



Epizootiological study of rodent-borne hepatitis E virus HEV-C1 in small mammals in Hanoi, Vietnam

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ABSTRACT. There is concern about the zoonotic potential of rodent-borne hepatitis E virus, designated as HEV-C1. However, epizootiological information about HEV-C1 is limited. To address this issue, serum samples from 443 small mammals captured at 5 sites in Hanoi, Vietnam, were examined for anti-HEV-C1 IgG antibodies. In addition, livers of seropositive animals were examined for viral RNA. Anti-HEV-C1 antibodies were detected in 57 (12.9%) of the 443 serum samples. Seropositive animals were found in all of the sites (4.7% to 22.2%). Anti-HEV-C1 antibodies were detected from 48 (12.3%) of 389 *Rattus norvegicus* and 9 (19.6%) of 46 *R. tanezumi*, but were not detected from 8 *Suncus murinus*. Viral RNAs were detected from 13 (22.8%) of the 57 seropositive rodents. The detection rate of viral RNA in seropositive *R. tanezumi* (66.7%, 6/9) was significantly higher than that in seropositive *R. norvegicus* (14.6%, 7/48). The results suggest that *R. tanezumi* is more susceptible than *R. norvegicus* to HEV-C1 infection. Phylogenetic analysis revealed that Vietnamese strains were divided into 3 clusters in genetic group 2 of HEV-C1. Multiple clusters of viruses were detected at several sites without species specificity, suggesting that 3 clusters of HEV-C1 co-circulate in Hanoi, Vietnam.

KEY WORDS: hepatitis E virus, *Rattus tanezumi*, rodent, Vietnam, zoonosis

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Hepatitis E virus (HEV) is one of the major causes of acute hepatitis. HEV is transmitted primarily via the fecal-oral route and often causes water-borne epidemics in developing countries, while sporadic cases have occurred in industrialized countries, where the transmission is primarily zoonotic [7]. High mortality rates have been reported in pregnant women [1]. Chronic HEV infection in immunocompromised patients and transmission via blood transfusion and transplantation have also become serious issues [15].

HEV is classified as a member of the family *Hepeviridae*, genus *Orthohepevirus*. The genus includes four species: *Orthohepevirus A*, *B*, *C* and *D*. *Orthohepevirus A* includes strains from humans, pigs, wild boars, deer, mongooses, rabbits and camels. *Orthohepevirus B* includes strains from chickens. *Orthohepevirus C* includes strains from rats, ferrets and minks. *Orthohepevirus D* includes strains from bats [19]. Genotypes 1 to 4 HEV (HEV-1 to -4) of *Orthohepevirus A* are known to cause disease in humans. HEV-1 and HEV-2 infect only humans, while HEV-3 and HEV-4 can spread from animals to humans [9]. However, the zoonotic potential of other orthohepeviruses derived from various animals remains unclear.

HEV-C1, formerly called rat HEV, is a novel HEV belonging to *Orthohepevirus C*. HEV-C1 was first discovered from wild rodents in Germany in 2009 [6]. Following that report, HEV-C1 has been found in Vietnam, United States, Indonesia and China [10, 12, 13, 17], suggesting a worldwide distribution of HEV-C1. It is still unknown whether HEV-C1 can spread from wild rodents to humans. Purcell *et al.* [17] reported that rhesus monkeys did not develop viremia or antibodies even after intravenous inoculation of a 10^{5.2} 50% infectious dose of HEV-C1. On the other hand, Dremsek *et al.* [2] reported that some sera from healthy forestry workers in Germany reacted more strongly to HEV-C1 antigen than to HEV-3 antigen. We have also found that some sera obtained from patients with fever of unknown origin in Hanoi, Vietnam, showed higher reactivity against HEV-C1 antigen than HEV-1 antigen [18]. Successful propagation of HEV-C1 in human hepatoma cell lines has also been reported [4]. These results

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suggest that there is a potential risk of HEV-C1 infection in humans. However, epizootiological information about HEV-C1 in natural reservoirs is limited.

The aim of this study was to obtain epizootiological information about the prevalence, reservoir host species and genetic diversity of HEV-C1 in wild rodents in Hanoi, Vietnam. Serum samples from 443 small mammals captured at 5 sites in Hanoi were examined for anti-HEV-C1 IgG antibodies. Subsequently, we tried to detect viral RNA from liver homogenates of seropositive animals. Phylogenetic analysis was performed to determine the genetic diversity of HEV-C1.

