV, but the prevalence of IgG was 6.9% in 2007, 9.7% in 2008, and 11.8% in 2009, although differences among these years were not significant ($p>0.05$) (Table 2). No HEV RNA was detected in serum samples, and none of the staff had symptomatic hepatitis E during the 6-year study.

Conclusions

We conducted long-term monitoring of HEV infection in monkeys and report natural infection and transmission of HEV in a monkey facility. We sought to determine the source of the HEV outbreak and where HEV was intro-

Table 2. Prevalence of IgM against hepatitis E virus in serum samples from animal handlers at monkey facility, Japan, 2007–2009*

Year	No. positive/no. tested (%)
2007	2/29 (6.9)
2008	3/31 (9.7)
2009	4/34 (11.8)

*All samples were negative for IgG against hepatitis E virus and for virus RNA.

duced to colony D. At our research institute, each monkey colony is bred in a separate outdoor breeding facility built on a mountain, and the monkeys live in an environment similar to their natural habitat. Because each outdoor feeding facility is isolated by a double fence, natural reservoirs of HEV (wild boars and deer) cannot enter it. Phylogenetic analysis of monkey HEV strains indicated that this virus was genotype 3, and BLAST analysis showed that the monkey isolate is closest to HEV strains isolated from pigs in Japan. Nucleotide identities were 92%–93% (AB248521, AB248522, and AB481226). However, no evidence indicates that HEV is transmitted from pigs or wild boars to monkeys.

A notable finding in this study was the persistence of HEV infection. Generally, HEV infection is self-limiting and symptoms are transient. Persistent HEV infection occurs in solid-organ transplant recipients who have received immunosuppressive drugs (*8*) or in patients with other conditions associated with immunosuppression, such as HIV infection (*9*) and hematologic malignancies (*10,11*). However, there is no evidence of immunosuppression in monkey M1543, and the cause of the persistent HEV infection in this monkey is unknown.

The fact that the infectious HEV strain was detected in a monkey facility and caused an HEV outbreak cast doubt and apprehension on the safety of handling monkeys. Although no staff member showed development of symptomatic hepatitis E, precautions should be taken by facility workers who work with monkeys to prevent infection with HEV.

Acknowledgments

We thank Noriko Tsuneda for editorial assistance and Naomi Oohara for technical assistance.

This study was supported in part by a grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (17630007 to H.Y.) and in part by grants for Research on Emerging and Re-emerging Infectious Diseases, Research on Hepatitis, and Research on Food Safety from the Ministry of Health, Labour, and Welfare of Japan.

Dr Yamamoto is an associate professor at the University of Toyama, Toyama, Japan. His research interests are zoonoses, especially surveys of simian herpes B virus and hepatitis E virus infections of laboratory animals; and mouse and monkey Mx genes.

References

1. Emerson SU, Purcell RH. Hepatitis E virus. Rev Med Virol. 2003;13:145–54. http://dx.doi.org/10.1002/rmv.384
2. Meng XJ. Novel strains of hepatitis E virus identified from humans and other animal species: is hepatitis E a zoonosis? J Hepatol. 2000;33:842–5. http://dx.doi.org/10.1016/S0168-8278(00)80319-0
3. Tei S, Kitajima N, Takahashi K, Mishiro S. Zoonotic transmission of hepatitis E virus from deer to human beings. Lancet. 2003;362:371–3. http://dx.doi.org/10.1016/S0140-6736(03)14025-1
4. Li TC, Chijiwa K, Sera N, Ishibashi T, Etoh Y, Shinohara Y, et al. Hepatitis E virus transmission from wild boar meat. Emerg Infect Dis. 2005;11:1958–60. http://dx.doi.org/10.3201/eid1112.051041
5. Li TC, Yamakawa Y, Suzuki K, Tatsumi M, Razak MA, Uchida T, et al. Expression and self-assembly of empty virus-like particles of hepatitis E virus. J Virol. 1997;71:7207–13.
6. Li TC, Suzaki Y, Ami Y, Dhole TN, Miyamura T, Takeda N. Protection of cynomolgus monkeys against HEV infection by oral administration of recombinant hepatitis E virus-like particles. Vaccine. 2004;22:370–7. http://dx.doi.org/10.1016/j.vaccine.2003.08.004
7. Li TC, Ochiai S, Ishiko H, Wakita T, Miyamura T, Takeda N. A retrospective study on imported hepatitis E in Japan. Travel Med Infect Dis. 2012;10:80–5. http://dx.doi.org/10.1016/j.tmaid.2012.02.003
8. Kamar N, Selves J, Mansuy JM, Ouezzani L, Peron JM, Guitard J, et al. Hepatitis E virus and chronic hepatitis in organ-transplant recipients. N Engl J Med. 2008;358:811–7. http://dx.doi.org/10.1056/NEJMoa0706992
9. Dalton HR, Bendall RP, Keane FE, Tedder RS, Ijaz S. Persistent carriage of hepatitis E virus in patients with HIV infection. N Engl J Med. 2009;361:1025–7. http://dx.doi.org/10.1056/NEJMc0903778
10. le Coutre P, Meisel H, Hofmann J, Rocken C, Vuong GL, Neuburger S, et al. Reactivation of hepatitis E infection in a patient with acute lymphoblastic leukaemia after allogeneic stem cell transplantation. Gut. 2009;58:699–702. http://dx.doi.org/10.1136/gut.2008.165571
11. Ollier L, Tieulie N, Sanderson F, Heudier P, Giordanengo V, Fuzibet JG, et al. Chronic hepatitis after hepatitis E virus infection in a patient with non-Hodgkin lymphoma taking rituximab. Ann Intern Med. 2009;150:430–1.

Address for correspondence: Tian-Cheng Li, Department of Virology II, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashi-Murayama, Tokyo 208-0011, Japan; email: litc@nih.go.jp

Search past issues of EID at wwwnc.cdc.gov/eid

Characterization of Full Genome of Rat Hepatitis E Virus Strain from Vietnam

Tian-Cheng Li, Yasushi Ami, Yuriko Suzaki, Shumpei P. Yasuda, Kumiko Yoshimatsu, Jiro Arikawa, Naokazu Takeda, and Wakita Takaji

We amplified the complete genome of the rat hepatitis E virus (HEV) Vietnam strain (V-105) and analyzed the nucleotide and amino acid sequences. The entire genome of V-105 shared only 76.8%–76.9% nucleotide sequence identities with rat HEV strains from Germany, which suggests that V-105 is a new genotype of rat HEV.

Hepatitis E virus (HEV) is a positive-sense single-stranded RNA virus (*1*), classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* (*2*). Hepatitis E, caused by HEV infection, is a serious public health concern in developing countries and is recognized as sporadic and endemic acute hepatitis (*3*). To date, at least 4 genotypes of HEV have been isolated from humans (*4*). In addition, HEV has been isolated from other mammals, including pigs, wild boars, wild deer, rabbits, ferrets, bats, chickens, and wild rats (*5–9*). Much direct evidence indicates that HEV is transmitted from pigs or wild boars to humans, and therefore hepatitis E caused by genotypes 3 and 4 is recognized as a zoonotic disease (*6,8,10*).

Rat HEV was first isolated from Norway rats in Germany (*7,11*). Since then, rat HEV strains have been isolated from wild rats in other areas of Germany and detected in wild rats in the United States and Vietnam (*12–14*). Those results suggest that rat HEV infection is not restricted to Germany but is broadly distributed in wild rats throughout the world. The nucleotide sequences of the rat HEV isolated in Germany and the United States are similar; however, the partial sequences of the Vietnam rat HEV strain (V-105, JN040433) have been found to have 78.18%–79.43% identities with isolates from Germany, R63 and R68 (*14*). To confirm whether new genotypes of rat HEV exist, we amplified the entire genome of the rat HEV V-105 strain and analyzed the sequences. We confirmed that the rat HEV strain isolated in Vietnam belongs to a new genotype of rat HEV.

Author affiliations: National Institute of Infectious Diseases, Tokyo, Japan (T.-C. Li, Y. Ami, Y. Suzaki, W. Takaji); Graduate School of Medicine, Hokkaido University, Sapporo, Japan (S.P. Yasuda, K. Yoshimatsu, J. Arikawa); and Osaka University, Osaka, Japan (N. Takeda)

DOI: http://dx.doi.org/10.3201/eid1901.121007

The Study

The rat HEV used in this study was isolated from a 10% lung homogenate of a wild rat from Vietnam, which was positive for rat HEV RNA by reverse transcription PCR (RT-PCR) (*14*). Because of the limited availability of rat specimens that are positive for HEV RNA, we first transmitted the rat HEV to a laboratory rat (Wistar) to produce a large amount of virus for RNA extraction and genome amplification. After intravenous inoculation of the rat, fecal specimens positive for HEV RNA were collected, and a 10% suspension was prepared as described (*15*). RT-PCR was performed by using Superscript II RNase H^- (Invitrogen, Carlsbad, CA, USA) and primer TX30SXN (*14*). The full-length genome of the V-105 strain was amplified by RT-PCR with primers based on the nucleotide sequences of GU345042 and JN040433 (Table 1). All PCR products were purified by using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA) and cloned into TA cloning vector pCR2.1 (Invitrogen). The nucleotide sequencing was carried out by using an ABI 3130 Genetic Analyzer automated sequencer (Applied Biosystems, Foster City, CA, USA).

Because 901 nt of V-105, corresponding to nt 4108–5008 of the R63 genome, were already known (*14*), primers F13 and open reading frame (ORF) 1–R12 were designed. An ≈2,100-nt fragment of the C-terminus of the rat HEV V-105, nt 4923-poly (A) tail, was amplified with a pair of primers, F13 and TX30SXN, by the first RT-PCR. The ORF1 region was amplified with primers ORF1-F1 and ORF1-R12. Two fragments, 440 nt (nt 11–450) and 1,182 nt (nt 2990–4171), were amplified by nested PCR with 2 sets of primers, ORF1-F2/ORF1-R1 and ORF1-F7/ORF1-R12, respectively. On the basis of the nucleotide sequences of those amplified fragments, ORF1-F9, ORF1-F16, ORF1-R16, ORF1-F18, and ORF2-R21 were designed, and 3 fragments, 1,830 nt (nt 388–2217), 996 nt (nt 2080–3075), and 1,110 nt (nt 3991–5100), were amplified with 3 sets of primers, ORF1-F9/ORF1-R10, ORF1-F16/ORF1-R16, and ORF1-F18/ORF2-R21, respectively.

To amplify the N-terminus nonstructural region of V-105, we synthesized cDNA with primer ORF1-R14, and a DNA anchor (P-CACGAATTCACTATCGATTCTGGAACCTTCAGAGG-NH_3) was linked to the N-terminus of the cDNA by T4 RNA Ligase I (BioLabs, Tokyo, Japan). By using this anchor-cDNA as the template, the first and the nested PCRs were carried out with 2 sets of primers, anchor-1/ORF1-R14 and anchor-2/ORF1-R13, respectively.

Table 1. Oligonucleotides used in amplifying the complete genome of the rat HEV Vietnam strain, V-105*

Primers	Product length, l
Forward ORF1-F1 (1-21)† 5'-GCAACCCCCGATGGAGACCCA-3'‡ Reverse ORF1-R12 (4149-4171) 5'-GGCGGCCTCGAACTTCTCCTGAA-3'	§
Forward ORF1-F2 (11-30) 5'-ATGGAGACCCATCAGTATGT-3'† Reverse ORF1-R1 (431-450) 5'-GTGCAAAAGGAAAGATCAGT-3'	440
Forward ORF1-F9 (388-408) 5'-AGCTAACAACATCCGCCGTTG-3' Reverse ORF1-R10 (2197-2217) 5'-TGGGTTCGGTCGAAGGCCTCT-3'†	1,830
Forward ORF1-F16 (2080-2100) 5'-TGCAGCCGTTTATGAGGGAGA-3' Reverse ORF1-R16 (3055-3075) 5'-CGCCATTCTGTGGGTTCTAGA-3'	996
Forward ORF1-F7 (2990-3009) 5'-GACCCAAGGCAGATCCCTGC-3'† Reverse ORF1-R12 (4149-4171) 5'-GGCGGCCTCGAACTTCTCCTGAA-3'	1,182
Forward ORF1-F18 (3991-4011) 5'-ATTCACCACAGACGAGCCAGT-3' Reverse ORF2-R21 (5079-5100) 5'-GGTGATAGCCAATTGGTAAGCT-3'	1,110
Forward F13 (4896-4915) 5'-AATAACACTCTGGGCTGTAG-3' ReverseTX30SXN 5'-GACTAGTTCTAGATCGCGAGCGGCCGCCCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3'	2,092
Forward primer Anchor-1: 5'-CCTCTGAAGGTTCCAGAATCGATAG-3' Reverse primer ORF1-R14 (276-296) 5'-TAGACCTAGGGTGCGCACCGA-3'	§
Forward primer Anchor-2: 5'-GAATCGATAGTGAATTCGTG-3' Reverse primer ORF1-R13 (200-220) 5'-AACACGCTGTACCGGATGCGA-3'	240

*HEV, hepatitis E virus; ORF, open reading frame.
†Numbers in a parentheses show the positions of primers corresponding to the entire genome of rat HEV V-105.
‡Primer designed based on rat HEV (GU345042).
§The PCR product was not detected.

