indigenous HEV via intake of meat/viscera from domestic pigs or wild animals, such as boar and deer, has been recently increasingly recognized.[13–18] Additionally, HEV infection via blood transfusion has been rarely reported in Japan.[19]

The serological diagnoses of HAV and HEV infections have been already confirmed based on tests of anti-HAV IgM and anti-HEV IgM in many industrialized countries. In Japan, because the anti-HEV IgM and/or HEV RNA for the identification of HEV infection could not have been routinely examined before the 1990s, AH and ALF with HEV infection were considered to be "non-B, non-C", except for the cases whose stored blood samples were later tested for the presence of HEV RNA.[20] Since the anti-HEV IgA antibody became routinely detectable by the enzyme-linked immunoassay with purified recombinant HEV ORF2 protein (genotype 4) used as the solid-phase antigen,[21] in the sera from patients with acute liver injury (ALI) in October 2010, more than 100 patients have been annually reported to have AH-E.[7] However, these numbers of AH-E cases reported at present would be underestimated, because a nationwide survey of HEV infection in the general population of Japan suggested that approximately 150 000 adults contract HEV infection annually.[7,22]

Hepatitis A virus has six genotypes (I–VI), with genotypes I, II and III found in humans. These are further divided into subgenotypes IA and IB, IIA and IIB and IIIA and IIIB, respectively.[23–25] There are four recognized genotypes (1–4) of HEV that infect humans.[26] The prevalence of the genotypes/subgenotypes of HAV and HEV is known to be different across the world.[23,26] In Japan, subgenotype IA of HAV and genotypes 3 and 4 of HEV are predominant.[7,27–29] Additionally, genotypes 3 and 4 of HEV have been recognized to be zoonotic and autochthonous viruses.[7] Japan-indigenous genotype 3 HEV strains have been provisionally classified into three lineages, 3jp, 3us and 3sp, where "jp" stands for Japan-type, "us" for US-type and "sp" for Spanish (European) type.[3,30] The 3jp, 3us and 3sp lineages correspond to subgenotypes 3b, 3a and 3e, respectively, as proposed by Lu *et al.*[31] Our previous studies have indicated that the majority of sporadic AH and ALF cases had genotype IA AH-A, and genotype 3 AH-E in Iwate on Honshu Island,[32] and that genotype 4 HEV is predominant in the AH and ALF patients in Hokkaido.[14] However, a recent study on the spatial distribution of HEV genotypes suggested the circulation of genotype 4 HEV not only in Hokkaido, but also in the other districts.[7]

In the present study, using the registration system for a prospective cohort study on ALI started in 2004, we examined whether the annual prevalence of sporadic AH-A and AH-E, and the distribution of viral genotypes have changed in Iwate and three neighboring prefectures (Aomori, Akita and Miyagi) in the northern part of Honshu Island, Japan, during the last 10 years.

METHODS

Patients and registration system

A TOTAL OF 487 patients with ALI due to various viral and non-viral causes were registered in a prospective cohort study involving 42 hospitals in Iwate ($n = 31$), Aomori ($n = 5$), Akita ($n = 4$) and Miyagi ($n = 2$) prefectures in the northern part of Honshu (North Tohoku), Japan, located within 250 km of Iwate Medical University Hospital (the study group office). This cohort study was started in 2004 to validate a predictive model of hepatic encephalopathy (HE) in ALI,[33] including an evaluation of the therapies and prognosis of the patients. Briefly, when the ALI patients showing lower activity (<80%) for the prothrombin time (PT) visited each hospital, their history and laboratory data were immediately faxed to the study group office at Iwate Medical University. After the patient's probability of subsequent development of HE was calculated according to our previously established HE-predicting model,[33] the obtained data and the therapeutic options including whether the patients should be transferred to Iwate Medical University Hospital were returned to the patient's hospital by fax. Finally, the study group office collected the information about the causes, treatment and prognosis of registered patients with ALI by fax and/or telephone interview, except for the patients who were transferred to Iwate Medical University Hospital.