MATERIALS AND METHODS

Sample collection

A total of 443 small mammals (389 *Rattus norvegicus*, 46 *R. tanezumi* and 8 *Suncus murinus*) were captured using traps placed at a bus station, two hospitals (Hospitals A and B) and two markets (Markets A and B) in Hanoi, Vietnam, from August 2012 to December 2013 (Table 1). Sera and livers were collected from the animals and stored at -20°C and -80°C , respectively, until testing.

Enzyme-linked immunosorbent assay (ELISA)

To detect anti-HEV-C1 IgG antibody, ELISA was performed using virus-like particles (VLPs) of HEV-C1 as an antigen as described previously [10]. VLPs of HEV-C1 were produced by a baculovirus expression system and kindly provided by Dr. Tian-Cheng Li in the National Institute of Infectious Diseases, Tokyo, Japan. Ninety-six-well EIA/RIA plates (Corning, Corning, NY, U.S.A.) were coated with the VLPs ($1\ \mu\text{g}/\text{ml}$) overnight at 4°C . After blocking with phosphate buffered saline (PBS) containing 3% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, U.S.A.) for 1 hr at 37°C , sera were diluted to 1:200 with PBS containing 0.5% BSA and 0.05% Tween 20 and added to the plates for 1 hr at 37°C . Bound antibodies were detected with horseradish peroxidase (HRP)-conjugated goat anti-rat IgG (H+L) antibody (KPL, Gaithersburg, MD, U.S.A.) for rodent sera or HRP-conjugated Protein A (EY Laboratories, San Mateo, CA, U.S.A.) for shrew sera for 1 hr at 37°C . Reactivity of shrew sera against Protein A has been confirmed in a previous study [16]. Coloring reaction was performed with *o*-phenylenediamine dihydrochloride (Sigma-Aldrich) at room temperature for 30 min, and the reaction was stopped with 10% H_2SO_4 . Optical density (OD) at 492 nm was measured by using a SpectraMax 340 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, U.S.A.). The plates were washed with PBS containing 0.05% Tween 20 three times at the end of each step before the coloring step. The cutoff value was set at $\text{OD}=0.6$, which was determined by histogram analysis.

Detection of viral RNA

Since it has been reported that detection rate of viral RNA in seropositive rodents was remarkably higher than that in seronegative rodents [5, 13, 14], we examined livers of seropositive rodents for viral RNA. Total RNA was extracted from liver homogenate by using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. cDNA was synthesized from total RNA using SuperScript II Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, U.S.A.) and random hexamer according to the manufacturer's instructions. cDNAs of the ORF1 and ORF2 junctional region were amplified via nested PCR by using Platinum Taq DNA Polymerase High Fidelity (Thermo Fisher Scientific) and primer pairs: a primer pair of Rat HEV-F4S ($5'$ -TTTGCCCTTGTTMGGGCMATAGAGAA- $3'$) and Rat HEV-R3S ($5'$ -GCTGTCAWYGGCGACTGCCCGGCATCGGG- $3'$) and a primer pair of Rat HEV-F5S ($5'$ -GCCAACCTGCCYGARTGGTGYTTTTATGG- $3'$) and Rat HEV-R4S ($5'$ -CAGCGGCACGAACAGCARAAGCASGAGC- $3'$). For 3 representative strains, cDNAs of the entire ORF2 gene and the $3'$ non-coding region were also amplified by using the following primer pairs: Rat HEV-F8S ($5'$ -TACAGYTKGAGGGGARRARGGAATAACAYTC- $3'$) and Rat HEV-R7S ($5'$ -GCATARTTIGARGCCTCAGTYGCCATRATRITGCG- $3'$), Rat HEV-F9S ($5'$ -TACCCGATGCCGGSCAGTCGCCRATGAC- $3'$) and Rat HEV-R8S ($5'$ -TAAAGYTTRACIGAIGGYTCICCRTTCTCVGAC- $3'$), Rat HEV-F10S ($5'$ -GGTGRGGIGAYATAGGYAAGGGYATWGTICAG- $3'$) and Rat HEV-R10S ($5'$ -AGACACTGTGCGCKGCGYCKRYTGACARIGCCTG- $3'$), and Rat HEV-F11S ($5'$ -TGCCCWGCCTGTGCTYTGCCGGGCTHTC- $3'$) and TX30SXN [8].