The V-105 genome consisted of 6,927 nt plus a poly (A) tail of a still-undetermined length (GenBank accession no. JX120573). The genomic structure of V-105 was, from the N-terminus toward the C-terminus, the N 5′–untranslated region (UTR) at nt 1–10, ORF1at nt 11–4900, ORF3 at nt 4917–5225, ORF2 at nt 4928–6862, the 3′-UTR at nt 6863–6927, and the poly (A) tail starting at nt 6928. ORF2 and ORF3 encode 644 aa and 102 aa, respectively, as do R63 and R68. However, ORF1 of V-105 encodes 1,629 aa, which is 7 aa shorter than either R63 or R68. The V-105 genome possessed 2 aa insertions (Ser-Pro) between the aa residues 591 and 592 and 9 aa deletions (Ser-Pro-Pro-Gly-Pro-Pro-Pro-Ala-Gly) between aa residues 852 and 853, corresponding to those of R63. The 3′-UTR was 65 nt as were R63 and R68. Unlike R63 and R68, only 1 additional putative ORF, corresponding to ORF4 (nt residues 27–578), was found in V-105, suggesting that other putative ORFs, ORF5 and ORF6 found in R63 and R68, are not common in rat HEV.

When the V-105 genome was compared with reported HEV genomes, the V-105 genome shared identities of only 50.5% with avian HEV, 53.6% with rabbit HEV, 53.7%–54.0% with wild boar HEV, and 53.1%–53.5% with HEV genotypes 1–4. In contrast, V-105 shared relatively high nucleotide sequence identities (76.8%–76.9%) with rat HEV strains (R63 and R68) (Table 2). The nucleotide and amino acid sequences of ORF1, ORF2, and ORF3 of V-105 were compared with those of other HEV genotypes, and the identities among them are shown in Table 2. Together, these results suggest that V-105 is more similar to rat HEV than to other HEV genotypes.

Phylogenetic trees were generated on the basis of the nucleotide sequences derived from the entire genome and ORF3 of the genotypes 1–4, wild boar, rabbit, chicken, and rat HEV isolates. These trees demonstrated that V-105 does not belong to any known genotype and should probably be classified into a new genotype (Figure).

Table 2. Nucleotide and deduced amino acid sequence identities between human, wild boar, rabbit, rat, and avian HEV strains, compared with Vietnam rat HEV V-105 strain*

HEV strain (GenBank accession no.)	Vietnam rat HEV strain						
	Entire genome	Nucleotides, %			Amino acids, %		
		ORF1	ORF2	ORF3	ORF1	ORF2	ORF3
Genotype 1 (NC_001434)	53.5	50.7	60.8	51.0	54.1	55.5	33.3
Genotype 2 (M74506)	53.3	51.2	59.1	51.4	53.1	54.6	30.6
Genotype 3 (AF060668)	53.3	50.8	59.2	53.8	51.6	57.1	26.5
Genotype 4 (AJ272108)	53.1	50.9	58.9	52.6	50.7	55.4	24.5
Wild boar HEV (AB573435)	53.7	51.5	59.6	53.4	50.3	56.3	28.2
Wild boar HEV (AB602441)	54.0	51.5	59.6	53.4	50.3	56.3	28.2
Rabbit HEV (FJ906895)	53.6	51.3	59.3	51.5	52.1	56.2	26.2
Rat HEV (GU345042)/R63	76.9	75.7	79.6	80.6	87.0	91.6	66.7
Rat HEV (GU345043)/R68	76.8	75.5	79.8	80.9	86.4	92.1	66.7
Avian (chicken) HEV (AY535004)	50.5	49.7	54.2	47.0	44.7	47.4	33.9

*HEV, hepatitis E virus; ORF, open reading frame.

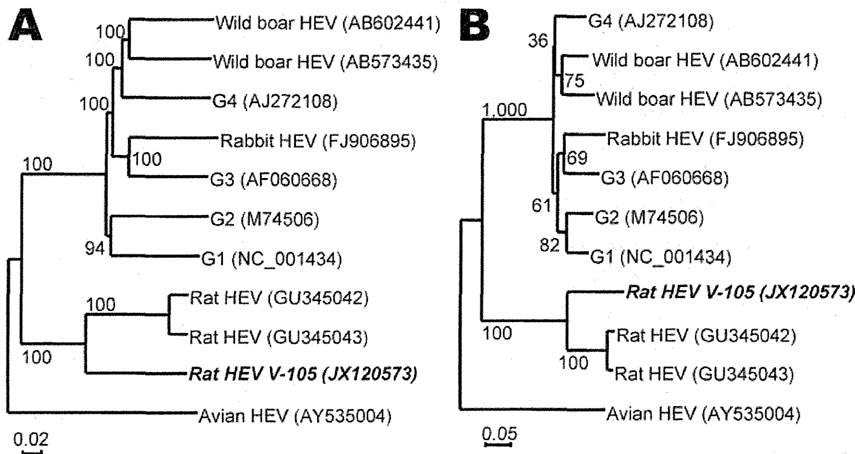


Figure. Phylogenetic relationships among genotypes 1–4, wild boar, rabbit, avian (chicken), and rat hepatitis E virus (HEV) isolates. The nucleic acid sequence alignment was performed by using ClustalX 1.81 (www.clustal.org). The genetic distance was calculated by the Kimura 2-parameter method. A phylogenetic tree with 1,000 bootstrap replicates was generated by the neighbor-joining method, based on the entire genome (A) and open reading frame 3 (B) of the genotypes 1–4, wild boar, rabbit, avian (chicken), and rat HEV isolates. Scale bar indicates nucleotide substitutions per site. **Boldface** indicates isolate used in this study.

Conclusions

In this study we successfully amplified the entire genome of an HEV strain isolated from a wild rat in Vietnam. Phylogenetic analyses and nucleotide and amino acid sequence comparisons demonstrated that the complete rat HEV genome sequences were consistently well separated from those of mammalian genotypes 1–4, wild boar, rabbit, and chicken HEV and close to those of the rat HEV strains. Although the entire genome of V-105 shared nucleotide sequence identities of only 76.8%–76.9% with the isolates from Germany (R63 and R68), the ORF1 and ORF3 amino acid identities between V-105 and these isolates were 86.4%–87.0% and 66.7%, respectively, which suggests that V-105 can be classified into a new genotype of rat HEV. However, ORF2 has relatively high amino acid identities with R63 and R68 (91.6%–92.1%), indicating that the V-105 and rat HEV isolates from Germany share similar antigenicity. In fact, rat HEV–like particles derived from R63 are cross-reactive to serum from V-105–infected wild rats (*14*).

In conclusion, we isolated and identified rat HEV strain V-105 from a wild rat in Vietnam, and this strain was highly divergent from known rat HEV isolates. We propose that the strain from Vietnam, V-105, is a new member of the rat HEV genotype.

This study was supported in part by grants for Research on Emerging and Re-emerging Infectious Diseases, Research on Hepatitis, and Research on Food Safety from the Ministry of Health, Labor, and Welfare, Japan.

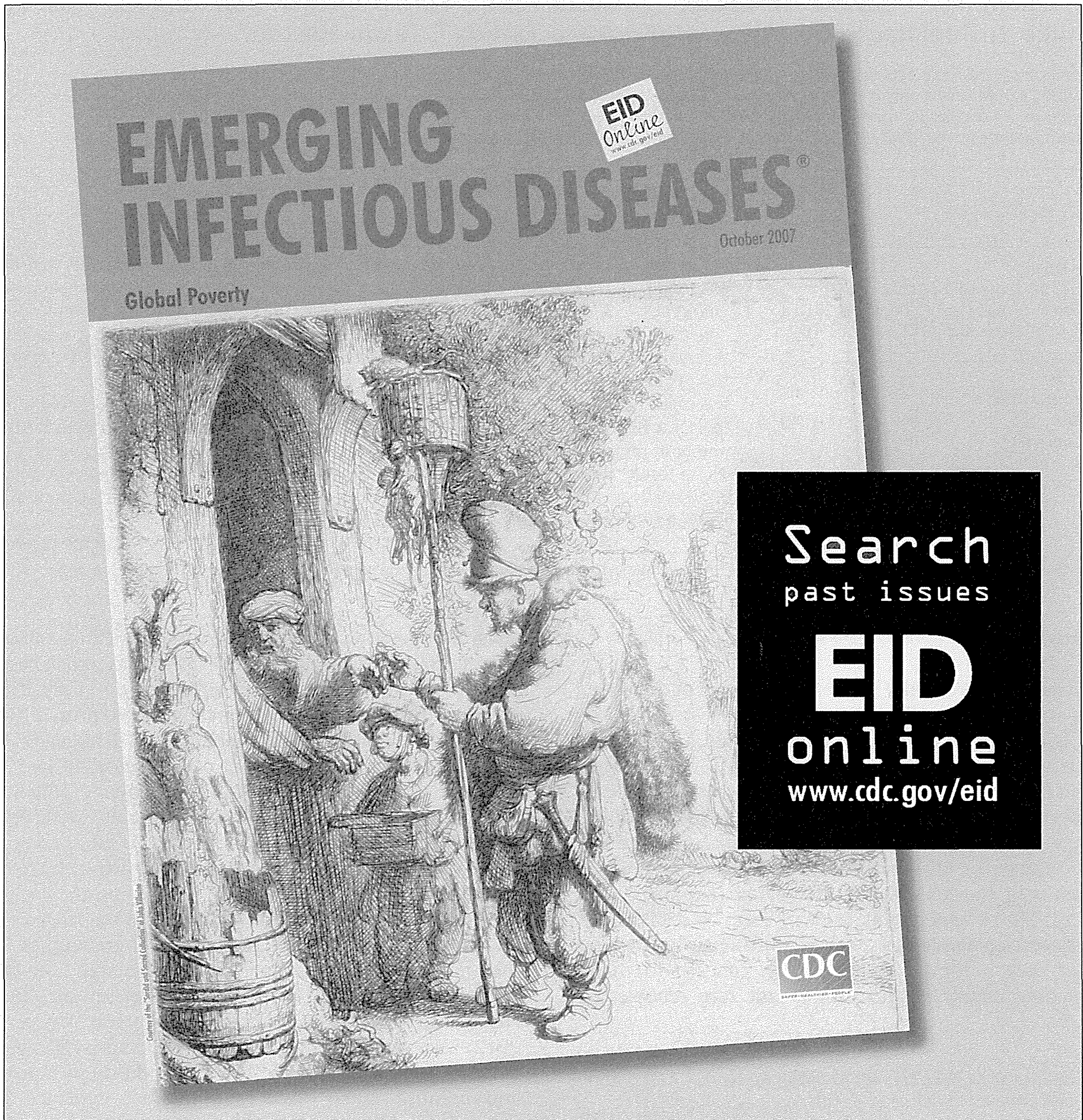
Dr Li is a senior researcher at the National Institute of Infectious Diseases, Tokyo. His research focuses on epidemiology, expression of viral proteins, and the 3-dimensional structure of HEV.