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committees of Iwate Medical University and each institution. Informed consent was obtained from each patient and/or their families. Simultaneously, when the ALI patients with unknown etiologies agreed to provide a blood sample, approximately 5 mL of peripheral blood was obtained, and separated serum samples were stored at −20°C or below until the assays of the HEV antibody and HEV RNA levels were performed in the study group office. Finally, the serum HEV antibody titer, HEV RNA and genotypes of HAV and HEV were measured in the Division of Virology, Department of Infection and Immunity, Jichi Medical University School of Medicine.

Identification of the causes and clinical disease types of ALI

The causes of ALI were classified into acute viral hepatitis, acute hepatic drug reactions, mushroom hepatotoxicity, acute-onset autoimmune hepatitis, alcoholic hepatic injury without previously recognized chronic status, acute cardio-circulatory disturbance including septic shock, malignant infiltration of the liver and unknown.[34] The "unknown" category included cases with a lack of medical records or without a full serological examination of virus markers except for HEV. Viral hepatitis was diagnosed based on the following examinations: HAV, positive for anti-HAV IgM; hepatitis B virus (HBV), positive for both hepatitis B surface antigen and anti-HBV core IgM; hepatitis C virus (HCV), positive for anti-HCV; HEV, positive for both anti-HEV IgM and IgA;[21] cytomegalovirus (CMV), anti-CMV IgM positive; Epstein–Barr virus (EBV), positive for both anti-EBV IgM and anti-EBV nuclear antigen; and parvovirus B19 (PPVB19), positive for anti-PPVB19 IgM. The HBV-associated infections included self-limiting AH, an acute flare of an asymptomatic HBV carrier without pre-existing symptomatic chronic liver disease and *de novo* hepatitis B. In this cohort study, the serological marker of hepatitis D virus (HDV) infection was not examined, because HDV infection, including co-infection with HBV, has never been experienced in our geographic regions. When the serum anti-HAV IgM or anti-HEV IgM and/or IgA findings were positive, HAV RNA and HEV RNA were examined by nested reverse transcription polymerase chain reaction (RT–PCR) methods, as described below, to confirm the ongoing HAV or HEV infection.

In addition, we carefully and precisely confirmed the clinical disease types, namely, self-limiting AH, ALF without overt HE, ALF with HE, subacute liver failure with HE, acute-on-chronic liver failure and late-onset hepatic failure (LOHF) based on the criteria of those diseases in Japan.[34–36]

Detection of HAV RNA and determination of the HAV genotypes/subgenotypes

Total RNA extracted from 100 μL of serum was reverse transcribed, and subsequent nested PCR was performed with primers derived from the areas of the VP1–2B region of the HAV genome, which is well conserved across all three genotypes (I–III), using the method described previously.[37] The amplification product of the first-round PCR was 548 base pairs (bp), and that of the second-round PCR was 522 bp. The PCR product of the second-round PCR was subjected to electrophoresis on an agarose gel, and a sample with a visible band at 522 bp was considered to be positive for HAV RNA. The HAV genotype was determined by a phylogenetic analysis of the amplified HAV sequence (481 nucleotides [nt]: primer sequences at both ends excluded).

Detection of HEV RNA and determination of the HEV genotypes

Total RNA extracted from 100 μL of serum was reverse transcribed, and subsequent nested PCR was performed with primers derived from the areas of the ORF2 region that are well conserved across all four genotypes (1–4), using the method described previously.[38] The size of the amplification product of the first-round PCR was 506 bp, and that of the amplification product of the second-round PCR was 457 bp. The PCR product of the second-round PCR was subjected to electrophoresis on an agarose gel, and a sample with a band of 457 bp was considered to be positive for HEV RNA. The HEV genotype was determined by a phylogenetic analysis of the amplified HEV sequence (412 nt: primer sequences at both ends excluded).