Sequence analysis

The PCR products were purified with a MinElute Gel Extraction Kit (Qiagen, Venlo, Netherlands) and subjected to nucleotide sequencing with a BigDye Terminator v3.1 Cycle Sequencing Kit and ABI PRISM 3130xl Genetic Analyzer (Thermo Fisher Scientific) according to the manufacturer's instructions. A phylogenetic tree was constructed by the neighbor-joining method with GENETYX-MAC (ver. 15.0.1), CLUSTAL W (ver. 1.83) and Njplot (ver. 2.3). Bootstrap resampling analysis was performed using 1,000 replicates. Accession numbers of nucleotide sequences determined in this study are LC164672 to LC164684.

Quantification of viral RNA

Total RNAs extracted from viral RNA-positive liver homogenates were reverse-transcribed into cDNAs as described above. The cDNAs were subjected to real-time PCR using Lightcycler 480 Probe Master (Roche, Basel, Switzerland), primers and probe designed by Jirintai *et al.* [4], and Lightcycler 480 II (Roche) according to the manufacturer's instructions.

Table 1. Prevalence rates of anti-HEV-C1 IgG antibodies in small mammals in Hanoi

Site	Species			Total
	<i>R. norvegicus</i>	<i>R. tanezumi</i>	<i>S. murinus</i>	
Bus station	12/98 (12.2%)	0/7 (0%)	0	12/105 (11.4%)
Hospital A	7/40 (17.5%)	8/32 (25.0%)	0	15/72 (20.8%)
Hospital B	2/31 (6.5%)	0/4 (0%)	0/8 (0%)	2/43 (4.7%)
Market A	25/212 (11.8%)	1/2 (50.0%)	0	26/214 (12.1%)
Market B	2/8 (25.0%)	0/1 (0%)	0	2/9 (22.2%)
Total	48/389 (12.3%)	9/46 (19.6%)	0/8 (0%)	57/443 (12.9%)

Table 2. Prevalence rates of viral RNA among seropositive rodents

Species	Positive rate of viral RNA
<i>R. norvegicus</i>	7/48 (14.6%)
<i>R. tanezumi</i>	6/9 (66.7%) ^{a)}
Total	13/57 (22.8%)

a) Significant difference in positive rate of viral RNA ($P < 0.05$).

Viral isolation

Viral RNA-positive liver homogenates were subjected to viral isolation using Huh-7 cells as described by Jirintai *et al.* [4]. Supernatant from the culture medium at 3 weeks post-inoculation was inoculated into new Huh-7 cells. Presence of virus in culture supernatant was confirmed by real-time PCR as described above.

Statistical analysis

Pearson's chi-square test was used for comparison of seroprevalences and detection rates of viral RNA among different groups. Student's *t*-test was used for comparison of variables. A *P* value < 0.05 was considered statistically significant.

RESULTS

Prevalence rate of anti-HEV-C1 IgG antibodies

Sera were examined for anti-HEV-C1 IgG antibodies in ELISA. Anti-HEV-C1 antibodies were detected in sera from 48 (12.3%) of the 389 *R. norvegicus* and 9 (19.6%) of the 46 *R. tanezumi*, but were not detected in sera from any of the 8 *S. murinus*. Overall, 57 (12.9%) of the 443 animals were positive for anti-HEV-C1 antibodies (Table 1). There was a significant difference in seropositivity between male and female *R. norvegicus* (16.7% versus 9.4%, $P < 0.05$), but there was no significant difference in seropositivity between male and female *R. tanezumi* (16.7% versus 22.7%). The prevalence rates in the trapping sites were 11.4% (12/105) at the bus station, 20.8% (15/72) in Hospital A, 4.7% (2/43) in Hospital B, 12.1% (26/214) in Market A and 22.2% (2/9) in Market B (Table 1). The prevalence rate in Hospital A, where *R. tanezumi* was exceptionally abundant, was relatively high. Average OD value of seropositive *R. tanezumi* was significantly higher than that of seropositive *R. norvegicus* (2.17 versus 1.35, $P < 0.05$).