References

1. Tam AW, Smith MM, Guerra ME, Huang CC, Bradley DW, Fry KE, et al. Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. Virology. 1991;185:120–31. http://dx.doi.org/10.1016/0042-6822(91)90760-9
2. Emerson SU, Anderson D, Arankalle A, Meng XJ, Purdy M, Schlauder GG, et al. Hepevirus. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA, editors. Virus taxonomy: eighth report of the International Committee on Taxonomy of Viruses. London: Elsevier/Academic Press; 2005. p. 853–7.
3. Emerson SU, Purcell RH. Hepatitis E virus. Rev Med Virol. 2003;13:145–54. http://dx.doi.org/10.1002/rmv.384
4. Jameel S. Molecular biology and pathogenesis of hepatitis E virus. Expert Rev Mol Med. 1999;1:1–16.
5. Raj VS, Smits SL, Pas SD, Provacia LB, Moorman-Roest H, Osterhaus AD, et al. Novel hepatitis E virus in ferrets, the Netherlands. Emerg Infect Dis. 2012;18:1369–70. http://dx.doi.org/10.3201/eid1808.111659
6. Li TC, Chijiwa K, Sera N, Ishibashi T, Etoh Y, Shinohara Y, et al. Hepatitis E virus transmission from wild boar meat. Emerg Infect Dis. 2005;11:1958–60. http://dx.doi.org/10.3201/eid1112.051041
7. Johne R, Heckel G, Plenge-Bönig A, Kindler E, Maresch C, Reetz J, et al. Novel hepatitis E virus genotype in Norway rats, Germany. Emerg Infect Dis. 2010;16:1452–5. http://dx.doi.org/10.3201/eid1609.100444
8. Meng XJ. Hepatitis E virus: animal reservoirs and zoonotic risk. Vet Microbiol. 2010;140:256–65. http://dx.doi.org/10.1016/j.vetmic.2009.03.017
9. Drexler JF, Seelen A, Corman VM, Fumie Tateno A, Cottontail V, Melim Zerbinati R, et al. Bats worldwide carry hepatitis E virus–related viruses that form a putative novel genus within the family *Hepeviridae*. J Virol. 2012;86:9134–47. http://dx.doi.org/10.1128/JVI.00800-12
10. Colson P, Borentain P, Queyriaux B, Kaba M, Moal V, Gallian P, et al. Pig liver sausage as a source of hepatitis E virus transmission to humans. J Infect Dis. 2010;202:825–34. http://dx.doi.org/10.1086/655898
11. Johne R, Plenge-Bonig A, Hess M, Ulrich RG, Reetz J, Schielke A. Detection of a novel hepatitis E–like virus in faeces of wild rats using a nested broad-spectrum RT-PCR. J Gen Virol. 2010;91:750–8. http://dx.doi.org/10.1099/vir.0.016584-0

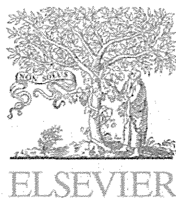
12. Johne R, Dremsek P, Kindler E, Schielke A, Plenge-Bonig A, Gregersen H, et al. Rat hepatitis E virus: geographical clustering within Germany and serological detection in wild Norway rats (*Rattus norvegicus*). Infect Genet Evol. 2012;12:947–56. http://dx.doi.org/10.1016/j.meegid.2012.02.021
13. Purcell RH, Engle RE, Rood MP, Kabrane-Lazizi Y, Nguyen HT, Govindarajan S, et al. Hepatitis E virus in rats, Los Angeles, California, USA. Emerg Infect Dis. 2011;17:2216–22. http://dx.doi.org/10.3201/eid1712.110482
14. Li TC, Yoshimatsu K, Yasuda SP, Arikawa J, Koma T, Kataoka M, et al. Characterization of self-assembled virus-like particles of rat hepatitis E virus generated by recombinant baculoviruses. J Gen Virol. 2011;92:2830–7. http://dx.doi.org/10.1099/vir.0.034835-0
15. Li TC, Suzaki Y, Ami Y, Dhole TN, Miyamura T, Takeda N. Protection of cynomolgus monkeys against HEV infection by oral administration of recombinant hepatitis E virus–like particles. Vaccine. 2004;22:370–7. http://dx.doi.org/10.1016/j.vaccine.2003.08.004

Address for correspondence: Tian-Cheng Li, Department of Virology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-murayama, Tokyo 208-0011, Japan; email: litc@nih.go.jp



Author's personal copy

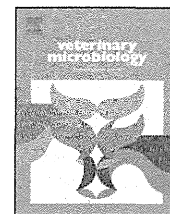
Veterinary Microbiology 163 (2013) 54–61



Contents lists available at SciVerse ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic



Susceptibility of laboratory rats against genotypes 1, 3, 4, and rat hepatitis E viruses

Tian-Cheng Li [a,*], Sayaka Yoshizaki [a], Yasushi Ami [b], Yuriko Suzaki [b], Shumpei P. Yasuda [c], Kumiko Yoshimatsu [c], Jiro Arikawa [c], Naokazu Takeda [d], Takaji Wakita [a]

[a] *Department of Virology II, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashi-murayama, Tokyo 208-0011, Japan*
[b] *Division of Experimental Animals Research, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashi-murayama, Tokyo 208-0011, Japan*
[c] *Department of Microbiology, Graduate School of Medicine, Hokkaido University, Kita-15, Nishi-7, Kita-ku, Sapporo 060-8638, Japan*
[d] *Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0781, Japan*

ARTICLE INFO

Article history:
Received 27 September 2012
Received in revised form 11 December 2012
Accepted 17 December 2012

Keywords:
Hepatitis E virus
Rat HEV
Laboratory rat
Wistar
Nude rat

ABSTRACT

To determine whether or not rats are susceptible to hepatitis E virus (HEV) infection, each of group containing three laboratory rats (Wistar) were experimentally inoculated with genotypes 1, 3, 4 and rat HEV by intravenous injection. Serum and stool samples were collected and used to detect HEV RNA and anti-HEV antibodies by RT-PCR and ELISA, respectively. The virus infection was monitored up to 3 months after inoculation. None of the serum or stool samples collected from the rats inoculated with G1, G3, or G4 HEV indicated positive sign for virus replication. Although no alteration was observed in ALT level, rat HEV RNA was detected in stools from both of the rats inoculated with rat HEV, and both rats were positive for anti-rat HEV IgG and IgM from 3 weeks after inoculation. These results demonstrated that rats are susceptible to rat HEV but not to G1, G3, and G4 HEV. We also confirm that the nude rats were useful for obtaining a large amount of rat HEV and that the rat HEV was transmitted by the fecal-oral route.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Hepatitis E virus (HEV) is the sole member of the genus *Hepevirus* in the family *Hepeviridae* (Meng et al., 2012). HEV is a small round non-enveloped virus, 27–34 nm in diameter, containing an approximately 7.2 kb single-strand RNA molecule as the genome, which encodes three discontinuous and partially overlapping open reading frames (ORFs) (Balayan et al., 1983; Tam et al., 1991). The 5′ end of the RNA contains a cap structure, and the 3′ terminus of the RNA is polyadenylated (Kabrane-Lazizi et al., 1999b; Magden et al., 2001; Tam et al., 1991). HEV causes both epidemic and sporadic acute hepatitis E, which is not only a serious public health concern in many developing countries, but also is not rare among the general population in developed countries (Emerson and Purcell, 2003; Nelson et al., 2011). Pregnant women have a high mortality risk, as high as 20% (Khuroo et al., 1981; Navaneethan et al., 2008). HEV is transmitted primarily via the fecal-oral route through contaminated drinking water. The full genome of G1 HEV was first identified in 1990 (Reyes et al., 1990). Since then, a large number of HEV have been isolated from human and other animals (Meng et al., 1997; Nakamura et al., 2006; Tei et al., 2003; Zhao et al., 2009), and the HEV isolates were grouped into at least four major genotypes, genotypes 1–4 (G1–G4) on the basis of the nucleotide and deduced amino acid sequences (Liu et al., 2008). In addition to these four genotypes are mammalian HEV strains that have been isolated from wild boar, bat, avian, and wild rat (Drexler et al., 2012; Haqshenas et al., 2001; Johne et al., 2010a; Raj et al., 2012; Takahashi et al., 2011). Because G3 and G4 HEV were isolated from pigs and wild boars in addition to humans, and because much direct and indirect evidence has

* Corresponding author. Tel.: +81 42 561 0771; fax: +81 42 565 4729.
E-mail address: litc@nih.go.jp (T.-C. Li).

0378-1135/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.vetmic.2012.12.014

Author's personal copy

indicated that HEV is transmitted from pigs or wild boars to humans, hepatitis E is recognized as a zoonotic disease (Li et al., 2005; Meng, 2010).

Rat HEV was recently isolated from Norway rats in Germany (Johne et al., 2010a). However, the antigenicity, pathogenicity, and epidemiology of this virus remain unclear due to the lack of a viable cell culture system to grow the virus. Although anti-G1 HEV antibodies have been detected in wild rats in USA and Japan (Favorov et al., 2000; Hirano et al., 2003), it is obscure whether or not HEV (G1–G4 HEV, and wild boar HEV) substantially replicates in rats. Although it has been reported that G1, G2 and G3 HEV do not infect laboratory rats (Purcell et al., 2011), an early report showed that human HEV is transmissible to Wistar rats (Maneerat et al., 1996). Furthermore, part of the G3 HEV genome was detected recently in various species of wild-caught rats (Lack and Volk, 2012). At present, the susceptibility and infectivity of HEV in rat remain unclear. On the other hand, rat HEV-specific antibodies have been found in humans, suggesting the zoonotic potential of rat HEV (Dremsek et al., 2012). However, infection experiments indicated that monkey and pigs are not susceptible to rat HEV (Cossaboom et al., 2012; Purcell et al., 2011). In this study, we inoculated laboratory rats (Wistar) with G1, G3, G4, and rat HEV, and monitored the virus growth to determine the rats' susceptibility to HEV infection.

2. Materials and methods

2.1. Preparation of HEV

The G1 HEV strain was derived from stool specimens from a cynomolgus monkey (*Macaca fascicularis*), which had been experimentally infected with an Indian strain (Li et al., 2004). The G3 HEV strain (DQ079632) was derived from stool specimens collected on a pig farm in Japan. The G4 HEV strain (DQ079628) was from a stool specimen collected from a wild boar caught in Aichi prefecture, Japan. The infectivity of G1, G3, and G4 HEV had been confirmed by experimental infections with cynomolgus monkeys (Li et al., 2008). The copy numbers of the G1, G3, and G4 HEV genome in the stool specimens were 5×10^4, 2×10^4, and 1×10^5 per ml, respectively, by real-time RT-PCR as described previously (Jothikumar et al., 2006). Rat HEV (V-105) was derived from rat serum and lung tissue collected from a Vietnamese wild rat (Li et al., 2011). Because the rat serum was collected to detect anti-hantavirus antibodies, it was heat-inactivated at 56 °C for 30 min. The rat lung tissue was homogenized with 10 mM phosphate-buffered saline (PBS) to prepare a 10% suspension, shaken at 4 °C for 1 h, and clarified by centrifugation at $10,000 \times g$ for 30 min. The supernatant was passed through a 0.45 μm membrane filter (Millipore, Bedford, MA). Both rat serum and lung suspensions were positive for rat HEV RNA by reverse-transcription polymerase chain reaction (RT-PCR); however, the amount of the rat HEV RNA was too low to be detected by real-time RT-PCR. All the specimens were stored at −80 °C until use.

2.2. Inoculation of rats and sample collection

Fifteen 5-week-old and six 20-week-old SPF rats (Wistar, Japan SLC), as well as two 5-week-old nude rats (Long–Evans-run/run, Japan SLC) were used in this study. All rats were female and negative for rat HEV RNA and anti-rat HEV antibodies, as determined by nested broad-spectrum RT-PCR and ELISA, respectively. To examine the laboratory rats' susceptibility to HEV, the rats were inoculated with HEV intravenously through the tail vein. To confirm the transmission route of rat HEV, two rats were fed in a cage in which had been placed 10 g of nude rats' stool samples containing 1.3×10^6 copy/g of rat HEV RNA. The rats were fed in the rat HEV-contaminated environment for one week and then moved to a new cage that was without rat HEV contamination. The serum samples were collected weekly for examining of HEV RNA as well as for HEV-specific IgG and IgM antibodies. Sera were also used to determine ALT values. Stool samples were collected daily after the inoculation to detect HEV RNA. Tissues of nude rats were collected after exsanguination, and a 10% tissue suspension was prepared as described above. The rats were monitored for 3–4 months after inoculation and were weighed daily. All the rat experiments were reviewed by the Institute's ethics committee and carried out according to the "Guides for animal experiments performed at NIID" under codes 111069 and 112011. Rats were individually housed in BSL-2 facilities.

2.3. A nested broad-spectrum RT-PCR

The RNA was extracted using the MagNA Pre LC system with MagNA Pre LC Total Nucleic Acid isolation (Roche Applied Science, Mannheim, Germany) according to the manufacturer's recommendations. Reverse transcription (RT) was performed at 42 °C for 50 min followed by 70 °C for 15 min in a 20 μl reaction mixture containing 1 μl of Superscript™ II RNase H⁻ reverse transcriptase (Invitrogen, Carlsbad, CA), 1 μl of the oligo(dT) primer, 1 μl of RNaseOUT™, 2 μl of 0.1 M dithiothreitol, 4 μl of 5 × RT buffer, 1 μl of 10 mM deoxynucleoside triphosphates, 5 μl of RNA, and 5 μl of distilled water.

A nested broad-spectrum RT-PCR analysis was performed to amplify a portion of the ORF1 genome, based on the method described previously with slight modification (Johne et al., 2010b). Five microliters of the cDNA was used for the first PCR in 50 μl of the reaction mixture containing an external forward primer, HEV-cs (5′-TCGCGCATCACMTTYTTCCARAA-3′), and an external reverse primer, HEV-cas (5′-GCCATGTTCCAGACDGTRTTCCA-3′). Each cycle consisted of denaturation at 95 °C for 30 s, primer annealing at 52 °C for 45 s, and an extension reaction at 72 °C for 60 s followed by final extension at 72 °C for 7 min. Two microliters of the first PCR product were used for the nested PCR with an internal forward primer, HEV-csn (5′-TGTGCTCTGTTTGGCCCNTGGTTYCDG-3′), and an internal reverse primer, HEV-casn (5′-CCAGGCTCACCRGARTGYTTCTTCCA-3′). Each cycle consisted of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 45 s, and an extension reaction at 72 °C for 60 s followed by final

Author's personal copy

extension at 72 °C for 7 min. The nested PCR products were separated by electrophoresis on 2% agarose gels.