Sequence analysis

The amplification products were sequenced directly on both strands by employing an Applied Biosystems 3130xl Genetic Analyzer (Life Technologies, Carlsbad, CA, USA) with a BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies). The sequence analysis was performed using the Genetyx software program (version 12.0.3; Genetyx, Tokyo, Japan), and multiple alignments were generated by the CLUSTAL Omega software program (ver. 1.2.0).[39] Phylogenetic trees were constructed by the neighbor-joining method,[40] using the Kimura two-parameter model and 1000 replicates of bootstrap resampling, as implemented in the MEGA6 software program (ver. 6.0.6: release #6140122).[41]

Statistical analysis

The statistical analysis was performed using the χ^2-test, Fisher's exact probability test and Mann–Whitney *U*-test where appropriate. $P \leq 0.05$ was considered statistically significant.

RESULTS

Prevalence of AH-A and AH-E among ALI patients

AMONG THE 487 patients registered in this cohort study, 135 were diagnosed with viral ALI and 212 patients were diagnosed with non-viral ALI, while 140

Table 1 Number of the ALI patients with viral or non-viral infection enrolled in the present study and prevalence of HAV and HEV infections, stratified by prefecture

Prefecture (no. of hospitals)	No. of ALI patients with:			
	Viral infection			Non-viral infection
	No. of patients	HAV	HEV	
Iwate (31)	116 (85.9%)†	7 (6.0%)	20 (17.2%)	175 (82.6%)‡
Aomori (5)	10 (7.4%)†	2 (20.0%)	0	24 (11.3%)‡
Akita (4)	7 (5.2%)†	0	3 (42.9%)	10 (4.7%)‡
Miyagi (2)	2 (1.5%)†	1 (50.0%)	0	3 (1.4%)‡
Total (42)	135	10 (7.4%)	23 (17.0%)	212

†Percentage of the total ALI patients with viral infection is indicated in parenthesis.
‡Percentage of the total ALI patients with non-viral infection is indicated in parenthesis.
ALI, acute liver injury; HAV, hepatitis A virus; HEV, hepatitis E virus.

patients were undiagnosed due to an unknown etiology (mostly non-viral), including those who had incomplete medical records (Table 1). In the 135 ALI patients with viral infections, which included self-limiting AH ($n = 25$), an acute flare of asymptomatic HBV carriers without pre-existing symptomatic chronic liver disease cirrhosis ($n = 46$) and *de novo* hepatitis B ($n = 4$), the prevalence of HBV infection was highest (55.6%, 75/135). Sporadic AH-E and AH-A were seen in 23 (17.1%) and 10 patients (7.4%), respectively. On the other hand, in the non-viral ALI cases, the numbers of patients with disease caused by alcoholic hepatic injury, autoimmune-related hepatic injury and drug-induced hepatic injury were 61 (28.6%), 36 (17.1%) and 59 (27.9%), respectively. In patients with disease caused by these other etiologies, acute cardio-circulatory disturbance, including septic shock, was the most common factor.

Although most of the cases studied were enrolled from Iwate, there were no appreciable differences of registration rate in patients with viral and non-viral ALI among the four prefectures (Table 1). In Iwate, HEV infection was significantly more prevalent than HAV infection (17.2% vs 6.0%, $P = 0.0209$), while the prevalence of HAV and HEV infections was similar in the non-Iwate area (15.8% vs 15.8%) (Table 1). However, when the prevalence of HAV and HEV infections was compared, there were no significant differences between the Iwate and non-Iwate areas (6.0% vs 15.7% [$P = 0.1323$] and 17.2% vs 15.8% [$P = 0.8760$], respectively).