Detection rate of viral RNA among seropositive rodents

Livers of seropositive rodents were examined for RNA of HEV-C1 by RNA extraction, reverse transcription and nested PCR. Viral RNAs were detected from 13 (22.8%) of the 57 seropositive rodents. The detection rate of viral RNA in seropositive *R. tanezumi* was 66.7% (6/9), which was significantly higher than the rate of 14.6% (7/48) in seropositive *R. norvegicus* (Table 2).

Quantity of viral RNA in livers of PCR-positive rodents

To compare viral loads between PCR-positive *R. tanezumi* and *R. norvegicus*, viral RNAs in their livers were quantified by real-time PCR. Viral RNAs were detected from all of the 6 *R. tanezumi* and 5 of the 7 *R. norvegicus*. Average copy number of viral RNA in the 6 *R. tanezumi* was 1.2×10^5 copy / μg RNA, which was comparable with that in the 5 *R. norvegicus* (1.9×10^5 copy / μg RNA).

Viral isolation from livers of PCR-positive rodents

To isolate virus from livers of PCR-positive *R. tanezumi* and *R. norvegicus*, liver homogenates were inoculated into Huh-7 cells. Presence of HEV-C1 in culture medium at 3 weeks post-inoculation was confirmed by real-time PCR. HEV-C1 was isolated from 5 (83.3%) of the 6 *R. tanezumi*, while HEV-C1 was isolated from 5 (71.4%) of the 7 *R. norvegicus*. There was no significant difference in quantity of viral RNA in culture medium of Huh-7 cells (data not shown).

Sequence analysis

Nucleotide sequences of cDNA amplified from the 13 rodents were successfully determined. BLAST analysis showed that all of the sequences had the highest homology to HEV-C1 sequences (89% to 96%), indicating that all of the Vietnamese strains detected in this study were HEV-C1. According to previous studies, HEV-C1 strains can be divided into 3 genetic groups (G1, G2 and G3) and 2 unassigned China clusters [12, 14]. In order to compare Vietnamese strains with all of the genetic groups and clusters, sequences of the common genomic region corresponding to nt 4,151 to 4,366 in the HEV-C1 genome (JX120573) were used for phylogenetic analysis. A phylogenetic tree showed that the 13 Vietnamese strains could be divided into 3 clusters (Vietnam clusters 1 to 3) (Fig. 1A). HEV-C1 strain Vietnam-105, which was previously isolated from *R. tanezumi* in Hanoi [10], was included in Vietnam cluster 3. All of the Vietnam clusters were classified into G2 and separated from Indonesia clusters 1 and 3 and China clusters A1 and A2 in G2 (Fig. 1A). In order to conduct phylogenetic analysis based on longer sequences, Vietnam-Rt153-2013, Vietnam-Rn142-2013 and Vietnam-Rt335-2013 were selected as representatives for Vietnam clusters 1 to 3, respectively, and the nucleotide sequences of the entire ORF2 gene and the 3' non-coding region were determined. A phylogenetic tree based on sequences corresponding to nt 4,138 to 6,927 in the HEV-C1 genome (JX120573) confirmed that there were 3 Vietnam clusters in the G2 branch of HEV-C1 (Fig. 1B). Multiple Vietnam clusters of strains were detected from animals captured at the bus station and Hospital A in Hanoi (Table 3). Strains of Vietnam clusters 1 and 3 were detected from both of *R. norvegicus* and *R. tanezumi* (Table 3).

DISCUSSION

Information on the prevalence of HEV-C1 infection in wild animals has been limited. In this study, we conducted an investigation at 5 sites in Hanoi and revealed that HEV-C1 was widely distributed among wild rodents in Hanoi. The prevalence rate of IgG antibody to HEV-C1 in wild animals captured at the 5 sites was 12.9% (4.7% to 22.2%), similar to or slightly lower than previously reported rates: 18.8% in Hanoi and 22.7% in Haiphong in Vietnam [10], 24.5% (0% to 41.2%) in Germany [5], 78.4% in Los Angeles, U.S.A. [17], 18.1% (0% to 100%) in Lombok Island located west of Bali Island [13] and 37.1% (11.8% to 46.8%) in Lombok Island and Solo in Central Java [14] in Indonesia, and 23.3% (0% to 45.3%) in Guangdong Province, China [12]. Unexpectedly, the prevalence rate in animals captured at one of the hospitals (Hospital A) in Hanoi was relatively high (15/72, 20.8%). On the other hand, the largest number of antibody-positive rodents was captured in Market A (26/214, 12.1%), which is an old wet market. Although the pathogenicity of HEV-C1 in humans remains unclear, there is a potential risk to human health.