2.4. Quantitative real-time RT-PCR for detection of rat HEV

To determine rat HEV RNA titers, a TaqMan assay was performed by using the 7500 FAST Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations. The primers consisted of 900 nmol/L forward (5′-GTGGTGCTTTTATGGTGACTG-3′, nt 4123–4143) and 900 nmol/L reverse (5′-CAAACTCACTAAAATCATTCTCAAACAC-3′, nt 4196–4223), and 250 nmol/L probe (5′-6FAM-GTTCAGGAGAAGTTCGAGGCCGCCGT-TAMRA-3′, nt 4148–4173). One-step quantitative RT-PCR (qRT-PCR) cycling conditions were 15 min at 48 °C, a 10-min incubation at 95 °C, and 50 cycles for 15 s at 95 °C and 1 min at 60 °C. Standard RNA is a partial genome of V-105 (nt 3991–5100) that was synthesized by using the MEGA script kit (Applied Biosystems). A 10-fold serial dilution of standard RNA (10^7 to 10^1 copies) was used for the quantitation of viral copy numbers in reaction tubes. Amplification data were collected and analyzed with Sequence Detector software version 1.3 (Applied Biosystems). This quantitative real-time RT-PCR system, with a sensitivity of 10 copies, was used exclusively for rat HEV.

2.5. Detection of IgG and IgM antibodies

Anti-rat HEV IgG and IgM antibodies were detected by enzyme-linked immunosorbent assay (ELISA) as described previously (Li et al., 2011). Flat-bottom 96-well polystyrene microplates (Immulon 2; Dynex Technologies, Chantilly, VA) were coated with the purified rat HEV-LPs (1 μg/mL, 100 μl/well) and incubated overnight at 4 °C. The unbound HEV-LPs were removed, and the plates were washed twice with 10 mM phosphate-buffered saline containing 0.05% Tween 20 (PBS-T) and then blocked with 200 μl of 5% skim milk (Difco Laboratories) dissolved in PBS-T for 1 h at 37 °C. After the plates were washed four times with PBS-T, diluted rat (100 μl/well) serum samples were added in duplicate. The plates were incubated at 37 °C for 1 h and washed three times as described above. The wells were incubated with 100 μl of horseradish peroxidase-conjugated goat anti-rat IgG (H+L) (Zemed Laboratories, San Francisco, CA) (1:10,000 dilution) or horseradish peroxidase-conjugated goat anti-rat IgM (Jackson Immunoresearch Laboratories Inc., West Grove, PA) (1:100,000 dilution), diluted with PBS-T containing 1% skim milk. The plates were incubated at 37 °C for 1 h and washed four times with PBS-T. The substrate orthophenylenediamine (100 μl) (Sigma Chemical, St. Louis, MO) and H_2O_2 were added to each well. The plates were incubated in a dark room at room temperature for 30 min, and then 50 μl of 4 N H_2SO_4 was added to each well. Absorbance was measured at 492 nm. The cutoff values for IgG and IgM were determined as described previously (Li et al., 2011). A sample was considered positive when the absorbance exceeded the cutoff value. Anti-G1, G3, and G4 HEV IgG and IgM antibodies were detected by ELISA as described previously (Li et al., 2000).

2.6. Liver enzyme level

ALT value was monitored weekly by the Fuji Dri-Chem Slide GPT/ALT-PIII kit (Fujifilm, Saitama, Japan). The geometric mean ALT during the pre-inoculation period of each animal was used as the normal ALT value, and a two-fold or greater increase at the peak was considered a sign of hepatitis.

3. Results

3.1. Rats became infected with rat HEV but not with G1, G3, or G4 HEV

Fifteen 5-week-old SPF rats (Wistar) were randomly assigned to five groups (Table 1). RT-PCR and ELISA indicated that all animals were negative for HEV RNA and anti-rat HEV antibodies prior to inoculation. All rats were inoculated intravenously with 300 μl of each sample. The rats in groups 1, 2, and 3 were inoculated with the stool samples containing 10^4 copies of G1, G3, and G4 HEV, respectively. Two rats in group 4 and two in group 5 were inoculated similarly with 10% rat lung homogenate or RNA-positive rat serum, respectively. Rat-12 and Rat-15 received PBS and were used to monitor the fecal-oral transmission of the virus (Table 1).

In groups 1, 2, 3, and 5, all the rat serum samples collected from 1 to 13 weeks p.i. were negative for HEV RNA, anti-HEV IgG, and anti-HEV IgM antibodies. ALT elevation was not observed in these serum samples. Consistent with the above results, HEV RNA was not detected in stool samples in those groups (data not shown). These results indicated that G1, G3, and G4 HEV as well as heat-inactivated rat HEV were unable to infect laboratory rats.

In contrast, anti-rat HEV IgG and IgM antibodies were detected in Rat-10 and Rat-11 at 4 and 5 weeks p.i. in group 4 (Fig. 1). The IgG titers increased with time, peaking at 10 and 7 weeks p.i. in Rat-10 and Rat-11, respectively, before gradually decreasing. The RNA was detected in the stool

Table 1
Grouping of HEV inoculation.

Groups	Rat no.	Genotype	Specimens	Virus titers
Group 1	Rat-1	G1 HEV	Monkey stool	10^4
	Rat-2	G1 HEV	Monkey stool	10^4
	Rat-3	G1 HEV	Monkey stool	10^4
Group 2	Rat-4	G3 HEV	Pig stool	10^4
	Rat-5	G3 HEV	Pig stool	10^4
	Rat-6	G3 HEV	Pig stool	10^4
Group 3	Rat-7	G4 HEV	Wild boar stool	10^4
	Rat-8	G4 HEV	Wild boar stool	10^4
	Rat-9	G4 HEV	Wild boar stool	10^4
Group 4	Rat-10	Rat HEV	Rat lung tissue	ND[a]
	Rat-11	Rat HEV	Rat lung tissue	ND[a]
	Rat-12	–	PBS	–
Group 5	Rat-13	Rat HEV	Rat serum[b]	ND[a]
	Rat-14	Rat HEV	Rat serum[b]	ND[a]
	Rat-15	–	PBS	–

[a] Less than detection threshold.
[b] Headed at 56 °C for 30 min.

Author's personal copy

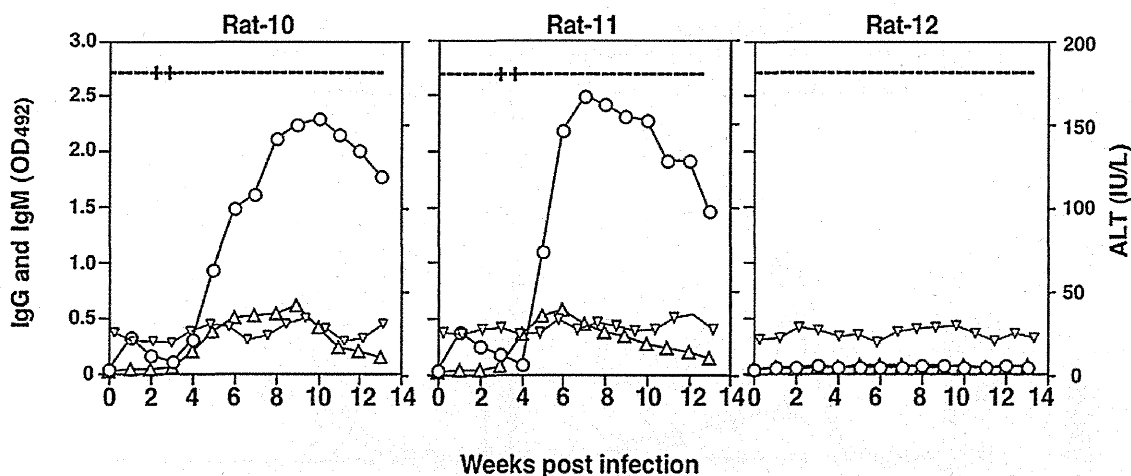


Fig. 1. Kinetics of biochemical, serological, and virological markers after intravenous inoculation. Laboratory rats (Wistar) were inoculated with rat HEV-positive lung suspension (Rats 10 and 11) or PBS (Rat-12). HEV RNA in the stool was monitored by RT-PCR: +, positive; –, negative. Anti-rat HEV IgG (○) and IgM (△) antibodies were detected by ELISA, and ALT (▽) elevation was monitored.

specimens from Rat-10 on days 17–20 and from Rat-11 on days 21–24 p.i. The copy numbers of the RNA were low and were detectable only by the nested RT-PCR (data not shown). Rat HEV RNA was undetectable in the rat serum. The nucleotide sequences of the amplified products from the stool specimens were identical to those detected from the lung suspension samples (data not shown). These results demonstrated that rat HEV is infectious to laboratory rats.

During the 3 months of the experiments, the ALT levels of all the rats were lower than 40 IU/L, demonstrating no changes in liver failure. The body weights of the infected rats increased similarly to those without infection, even in the period when rat HEV replication was extensive. No virus replication markers were detected in one of the non-inoculated rat (Rat-15). In this case, we found no evidence of oral transmission of rat HEV.

3.2. Infectivity of stools recovered from V-105-infected rats

To confirm the infectivity of rat HEV recovered from Rat-10 and Rat-11, stool samples taken from Rat-10 on day 18 p.i. and those from Rat-11 on day 22 p.i. were made to 10% stool suspensions. Two 20-week-old female rats (Rat-10a and Rat-10b) were intravenously inoculated with 300 μl of the suspension from Rat-10. Similarly, Rat-11a and Rat-11b were used for the stool suspension from Rat-11. As shown in Fig. 2, anti-rat HEV IgG and IgM antibodies were detected in the sera from all four rats at 3 weeks p.i. Rat HEV RNA was detected in stool from three of the rats, Rat-10a, Rat-10b, and Rat-11a, indicating that the rat HEV recovered from laboratory rats Rat-10 and Rat-11 are infectious. The ALT level did not increase during the experiment in any of the four rats (data not shown).

Because serum and stools recovered from laboratory rats contain extremely low copy numbers of rat HEV, two 5-week-old nude rats (athymic rats), NR1 and NR2, were intravenously injected with 300 μl of a 10% stool suspension derived from Rat-10 to produce a larger amount of rat HEV. In both nude rats, viral RNA was first detected at 3 weeks p.i. in sera and at 2 weeks p.i. in the stool (Fig. 3). The RNA increased, peaking at 12 weeks in serum with titers 1.6×10^3 copy/ml, and were constantly detected in feces with the titers 2×10^6–7×10^6 copy/g. The IgG was negative during the experimental period (data not shown).

To compare the viral loads in different tissues of the nude rats, the sera, intestinal contents, and tissues including liver, heart, spleen, lung, kidney, bladder, womb, salivary gland, and muscle were collected following the exsanguination at the 120 days p.i. The viral RNA titers were detected by real-time RT-PCR with a 10% suspension of each tissue or 100 μl of blood. In addition to the intestinal contents, high virus titers were observed in the liver: 3.1×10^7 and 3.9×10^7 copies/g in nude rats N1 and N2, respectively (Fig. 4). Other than the liver, the spleen was the only other site where HEV RNA was detected, with an extremely low titer, 2.1×10^2, suggesting that rat HEV replicates in liver and is secreted in intestine.

3.3. Rat HEV transmitted through fecal-oral route

Because human HEV is transmitted via a fecal-oral route, the possibility of the same transmission route was examined. Two 20-week-old female rats, OR1 and OR2, were fed in a cage with 10 g of stool samples containing 1.3×10^6 copy/g of rat HEV RNA derived from rat HEV-infected nude rats N1 and N2. After one week, both rats were moved to a clean cage and serum and stool samples were collected to monitor the RNA.