Annual number of AH-A and AH-E cases during the period 2004–2013

The annual number of patients with AH-A or AH-E during the period 2004–2013 is shown in Figure 1. The number of patients with AH-A or AH-E differed by year, ranging from zero to six patients, with the highest number (four) for AH-E in 2010 and 2011 and two cases each year for AH-A in 2006, 2009 and 2011. When the AH patients were divided into two groups (2004–2008 and 2009–2013) according to the year of onset, the number of AH-E patients was higher in the 2009–2013 group than in the 2004–2008 group (15 vs 8), although there was no appreciable difference in the prevalence between the two year groups (17.4% vs. 16.3%). The number of AH-A patients was similar between the two year groups, and the prevalence of AH-A was nearly stable (8.2% in 2004–2008 and 7.0% in 2009–2013). When the month of onset of illness was compared between the AH-A and AH-E patients, there was no particular season associated with

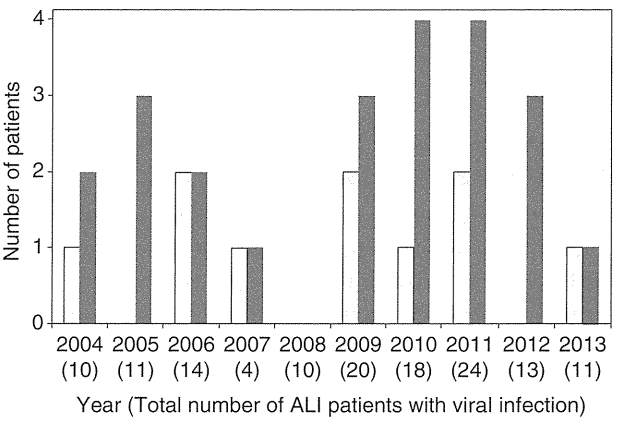

Figure 1 Annual number of acute hepatitis patients with hepatitis A and E during the period 2004–2013. ALI, acute liver injury; □, hepatitis A; ■, hepatitis E.

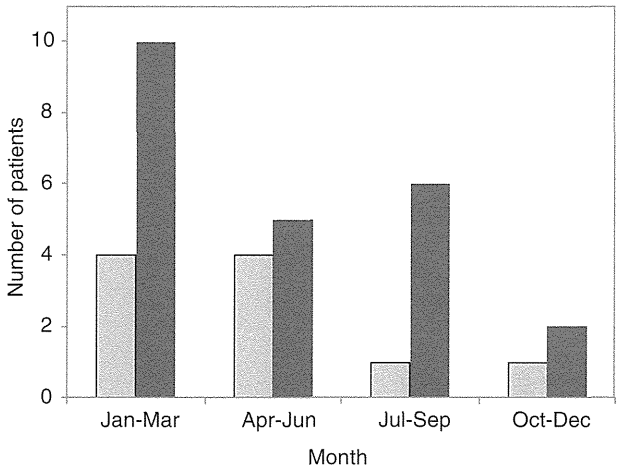

Figure 2 Number of acute hepatitis patients with hepatitis A and E according to the month of disease onset. ▨, hepatitis A; ■, hepatitis E.

the development of AH-A or AH-E, although the number of AH-A and AH-E patients was relatively higher from January to June (Fig. 2).

Distribution of the genotypes (subgenotypes) of HAV and HEV

With regard to the genotype (subgenotype) of HAV, both IA and IIIA were seen during the last 10 years, and there were no significant differences in the distribution of IA and IIIA between the two groups divided by the year of onset (IA [$n = 2$], IIIA [$n = 1$] in 2004–2008 and IA [$n = 2$], IIIA [$n = 2$] in 2009–2013). According to the medical records, patients A8 and A9 infected with subgenotype IIIA HAV were considered to have contracted HAV infection via a common transmission route by consuming raw or undercooked meat/viscera during the same period of time (Table 2). On the other hand, HEV genotype 3 was predominant throughout the observation period. Notably, although all patients during the period 2004–2008 had genotype 3 HEV, three patients (20%) were infected with genotype 4 HEV during the period 2009–2014 (Table 3).