It is unclear which species is important as a reservoir of HEV-C1. In this study, we showed that the prevalence rate of IgG antibody to HEV-C1 in *R. tanezumi* was higher than that in *R. norvegicus*. We also showed that the detection rate of viral RNA and average OD value in seropositive *R. tanezumi* was significantly higher than that in seropositive *R. norvegicus* (66.7% versus 14.6% and 2.17 versus 1.35). These results suggest that *R. tanezumi* is more susceptible than *R. norvegicus* to HEV-C1 infection. On the other hand, we also found that quantity of viral RNA and viral isolation rate in PCR-positive *R. norvegicus* were comparable with those in *R. tanezumi*. The results imply that HEV-C1 propagates transiently in *R. norvegicus*. It has been reported that HEV-C1 caused transient infection in laboratory rats (*R. norvegicus*) [11, 17]. Johne *et al.* [5] reported that 6 (16.7%) of 36 IgG-positive *R. norvegicus* in Germany had viral RNA, similar to the results of our study. Mulyanto *et al.* [14] reported a high detection rate of viral RNA in IgG-positive *R. rattus* (67/137, 48.9%), which is a species closely related to *R. tanezumi*. We speculate that so-called black rats have susceptibility to HEV-C1 infection. Interestingly, the proportion of *R. tanezumi* was exceptionally high in Hospital A. The high proportion of *R. tanezumi* may have increased the prevalence of HEV-C1 infection in the rodent population in that place. Although Guan *et al.* [3] reported that 27 (10.4%) of 260 *S. murinus* were positive for IgG antibody to HEV-C1 antigen, we could not detect seropositive *S. murinus*. Because of the low number of *S. murinus* examined in this study, we cannot draw a conclusion about the susceptibility of *S. murinus* to HEV-C1. Further studies are needed to examine the susceptibility of *S. murinus* using a greater number of samples from *S. murinus*.

We demonstrated that at least 3 clusters of HEV-C1 strains were circulating in Hanoi. The Vietnam clusters belonged to G2, but differed from Indonesia clusters 1 and 3 and China clusters A1 and A2 in G2. Taken together, the results of the present and previous studies suggest that there are diverse HEV-C1 strains other than G1 strains in Southeast Asia, although one strain of G1 HEV-C1 was detected in Indonesia [14]. Geographical clustering of HEV-C1 strains was shown in previous studies conducted in Germany, Indonesia and China [5, 12, 14]. However, in this study, multiple Vietnam clusters of HEV-C1 were detected from animals captured at the bus station and Hospital A, suggesting that 3 Vietnam clusters of HEV-C1 co-circulate in Hanoi. We speculate that some of the clusters had been imported from another place with the movement of infected rodents and/or virus-contaminated materials and had colonized. The ease of colonization will be a challenge for the control of HEV-C1.

In conclusion, diverse HEV-C1 strains were found to be widely distributed in an urban area of Hanoi. *R. tanezumi* is an important reservoir of HEV-C1. In order to evaluate the risk of HEV-C1 infection in humans, investigation of human specimens collected in an endemic area of HEV-C1 is needed.