As shown in Fig. 5, rat HEV RNA in stools was detected from 36 to 42 days p.i. in OR1 and from 13 to 20 days p.i in OR2. Anti-rat HEV IgG and IgM were both detected in the sera at 6 and 3 weeks p.i. in OR1 and OR2, respectively. The IgM antibody titers peaked at 7 and 5 weeks p.i. then decreased gradually. The IgG antibody titers peaked at 8 and 5 weeks, respectively, and remained at high levels until the end of the experiment. Rat HEV RNA in the serum samples was detected at 3 weeks p.i. only in OR2. These results indicated that rat HEV was transmitted through the fecal-oral route. Compared with intravenous inoculation, transmission by the fecal-oral route took more time and higher titers of rat HEV to establish infection. The anti-IgG

Author's personal copy

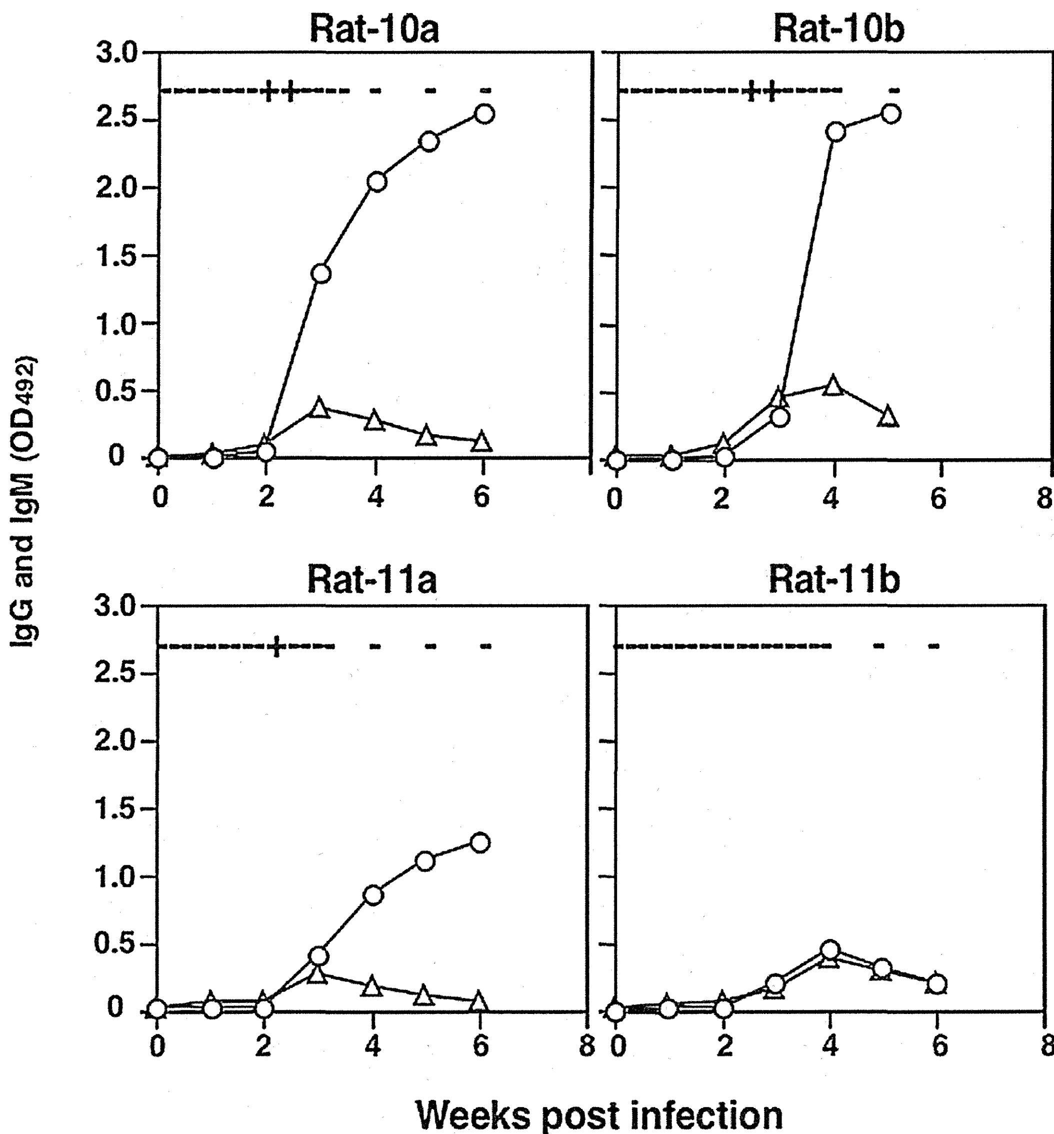


Fig. 2. Infectivity of the stool suspensions derived from the V-105-infected rats. Laboratory rats (Rats 10a and 10b) were inoculated with the stool suspension from Rat-10. Stool samples were collected daily and serum samples were collected weekly until 6 or 8 weeks. Similarly, Rats 11a and 11b were inoculated with the stool suspension from Rat-11. HEV RNA in the stool was monitored by RT-PCR: +, positive; –, negative. Serum IgG (○) and IgM (△) antibodies were detected by ELISA.

antibody induced by oral inoculation lasted longer than that induced by intravenous inoculation.

4. Discussion

Rats have long been suspected as a potential reservoir for HEV. Before rat HEV was isolated in Germany, anti-HEV IgG antibodies have been detected in various rat species, including Norway (*Rattus norvegicus*), black (*Rattus rattus*), and cotton (*Sigmodon hispidus*) rats (Arankalle et al., 2001; Favorov et al., 2000; Hirano et al., 2003; Kabrane-Lazizi et al., 1999a) by using the antigens derived from G1 HEV, suggesting that HEV or HEV-like virus infection occurred in wild rats. However, the source of the infection was confirmed in a few cases, and it is not clear whether the anti-HEV IgG was induced by HEV or other HEV-like viruses. In 2002, it was reported that the partial genome of the genotype 1 HEV was detected from wild rats in Nepal (He et al., 2002). However, that paper was retracted in 2006 because of a suspicion that the Nepal rodent HEV sequence was contaminated in the laboratory.

Recently, the capsid proteins of rat HEV were expressed by a recombinant baculovirus in insect Tn5 cells; these proteins were found to be self-assembled and to form virus-like particles (V-LPs). An ELISA was developed using rat HEV-LPs as antigens and was employed to examine the rat HEV-specific IgG and IgM responses. We found that 20.9% and 3.6% of wild rats in Vietnam were positive for rat HEV IgG and IgM antibodies, respectively. Furthermore, a new rat HEV strain, V-105, was isolated from rat HEV IgM-positive serum (Li et al., 2011).

To determine whether or not rat HEV can infect laboratory rats and to examine the susceptibility of laboratory rats to other HEV genotypes, we performed infection experiments using G1, G3, G4, and rat HEV with laboratory rats. No sign of viral replication of G1, G3, or G4

Author's personal copy

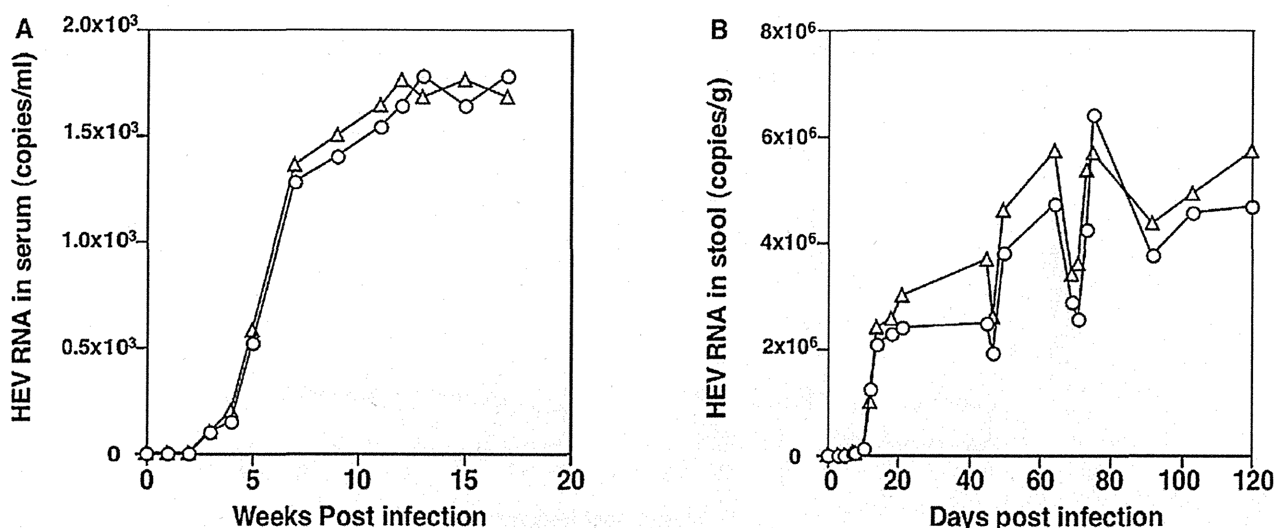


Fig. 3. Kinetics of rat HEV RNA in nude rats. Two nude rats were intravenously inoculated with rat HEV-containing stool suspension. HEV RNA in the serum (A) and stool (B) were monitored by real-time RT-PCR. Nude rat N1, ○; nude rat N2, △.

HEV was observed, and we concluded that the laboratory rats were resistant to G1, G3, and G4 HEV infection. Therefore, the anti-HEV IgG antibodies reported in various species of rats before may be cross-reactive antibodies induced by HEV or HEV-like virus other than human HEV. This result was consistent with that reported by Purcell et al., in which they confirmed that genotype 1. 2 and 3 HEV do not infect laboratory rats (Purcell et al., 2011). However, Maneerat et al. (1996) published a contradictory result, in which it has reported that human HEV (presumably genotype 1) was transmissible to Wistar laboratory rats, although others have not been able to reproduce this report. Recently Lack and Volk (2012) isolated strains of genotype 3 HEV from various species of wild-caught rats in the United States. Although the detailed sequences data are not available, the genetic trees from the Technical Appendix shows that isolated sequences are quite similar to those of G3 HEV strain AF082843, isolated from a pig in the United States. However, this strain failed to infect to laboratory rats (Purcell et al., 2011).

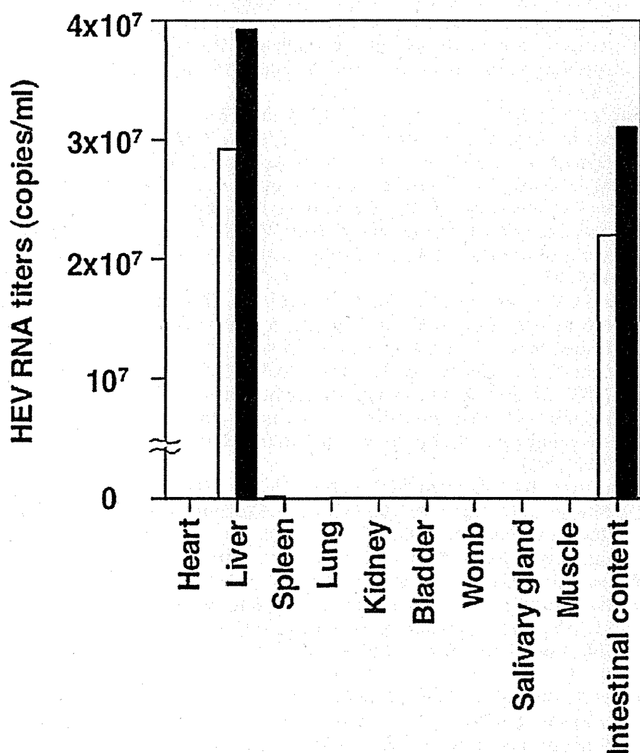


Fig. 4. Distribution of rat HEV in a nude rat. HEV RNA in different tissues from two nude rats, NR1 (white bars) and NR2 (black bars), are shown.

In contrast to G1, G3, and G4 HEV, HEV derived from wild rats infected the laboratory rats. Although no rat HEV RNA was detected in the serum samples, seroconversions were observed and rat HEV was detected in stool, demonstrating that laboratory rats are susceptible to rat HEV. We also confirmed that rat HEV was transmitted by the fecal-oral route. However, during the experimental period, rat weight increased as with normal rats and the ALT level did not change (Fig. 1). These results suggested that rat HEV, at least in low doses, might not be pathogenic to rats, as shown in this study, where i.v. inoculation or oral transmission did not induce any serious sign in the rats. We also found that the infectivity of rat HEV was lost after heat treatment at 56 °C for 30 min, suggesting that rat HEV can be inactivated relatively easily.

Because the wild rat serum has been heated at 56 °C for 30 min, we used rat lung homogenates containing rat HEV RNA to inoculate laboratory rats for the transmission study. We did not think rat HEV could be replicated at lung tissues, and we think that rat HEV RNA is included in the blood remaining in the lung tissues. In fact, after exsanguinations, the lung tissues from experimentally infected nude rats were all negative for HEV RNA. In addition, rat HEV RNA was not detected in heart, spleen, kidney, bladder, womb, salivary gland, or muscle, though it was detected in liver, suggesting that rat HEV replicates in rat liver.

In this study we confirmed that rat HEV infection was transmitted through the fecal-oral route. However, rat R-12 did not become infected with rat HEV (Fig. 1) even

Author's personal copy

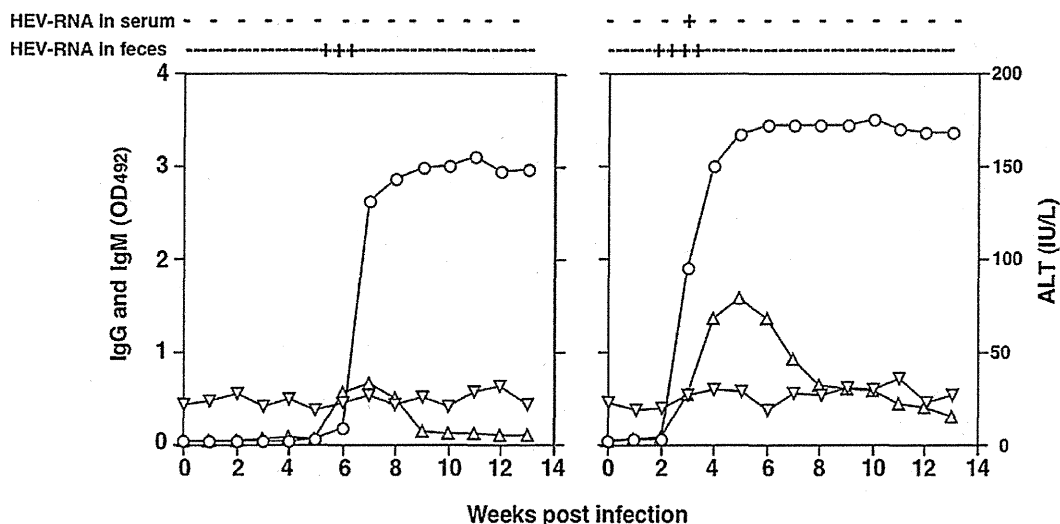


Fig. 5. Kinetics of biochemical, serological, and virological markers after oral inoculation. Laboratory rats were fed HEV-contaminated food for one week, and then viral RNA in the sera and stool were monitored by RT-PCR; +, positive; –, negative. Anti-rat HEV IgG (○) and IgM (△) antibodies were detected by ELISA, and ALT (▽) elevation was monitored.

though it was kept with HEV-infected rats, R-10 and R-11. The amount of rat HEV in the stools was too low to cause infection. Thus, HEV infection by oral inoculation may require more virus than that by intravenous inoculation. Our results clearly showed that the nude rats were useful to obtain a large amount of virus for further study of the cell culture and characterization of rat HEV.