Characteristics of patients with AH-A or AH-E

The demographic and virological features of the 10 AH-A patients and 23 AH-E patients are shown in Tables 2 and 3, respectively, and the clinical features and laboratory data are compared between the AH-A and AH-E patients as in Table 4. The AH-E patients were older than AH-A patients (57.3 vs 45.2 years) ($P = 0.0327$), but the liver function test results at the time of registration were similar between the AH-A and AH-E patients. With regard to the clinical disease types, the majority of patients with AH-A and AH-E showed self-limiting AH, while three patients (A2, E1 and E21) contracted ALF without overt HE and one patient (E18) developed LOHF. Finally, two AH-E patients diagnosed with self-limiting AH or LOHF died: patient E2 died due to complications of a hypersensitive drug reaction and CMV reactivation, as previously described in a case report,[42] and patient E18 died due to liver failure.

Table 2 Profiles of the 10 patients with HAV infection

Patient no.	Onset year	Sex	Age (years)	Location†	Clinical disease type and prognosis	HAV genotype	Name of HAV isolate
A1	2004	M	54	Iwate	AH, alive	IA	HA13-2172
A2	2006	M	22	Miyagi (India)	ALF without HE, alive	IIIA	HA13-2173
A3	2006	F	38	Iwate	AH, alive	NA	NA
A4	2007	M	40	Iwate (Indonesia)	AH, alive	IA	HA13-2348
A5	2009	M	54	Iwate	AH, alive	IA	HA13-2174
A6	2009	M	45	Aomori	AH, alive	NA	NA
A7	2010	M	29	Aomori	AH, alive	NA	NA
A8	2011	M	52	Iwate	AH, alive	IIIA	HA13-2175
A9	2011	M	49	Iwate	AH, alive	IIIA	HA13-2176
A10	2013	F	69	Iwate	AH, alive	IA	HA13-2171

†The location indicates the place where the HAV strains were obtained. The HAV strain from patient A2 was imported from India, and that from patient A4 was from Indonesia.
AH, self-limited acute hepatitis; ALF without HE, acute liver failure without hepatic encephalopathy; HAV, hepatitis A virus; NA, not available.

Table 3 Profiles of the 23 patients with HEV infection

Patient no.	Onset year	Sex	Age (years)	Location†	Clinical disease type and prognosis	HEV genotype‡	Name of HEV isolate
E1	2004	F	54	Iwate	ALF without HE, alive	3 (3us)	HE-JA04-0525
E2	2004	M	65	Iwate (Tokyo)§	AH, died	3 (3jp)	HE-JA42 (AB218721)
E3	2005	M	39	Iwate	AH, alive	3 (3jp)	HE-JA05-0325
E4	2005	M	50	Iwate	AH, alive	3 (3jp)	HE-JA05-1832
E5	2005	F	43	Iwate	AH, alive	3 (3jp)	HE-JA05-1015
E6	2006	M	52	Iwate	AH, alive	3 (3jp)	HE-JA06-0319
E7	2006	M	68	Iwate	AH, alive	3 (3jp)	HE-JA06-1647
E8	2007	M	71	Iwate	AH, alive	3 (3jp)	HE-JA07-0744
E9	2009	M	83	Akita	AH, alive	3 (3us)	HE-JA09-0490
E10	2009	M	57	Iwate	AH, alive	3 (3us)	HE-JA10-0027
E11	2009	M	53	Iwate	AH, alive	3 (3us)	HE-JA10-0037
E12	2010	F	59	Iwate	AH, alive	3 (3jp)	HE-JA10-0919
E13	2010	M	51	Iwate	AH, alive	3 (3jp)	HE-JA10-0934
E14	2010	M	74	Iwate	AH, alive	4	HE-JA10-0938
E15	2010	F	18	Iwate	AH, alive	3 (3jp)	HE-JA10-0921
E16	2011	M	75	Iwate	AH, alive	3 (3jp)	HE-JA11-0901
E17	2011	M	41	Iwate	AH, alive	3 (3jp)	HE-JA11-1396
E18	2011	M	72	Iwate	LOHF, died	4	HE-JF11-1400
E19	2011	F	51	Iwate	AH, alive	3 (3us)	HE-JA11-1409
E20	2012	M	46	Iwate	AH, alive	3 (3us)	HE-JA12-0421
E21	2012	M	61	Iwate	ALF without HE, alive	3 (3us)	HE-JA12-1020
E22	2012	M	55	Akita	AH, alive	4	HE-JA12-1019
E23	2013	M	79	Akita	AH, alive	3 (3jp)	HE-JA13-2014