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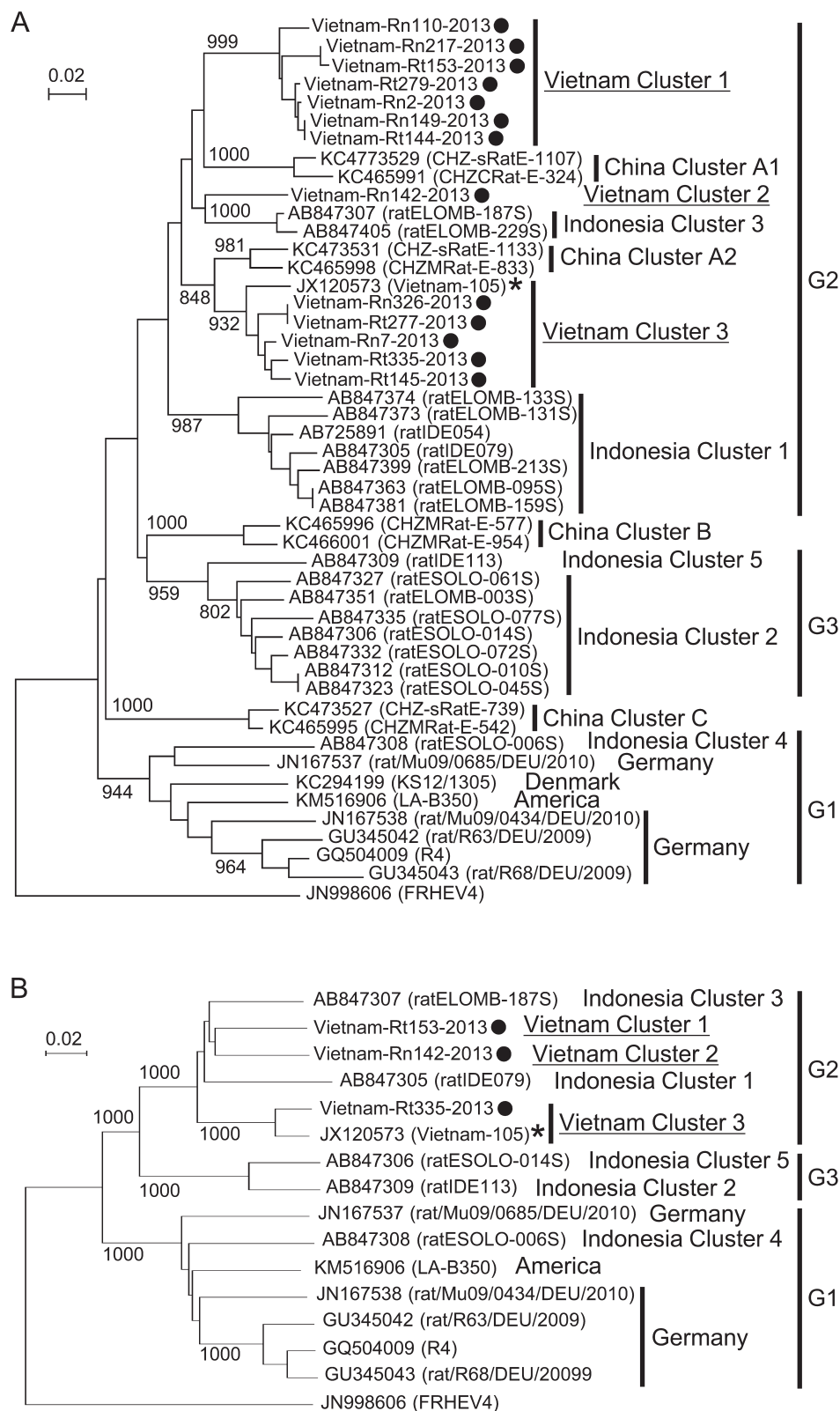


Fig. 1. Phylogenetic analysis of Vietnamese strains of HEV-C1. Phylogenetic trees were constructed by the neighbor-joining method based on sequences corresponding to nt 4,151 to 4,366 (A) and nt 4,138 to 6,927 (B) in the genome of the HEV-C1 strain Vietnam-105 (JX120573) using HEV-C2 strain FRHEV4 as an outgroup. More than 800 bootstrap values in the major branch are shown. Filled circles and asterisks indicate Vietnamese strains detected in the present and previous studies, respectively [8]. Each reference strain is presented with the accession number. G1, G2 and G3 represent Genetic groups 1, 2 and 3, respectively, which were described by Mulyanto *et al.* [14].

Table 3. Strains of Vietnam clusters detected from rodents in Hanoi

Vietnam cluster	Species	Site			Total
		Bus station	Hospital A	Market A	
1	<i>R. norvegicus</i>	1	2	1	4
	<i>R. tanezumi</i>	0	3	0	3
2	<i>R. norvegicus</i>	1	0	0	1
	<i>R. tanezumi</i>	0	0	0	0
3	<i>R. norvegicus</i>	1	1	0	2
	<i>R. tanezumi</i>	0	3	0	3

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