When IgG antibody kinetics were compared between intravenous inoculation and oral inoculation, we found that oral inoculation induced higher titers, which was maintained for a long time without decline (Fig. 5). These results give us a hint about the route of HEV vaccination. Thus far, phase II and III recombinant HEV vaccine trials have been reported by two research groups (Shrestha et al., 2007; Zhu et al., 2010). Both groups delivered the candidate HEV vaccine by intramuscular injection. Although the protective efficacy against clinically overt HEV infection was confirmed, the duration of protection afforded by this vaccine remains unknown. Shrestha et al. (2007) reported that only 56.3% of vaccine inoculators maintained a level of anti-HEV antibody for 2 years post-vaccination. As natural HEV infection occurs via the fecal-oral route, oral delivery of an HEV vaccine could induce the same immune responses as natural infection. It is necessary to consider whether the oral delivery of HEV vaccine induces immune responses that are stronger than those induced by intramuscular inoculation.

Conflict of interest statement

None declared.

Acknowledgments

This study was supported in part by grants for Research on Emerging and Re-emerging Infectious Diseases, Research on Hepatitis, and Research on Food Safety from the Ministry of Health, Labor, and Welfare, Japan.

References

Arankalle, V.A., Joshi, M.V., Kulkarni, A.M., Gandhe, S.S., Chobe, L.P., Rautmare, S.S., Mishra, A.C., Padbidri, V.S., 2001. Prevalence of anti-hepatitis E virus antibodies in different Indian animal species. J. Viral Hepat. 8, 223–227.
Balayan, M.S., Andjaparidze, A.G., Savinskaya, S.S., Ketiladze, E.S., Braginsky, D.M., Savinov, A.P., Poleschuk, V.F., 1983. Evidence for a virus in non-A, non-B hepatitis transmitted via the fecal-oral route. Intervirology 20, 23–31.
Cossaboom, C.M., Cordoba, L., Sanford, B.J., Pineyro, P., Kenney, S.P., Dryman, B.A., Wang, Y., Meng, X.J., 2012. Cross-species infection of pigs with a novel rabbit, but not rat, strain of hepatitis E virus isolated in the United States. J. Gen. Virol. 93, 1687–1695.
Dremsek, P., Wenzel, J.J., Johne, R., Ziller, M., Hofmann, J., Groschup, M.H., Werdermann, S., Mohn, U., Dorn, S., Motz, M., Mertens, M., Jilg, W., Ulrich, R.G., 2012. Seroprevalence study in forestry workers from eastern Germany using novel genotype 3- and rat hepatitis E virus-specific immunoglobulin G ELISAs. Med. Microbiol. Immunol. 201, 189–200.
Drexler, J.F., Seelen, A., Corman, V.M., Fumie Tateno, A., Cottontail, V., Melim Zerbinati, R., Gloza-Rausch, F., Klose, S.M., Adu-Sarkodie, Y., Oppong, S.K., Kalko, E.K., Osterman, A., Rasche, A., Adam, A., Muller, M.A., Ulrich, R.G., Leroy, E.M., Lukashev, A.N., Drosten, C., 2012. Bats worldwide carry hepatitis E virus-related viruses that form a putative novel genus within the family Hepeviridae. J. Virol. 86, 9134–9147.
Emerson, S.U., Purcell, R.H., 2003. Hepatitis E virus. Rev. Med. Virol. 13, 145–154.
Favorov, M.O., Kosoy, M.Y., Tsarev, S.A., Childs, J.E., Margolis, H.S., 2000. Prevalence of antibody to hepatitis E virus among rodents in the United States. J. Infect. Dis. 181, 449–455.
Haqshenas, G., Shivaprasad, H.L., Woolcock, P.R., Read, D.H., Meng, X.J., 2001. Genetic identification and characterization of a novel virus related to human hepatitis E virus from chickens with hepatitis-splenomegaly syndrome in the United States. J. Gen. Virol. 82, 2449–2462.
He, J., Innis, B.L., Shrestha, M.P., Clayson, E.T., Scott, R.M., Linthicum, K.J., Musser, G.G., Gigliotti, S.C., Binn, L.N., Kuschner, R.A., Vaughn, D.W., 2002. Evidence that rodents are a reservoir of hepatitis E virus for humans in Nepal. J. Clin. Microbiol. 40, 4493–4498.
Hirano, M., Ding, X., Li, T.C., Takeda, N., Kawabata, H., Koizumi, N., Kadosaka, T., Goto, I., Masuzawa, T., Nakamura, M., Taira, K., Kuroki, T., Tanikawa, T., Watanabe, H., Abe, K., 2003. Evidence for widespread infection of hepatitis E virus among wild rats in Japan. Hepatol. Res. 27, 1–5.
Johne, R., Heckel, G., Plenge-Bönig, A., Kindler, E., Maresch, C., Reetz, J., Schielke, A., Ulrich, R.G., 2010a. Novel hepatitis E virus genotype in Norway rats, Germany. Emerg. Infect. Dis. 16, 1452–1455.
Johne, R., Plenge-Bonig, A., Hess, M., Ulrich, R.G., Reetz, J., Schielke, A., 2010b. Detection of a novel hepatitis E-like virus in faeces of wild rats using a nested broad-spectrum RT-PCR. J. Gen. Virol. 91, 750–758.

Author's personal copy

Jothikumar, N., Cromeans, T.L., Robertson, B.H., Meng, X.J., Hill, V.R., 2006. A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. J. Virol. Methods 131, 65–71.
Kabrane-Lazizi, Y., Fine, J.B., Elm, J., Glass, G.E., Higa, H., Diwan, A., Gibbs Jr., C.J., Meng, X.J., Emerson, S.U., Purcell, R.H., 1999a. Evidence for widespread infection of wild rats with hepatitis E virus in the United States. Am. J. Trop. Med. Hyg. 61, 331–335.
Kabrane-Lazizi, Y., Meng, X.J., Purcell, R.H., Emerson, S.U., 1999b. Evidence that the genomic RNA of hepatitis E virus is capped. J. Virol. 73, 8848–8850.
Khuroo, M.S., Teli, M.R., Skidmore, S., Sofi, M.A., Khuroo, M.I., 1981. Incidence and severity of viral hepatitis in pregnancy. Am. J. Med. 70, 252–255.
Lack, J.B., Volk, K., Van Den Bussche, R.A., 2012. Hepatitis E virus genotype 3 in wild rats, United States. Emerg. Infect. Dis. 18, 1268–1273.
Li, T.C., Chijiwa, K., Sera, N., Ishibashi, T., Etoh, Y., Shinohara, Y., Kurata, Y., Ishida, M., Sakamoto, S., Takeda, N., Miyamura, T., 2005. Hepatitis E virus transmission from wild boar meat. Emerg. Infect. Dis. 11, 1958–1960.
Li, T.C., Suzaki, Y., Ami, Y., Dhole, T.N., Miyamura, T., Takeda, N., 2004. Protection of cynomolgus monkeys against HEV infection by oral administration of recombinant hepatitis E virus-like particles. Vaccine 22, 370–377.
Li, T.C., Suzaki, Y., Ami, Y., Tsunemitsu, H., Miyamura, T., Takeda, N., 2008. Mice are not susceptible to hepatitis E virus infection. J. Vet. Med. Sci. 70, 1359–1362.
Li, T.C., Yoshimatsu, K., Yasuda, S.P., Arikawa, J., Koma, T., Kataoka, M., Ami, Y., Suzaki, Y., Mai le, T.Q., Hoa, N.T., Yamashiro, T., Hasebe, F., Takeda, N., Wakita, T., 2011. Characterization of self-assembled virus-like particles of rat hepatitis E virus generated by recombinant baculoviruses. J. Gen. Virol. 92, 2830–2837.
Li, T.C., Zhang, J., Shinzawa, H., Ishibashi, M., Sata, M., Mast, E.E., Kim, K., Miyamura, T., Takeda, N., 2000. Empty virus-like particle-based enzyme-linked immunosorbent assay for antibodies to hepatitis E virus. J. Med. Virol. 62, 327–333.
Liu, Z., Meng, J., Sun, X., 2008. A novel feature-based method for whole genome phylogenetic analysis without alignment: application to HEV genotyping and subtyping. Biochem. Biophys. Res. Commun. 368, 223–230.
Magden, J., Takeda, N., Li, T., Auvinen, P., Ahola, T., Miyamura, T., Merits, A., Kaariainen, L., 2001. Virus-specific mRNA capping enzyme encoded by hepatitis E virus. J. Virol. 75, 6249–6255.
Maneerat, Y., Clayson, E.T., Myint, K.S., Young, G.D., Innis, B.L., 1996. Experimental infection of the laboratory rat with the hepatitis E virus. J. Med. Virol. 48, 121–128.
Meng, X.J., 2010. Hepatitis E virus: animal reservoirs and zoonotic risk. Vet. Microbiol. 140, 256–265.
Meng, X.J., Anderson, D.A., Arankalle, V.A., Emerson, S.U., Harrison, T.J., Jameel, S., Okamoto, H., 2012. Hepeviridae. In: King, A.M.Q.A.M.J., Carstens, E.B., Lefkowitz, E.J. (Eds.), Virus Taxonomy: Ninth Report of the ICTV. Elsevier/Academic Press, London, pp. 1021–1028.
Meng, X.J., Purcell, R.H., Halbur, P.G., Lehman, J.R., Webb, D.M., Tsareva, T.S., Haynes, J.S., Thacker, B.J., Emerson, S.U., 1997. A novel virus in swine is closely related to the human hepatitis E virus. Proc. Natl. Acad. Sci. U. S. A. 94, 9860–9865.
Nakamura, M., Takahashi, K., Taira, K., Taira, M., Ohno, A., Sakugawa, H., Arai, M., Mishiro, S., 2006. Hepatitis E virus infection in wild mongooses of Okinawa, Japan: demonstration of anti-HEV antibodies and a full-genome nucleotide sequence. Hepatol. Res. 34, 137–140.
Navaneethan, U., Al Mohajer, M., Shata, M.T., 2008. Hepatitis E and pregnancy: understanding the pathogenesis. Liver Int. 28, 1190–1199.
Nelson, K.E., Kmush, B., Labrique, A.B., 2011. The epidemiology of hepatitis E virus infections in developed countries and among immunocompromised patients. Expert Rev. Anti. Infect. Ther. 9, 1133–1148.
Purcell, R.H., Engle, R.E., Rood, M.P., Kabrane-Lazizi, Y., Nguyen, H.T., Govindarajan, S., St Claire, M., Emerson, S.U., 2011. Hepatitis E virus in rats, Los Angeles, California, USA. Emerg. Infect. Dis. 17, 2216–2222.
Raj, V.S., Smits, S.L., Pas, S.D., Provacia, L.B., Moorman-Roest, H., Osterhaus, A.D., Haagmans, B.L., 2012. Novel hepatitis e virus in ferrets, the Netherlands. Emerg. Infect. Dis. 18, 1369–1370.
Reyes, G.R., Purdy, M.A., Kim, J.P., Luk, K.C., Young, L.M., Fry, K.E., Bradley, D.W., 1990. Isolation of a cDNA from the virus responsible for enterically transmitted non-A, non-B hepatitis. Science 247, 1335–1339.
Shrestha, M.P., Scott, R.M., Joshi, D.M., Mammen Jr., M.P., Thapa, G.B., Thapa, N., Myint, K.S., Fourneau, M., Kuschner, R.A., Shrestha, S.K., David, M.P., Seriwatana, J., Vaughn, D.W., Safary, A., Endy, T.P., Innis, B.L., 2007. Safety and efficacy of a recombinant hepatitis E vaccine. N. Engl. J. Med. 356, 895–903.
Takahashi, M., Nishizawa, T., Sato, H., Sato, Y., Jirintai, Nagashima, S., Okamoto, H., 2011. Analysis of the full-length genome of a hepatitis E virus isolate obtained from a wild boar in Japan that is classifiable into a novel genotype. J. Gen. Virol. 92, 902–908.
Tam, A.W., Smith, M.M., Guerra, M.E., Huang, C.C., Bradley, D.W., Fry, K.E., Reyes, G.R., 1991. Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. Virology 185, 120–131.
Tei, S., Kitajima, N., Takahashi, K., Mishiro, S., 2003. Zoonotic transmission of hepatitis E virus from deer to human beings. Lancet 362, 371–373.
Zhao, C., Ma, Z., Harrison, T.J., Feng, R., Zhang, C., Qiao, Z., Fan, J., Ma, H., Li, M., Song, A., Wang, Y., 2009. A novel genotype of hepatitis E virus prevalent among farmed rabbits in China. J. Med. Virol. 81, 1371–1379.
Zhu, F.C., Zhang, J., Zhang, X.F., Zhou, C., Wang, Z.Z., Huang, S.J., Wang, H., Yang, C.L., Jiang, H.M., Cai, J.P., Wang, Y.J., Ai, X., Hu, Y.M., Tang, Q., Yao, X., Yan, Q., Xian, Y.L., Wu, T., Li, Y.M., Miao, J., Ng, M.H., Shih, J.W., Xia, N.S., 2010. Efficacy and safety of a recombinant hepatitis E vaccine in healthy adults: a large-scale, randomised, double-blind placebo-controlled, phase 3 trial. Lancet 376, 895–902.

日本食品微生物学会雑誌 Jpn. J. Food Microbiol., 29(1), 32-37, 2012

シンポジウム1 食品からのノロウイルス検出法の最前線

●Symposium 1 Current Topics on Detection of Norovirus in Foods

食品のノロウイルス検査の汎用化を目指したパンソルビン・トラップ法の開発

Development of PANSORBIN® Trap Method as a General Protocol to Detect Norovirus in Contaminated Food

斎藤博之†
（秋田県健康環境センター）
Hiroyuki SAITO
(Akita Prefectural Research Center for Public Health and Environment, Akita-shi, Akita 010-0874)

1. はじめに

平成22年の食中毒統計によると，全国で1年間に1,254例の食中毒事例が発生し，そのうち3割に相当する399例がノロウイルス（NoV）によって引き起こされている（原因物質の第1位）．同様に患者数においても，全食中毒被害者25,972名の過半数に相当する13,904名がNoVの感染によるものである．このように，ウイルス性食中毒の大部分を占める原因物質がNoVであることが判明しているにもかかわらずカキ以外の一般的な食品からウイルスを検出できた例は非常に少ない．そのため，原因食品や汚染経路の解明が困難で予防対策においても決め手を欠いているのが実状である．

平成9年に食品衛生法の中に初めてウイルスが登場し，ウイルス性食中毒という概念が確立したのは，歴史の一つの節目と言える．その後，糞便検体に対する検査技術は長足の進歩を遂げたものの，食品検体からのウイルス検出は困難を極め，「糞便は検査できるが，食品の検査は難しい」といった状態のまま10年が経過した．ウイルスが食品衛生法上の食中毒原因物質として規定されている以上，このような片肺飛行を続けることは社会的に大きなトラブルを招来する結果につながるため，食品からのウイルス検出技術の開発は焦眉の急とされていた．そこで平成19年から厚生労働科学研究費補助金（食品の安心・安全確保推進研究事業）による研究の一環として，食品中のNoVを検出するための実践的手法の開発に関する研究がスタートした．その結果，パンソルビン・トラップ法（パントラ法）を開発し，固形，液状，練り物，油物などの多種・多様な食品からNoVを検出することが可能となった．本稿では，足掛け4年にわたった開発の過程を振り返り，今後の実践使用に向けた道筋について考察する．

† 連絡先
〒010-0874 秋田県秋田市千秋久保田町6-6

2. 食品のウイルス検査が困難な理由

NoVはいまだ実験室内培養技術が確立されていないため，増菌培養に相当するプロセスが存在せず，食品検体から直接PCRなどで検出することを余儀なくされている．それは極めて困難な作業であるが，理由としては次のようなものがある．

1) 検体の数の問題

糞便検体と違って一部を代表検体として検査して，ほかを省略するといった間引きは原則としてできないため検体数が多くなる．一定範囲の食品をプールして検査を行う方針を採用した場合，次で示す検体の量の問題に直面する．

2) 検体の量の問題

食品検体を適当な緩衝液に懸濁して乳剤とした場合，その量は少なく見積もっても50 ml程度になる．一方，PCRで用いる検体（RNA抽出液）は50 μl程度であり，1,000倍に相当する減量濃縮が必要である．

3) 検体の質の問題

表面が平滑な固形食品では，緩衝液などで洗滌することで，比較的濁質の少ない形でNoVを回収できる可能性がある．しかし，練り物や油物などを乳剤にした場合は，どれほど遠心しても上清は濁ったままであり，フィルターでろ過しようにも目詰まりを起こしてうまくいかない．また，現在多用されているポリエチレングリコール（PEG）沈殿法では，NoVとは無関係の大量の沈殿が生じて手に負えないことも多い．

4) 必要機器・時間・コストの問題

PEG沈殿法では，原則としてオーバーナイトの処理が必要とされており，さらに10,000 rpm程度の遠心によって沈殿物を回収しなければならない．しかし，50 mlの容量を10,000 rpmで遠心するには高速冷却遠心

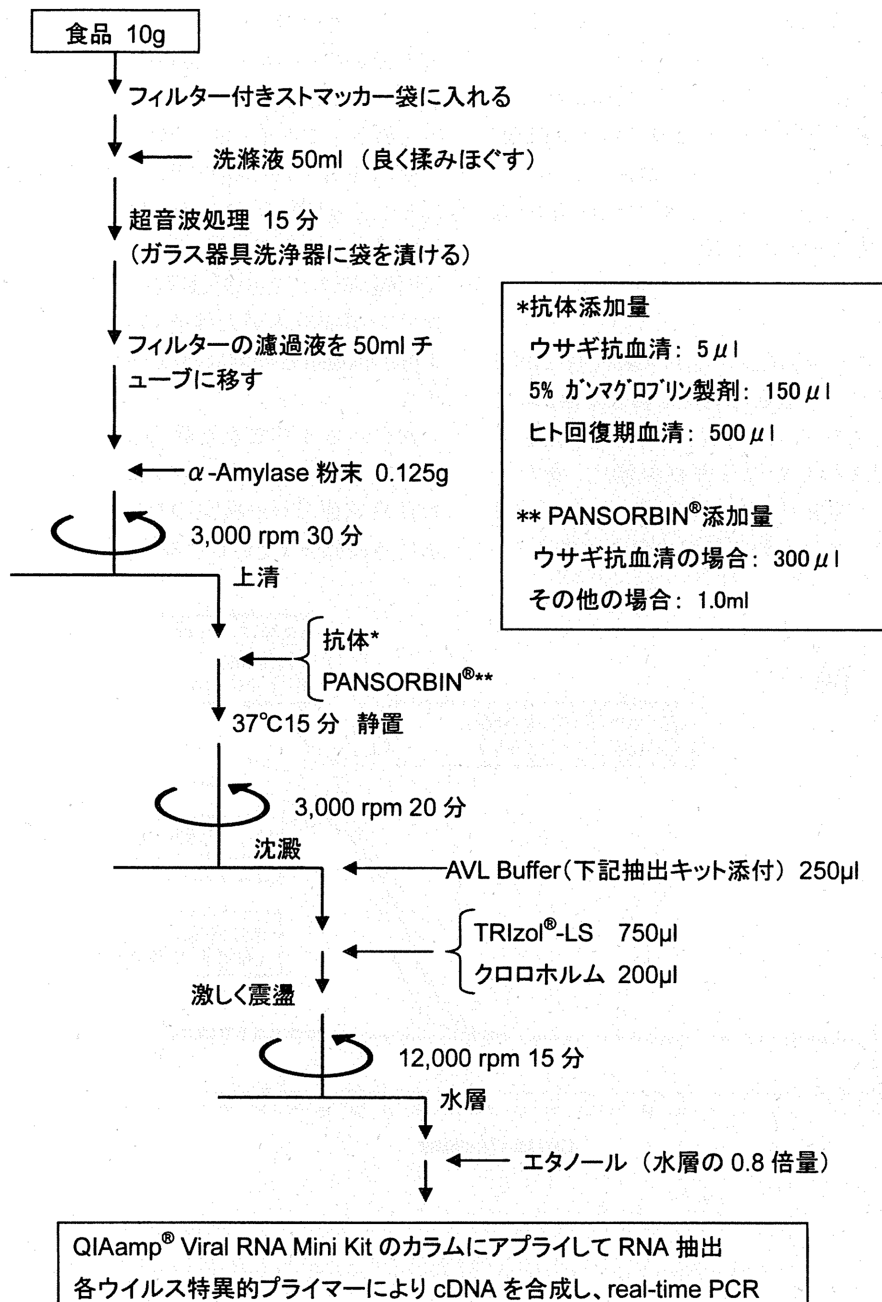


図1. パントラ法の操作手順

機を必要とし，遠心チューブも専用品を用いるようになっていることが多い．こうした専用遠心チューブはディスポーザブル使用を前提としていないため高価であり，また，洗って再利用するのはPCRを行う関係上不安が残る．

3. パントラ法の開発

パントラ法の基本原理は，食品乳剤中にNoVに特異的な抗体を添加して抗原抗体複合体を形成させ，さらにそれを黄色ブドウ球菌表面のプロテインAに吸着させて，菌体とともにNoVを沈殿・回収することである．最大の特長は，食品乳剤が濁ったままでよいという点にある．パントラ法の基本的な操作手順は図1に示すとおりである．最初に食品を50 mlの緩衝液（0.1 M Tris・HCl–0.5 M NaCl–0.1% Tween 20, pH 8.4）に懸濁した後で3,000 rpm, 30分の遠心を行うが，このとき用いる遠心機は一般の検査室にある普通のものであり，チューブもプラスチックのディスポーザブル製品である．この遠心条件で沈殿する固形物だけを除去しておけば，上清は濁っていてもかまわない．その後，抗体とパンソルビン（黄色ブドウ球菌をホルマリン固定して熱処理したもので，「PANSORBIN® Cells」の商品名でメルク社より購入できる）を添加してNoV-IgG-菌体の複合体を形成させる．その後，再び3,000 rpm, 20分の遠心を行うが，この条件で沈殿する食品由来の固形成分はすでに最初の遠心の際に除去されているため，結果としてNoVを吸

着した黄色ブドウ球菌だけが沈殿してくる．この段階で上清は濁ったままであることが多いが，NoVは菌体と一緒に沈殿物のほうに移行しているため，上清は捨ててよいことになる．沈殿した菌体を少量の緩衝液で再懸濁してから市販のキットを用いてRNA抽出を行えば，50 mlの食品乳剤から50 μlのPCRサイズのRNA溶液まで，効率よく減量濃縮できることになる．

4.　NoV回収試験

図1の基本プロトコールに従い，NoV GII/4型で汚染させた食品からウイルスの回収を試みた結果を図2に示す．添加抗体としてはNoV GII/4型のウイルス様粒子(VLPs)を免疫して作製したウサギ抗血清[3]，または5% ガンマグロブリン製剤（Baxter社の「Gammagard」）を用いた．ガンマグロブリン製剤使用の理由は後述（**6. パントラ法の汎用化**）する．NoVに対するreal-time PCRは既報[4]に従い，cDNAはPCRと同じプライマーを用いて合成した．モデル食品としては，炭水化物の多いものとしてポテトサラダを，油脂の多いものとして焼きそばを選んだ．図2では，どちらの食品においてもNoV GII/4型特異的ウサギ抗血清を添加したものは80%前後の回収率が得られており，抗体を加えない場合（非特異吸着）との差は明確である．また，ガンマグロブリン製剤を用いた場合の回収率は25%前後であり，特異的抗血清との差はPCRにおいて3サイクル以内である．

次に，さまざまな形状の食品をNoV GII/4型で汚染させたものから，パントラ法とPEG沈殿法でウイルスの回収試験を行い両者の比較を行った．PEG沈殿法における回収率を1とした場合のパントラ法の回収率比を

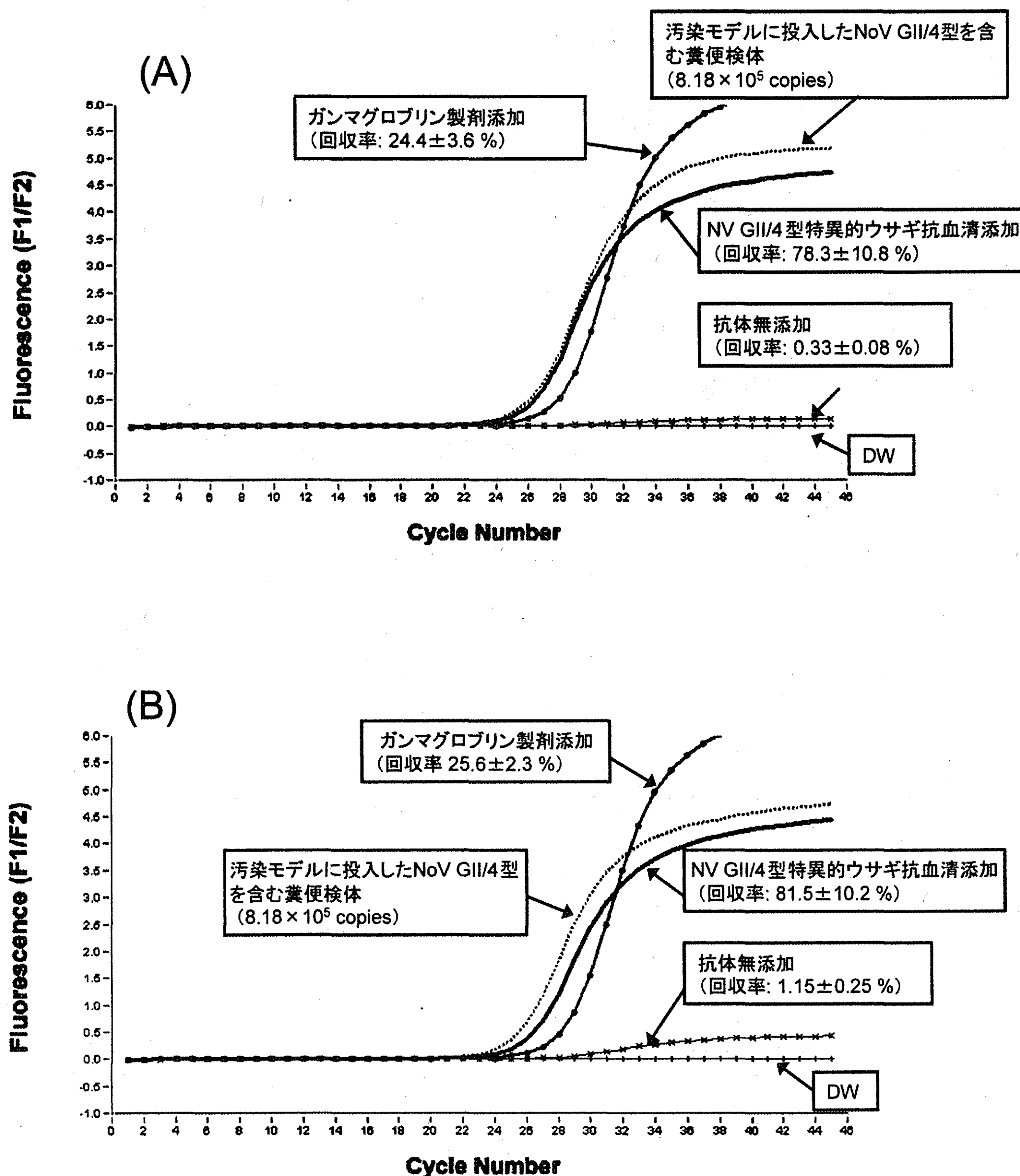


図2.　NoV GII/4型による汚染モデル食品からのウイルス回収試験
（A）ポテトサラダ，（B）焼きそば．回収率の数値は3回の試験の平均と標準偏差で示す．

表1に示す．まぐろ刺身やきのこの白和え（豆腐）のような，表面が平滑な食品においては，両者の差はあまりない．しかし，ゴボウサラダ（マヨネーズ和え）やれんこんのキンピラ（油脂を多用）など，表面汚染に止まらない形状の食品では，パントラ法の効果が顕在化してくる．事実，これらの食品にPEG沈殿法を用いた場合には，冒頭でも述べたとおり大量の濁質が操作上の障害となった．それに対し，パントラ法を用いた場合には，どのような形状の食品であっても共通の手順で検査を進めることができた．

5. 検出限界に関する検討

上記の評価試験ではウイルスの回収率を指標としたため，食品1 g当たり10^5コピー程度の汚染モデルを作成したが，実際の食中毒事例はこれよりはるかに少ないウイルス量で引き起こされている．そこで，もう一つの評価指標として検出限界について検討したものが表2である．段階希釈したウイルスを添加することで，さまざまな汚染レベルの食品モデルを作成した．また，ウイルス量が極少の場合には一般にnested-PCRのような一段高感度な手法が用いられる．現在NoVにおいて用いられているプライマーを使うことを前提とするならば，最初にプライマーCOG2F[4]とG2SKR[6]でconventional PCRを行い，その増幅産物に対して，さらに内側のプライマーで再増幅する形となる．表2では2回目の増幅にreal-time PCRを用いて，カーブが立ち上がったものについて＋と判定している．このような反応系ではreal-time PCRであっても結果は定性扱いとなるが，ゲル電気泳動したものをハイブリダイゼーションで確認したのと同等の意味があるため，タイムプレッシャーを受ける局面では便利である．このようにしてパントラ法の検出限界を求めたところ，NoV GII/4型特異的ウサギ抗血清を用いた場合は食品1 g当たり10コピー，ガンマグロブリン製剤を用いた場合は35コピーまで検出可能であった．なお，最終的な検出限界の数値はパントラ法の効果だけではなく，PCRの反応効率も包含したものであることに留意する必要がある（前述の回収率による評価は，ウイルス投入量に対する回収量を比較しているためPCRの反応効率は影響しない）．したがって，検出限界値はさまざまな要因（遺伝子型，流行ウイルスの遺伝子変異，新しいプライマーの開発など）で変動する可能性がある．

表1. さまざまな食品におけるパントラ法とPEG沈殿法の回収率の比較

食品の種類	パントラ法の回収率比（対PEG沈殿法）
ゴボウサラダ	1,000
れんこんのキンピラ	840
マカロニサラダ	240
鶏五目煮	39
ナポリタン	17
ポテトサラダ	15
まぐろ刺身	3.4
きのこの白和え	2.0

PEG沈殿法による回収率を1とした場合のパントラ法による回収率比を示す（既報[11]より抜粋）．パントラ法の添加抗体はNoV GII/4型特異的ウサギ抗血清を使用した．

6. パントラ法の汎用化

開発段階ではさまざまな反応条件を最適化するために系を単純化する必要があり，NoV GII/4型で汚染させた市販食品をモデル食品とした．添加する抗体としてNoV GII/4型のVLPsを免疫して作製したウサギ抗血清を用いて検討した．しかし，このようにして作製された抗血清は理論上の裏づけはあるものの絶対量が少なく，全国の食中毒事例に対応するには全く足りていない．また，現在のところNoV GII/4型を含めて19遺伝子型に対する特異的抗血清が利用可能であるが，その他の遺伝子型には対応できない．一方，実際に食品検体が搬入される時点では遺伝子型は不明なため選択が困難であるという実践的な問題もある．さらに，サポウイルス（SaV）などNoV以外の食中毒起因ウイルスへの対応も必要である．

ヒトは成長する過程で，さまざまなウイルスの感染を受けてそれらに対する抗体を持つに至っている．NoVに対しても同様であることが抗体保有調査の結果から判明している．したがって，添加抗体として市販ヒト血清を用いれば量的制約を受けることなしに，多くの型のNoVや他の食中毒起因ウイルスにも対応できることが期待される．しかし一方で，保有抗体レベルの個人差をどのように平準化させるかという新たな問題が生ずる．そこで，1万人単位でプールされた血漿から製造・販売されているガンマグロブリン製剤を利用することでこの

表2. Semi-nested PCRによる検出限界

食品の種類	添加抗体 ＼ 汚染レベル（copies/g・食品）	3.50×10^4	3.50×10^3	3.50×10^2	1.05×10^2	35	10	3
ポテトサラダ	NoV GII/4型特異的ウサギ抗血清	＋	＋	＋	＋	＋	＋	－
	ガンマグロブリン製剤	＋	＋	＋	＋	＋	－	－
焼きそば	NoV GII/4型特異的ウサギ抗血清	＋	＋	＋	＋	＋	＋	－
	ガンマグロブリン製剤	＋	＋	＋	＋	＋	－	－

表3．ガンマグロブリン製剤の各ウイルスとの反応性

添加抗体	各ウイルスの回収率(%)																		
	NoV													SaV				HAV	AdV41
	GI/3	GI/4	GI/8	GI/9	GI/14	GII/2	GII/3	GII/4	GII/5	GII/6	GII/12	GII/13	GII/18	GI	GII	GIV	GV		
ガンマグロブリン製剤	12.6	12.7	2.7	7.0	27.8	45.1	12.4	45.7	22.4	11.9	43.0	55.5	9.4	8.0	30.2	16.9	35.3	13.7	38.4
特異的抗血清	50.4	14.3	13.0	N.T.	N.T.	93.4	14.8	77.3	36.4	18.4	65.8	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.

50 m*l*のウイルス希釈液からのパントラ法による回収率を示す．各ウイルスのreal-time PCRについては既報（NoV[4]，SaV[9]，HAV[2]，AdV41[7]）に従った．

問題を解決することができた．これまでのところ，ガンマグロブリン製剤は，NoVでは13遺伝子型（GI/3, GI/4, GI/8, GI/9, GI/14, GII/2, GII/3, GII/4, GII/5, GII/6, GII/12, GII/13, GII/18），SaVではヒトに感染する4種類すべての型，ほかにA型肝炎ウイルス（HAV）とアデノウイルス41型（AdV41）において有効であることを確認している（表3）．なお，ガンマグロブリン製剤は多くの製薬会社から市販されており，濃度も5%（静注用）～15%（筋注用）とさまざまである．図1では5%ガンマグロブリン製剤の添加量を示してあるが，ほかの濃度のものを用いる場合は変更する必要がある（10%製剤は75 *μl*, 15%製剤は50 *μl*）．多く入れ過ぎると，パンソルビンの抗体結合能を超えるため，逆に回収率が低下する結果となる．また，ここでは米国Baxter社のGammagardを用いたが，国内製のガンマグロブリン製剤よりも回収率が高く，より多くの種類のウイルスに対して有効であることが確認されている[10]．理由としては，米国では血漿採取における事実上の売血制度が存続しているため（各製薬会社がFDAの許認可のもとで有償採取），ドナーの中で抗体保有者の占める割合が高いことが考えられる[1]．ガンマグロブリン製剤は医薬品として流通しているため容易に入手可能であるが，薬事法で定められている「特定生物由来製品」に該当するためラベルに記載されている製造番号を記録して20年間保管しなければならない（照会が入ったときに情報開示の義務がある）．ウサギ抗血清，ガンマグロブリン製剤以外の捕捉抗体としては，感染者の回復期血清を利用してもウイルスが回収可能であることが確認されている[10]．血清診断のため血清が採取されることが多いA型肝炎では有用性が高い．また，当事者の協力が得られることが大前提となるが，不幸にして社会問題に発展したようなケースでは原因究明のための選択肢の一つとして意義がある．こうした回復期血清を用いたウイルスの回収法は，将来未知のウイルスが流行した場合の初期対応としても有効であるものと考えられる．一方，ウイルス特異的なウサギ抗血清は，理論上の裏づけが強固であるという利点を生かして，精度管理に用いるようにすれば量的な問題も回避できるであろう．

7．その他の留意事項

これまでにパントラ法に関するさまざまな検討を行ってきたが，その過程で浮上してきた留意事項を3点指摘しておきたい．その第一は，食品のウイルス検査において最大の障害となる成分は炭水化物であるという点である．油脂成分に関してはウイルス粒子を適切に洗い出すことができれば（そのため超音波による乳化プロセスを組み込んでいる），PCRの反応そのものに影響は少ない．PCRの普及初期には，反応チューブにミネラルオイルを添加していたことを考えると理解しやすい．炭水化物は物理的・化学的挙動が核酸（ポリリボース骨格）と同じであるため，さまざまな分画操作を行っても最後まで混入してくる．また，予期せずに沈殿を生じることがあり，それを遠心などで除去するとウイルスのRNAまでもが共沈して失われることが多い．炭水化物の沈殿を生じやすいポイントは精製カラムにアプライする直前のエタノール添加の段階である．また，抽出したRNA溶液が透明であったとしても，超低温フリーザーで保管して，後日解凍した際に不溶性の沈殿が生じていることがある．図1のプロトコールでは，ストマッカー袋のフィルターを利用することと，α-Amylase粉末（枯草菌由来の安価なものでよい）を加えて分解することでこの問題を解決している．さらに，cDNA合成直前のDNase処理（後述）の段階で再度α-Amylase（酵素として精製されたもの）を加えることでその後のPCRでの反応効率を上げることができる．

留意事項の2点目は，cDNA合成の際に用いる逆転写プライマーとして，ウイルス特異的なもの（PCRと同じでよい）を使用したほうがよいことである．一般には逆転写反応にランダムプライマーやoligo（dT）プライマーが用いられるが，パントラ法で抽出したRNA溶液には，黄色ブドウ球菌のRNAが大量に含まれているため，PCRの感度に影響するほどの非特異的な逆転写反応が起こる．混入してくる黄色ブドウ球菌のDNAとRNAは，微量のウイルスRNAを保持するためのキャリアとして働くため，プロトコールの途中まではむしろプラスの効果がある．cDNA合成の直前にDNase処理を行うのが一般的であるため，DNAに関してはその段階で除去される．残るRNAに関しては，上記のようにウ