†The location indicates the place where the patient contracted the HEV infection.
‡Genotype 3 was further divided into three subgenotypes (3jp, 3us and 3sp) in the present study: 3jp stands for Japan-type, 3us stands for US-type and 3sp stands for European (Spanish) type.
§This patient developed acute hepatitis E in Tokyo and was admitted to a hospital in his home town in Iwate soon after the onset of the disease.
AH, self-limited acute hepatitis; ALF without HE, acute liver failure without hepatic encephalopathy; HEV, hepatitis E virus; LOHF, late-onset hepatic failure.

Table 4 Clinical summary of the patients with acute hepatitis A or E

Features	HAV ($n = 10$)	HEV ($n = 23$)	*P*
Male : female	8:2	18:5	NS
Mean age, years (range)	45.2 (22–69)	57.3 (18–83)	0.0327
Possible transmission route			
Intake of fresh oysters or marine products	2	0	NS
Intake of or contact with meat/viscera from animals	2	6†	NS
Intake of natural spring water	0	2†	NS
Unknown	6	16	NS
Location of transmission			
Domestic	8	23	NS
Imported	2 (India, Indonesia)	0	NS
Genotypes and subgenotype	IA : IIIA = 7:3	3:4 = 20 (3jp, 13; 3us, 7):3	
Liver function tests at the time of registration, mean value (range)			
Total bilirubin (mg/dL)	4.45 (2.52–13.2)	3.8 (0.5–22.2)	NS
AST (IU/L)	2459 (190–10763)	1267 (108–8861)	NS
ALT (IU/L)	3081 (590–8150)	1483 (175–5753)	NS
Prothrombin time (INR)	1.36 (0.86–1.51)	1.19 (0.88–2.26)	NS
Clinical disease type			
AH	9	20	NS
ALF without hepatic encephalopathy	1	2	NS
LOHF	0	1	NS
Prognosis			
Alive : died	10:0	21:2	NS

†One case reported intake of uncooked meat and natural spring water.
AH, acute hepatitis; ALF, acute liver failure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HAV, hepatitis A virus; HEV, hepatitis E virus; INR, international normalized ratio; LOHF, late-onset hepatic failure; NS, not significant.

Phylogenetic analysis of HAV strains

Stored serum samples were available from seven out of the 10 AH-A patients, and seven HAV isolates were obtained in the present study (Table 2). Figure 3 shows the neighbor-joining tree of the 481-nt sequence in the VP1–2B segment of the 68 HAV isolates including 61 representative isolates of IA, IB, IIA, IIB, IIIA and IIIB, whose entire genomic sequences were known, and those obtained in the present study, using a simian HAV isolate (D00924) of genotype V as an outgroup. Although no significant bootstrap support was obtained, possibly because of the weak phylogenetic signal of the short region, HA13-2172 from patient A1, HA13-2174 from patient A5 and HA13-2171 from patient A10 were grouped into a cluster with Japan-indigenous IA strains (sublineage IA-1) which shared the highest identities of 99.5–99.7% with the reported IA-1 strains,[43,44] within the 481-nt VP1–2B sequence. In support of the patient's history of travel to Indonesia, HA13-2348 obtained from patient A4 was segregated into a cluster consisting of the Indonesian strains, with a bootstrap value of 98%, and shared the highest nucleotide sequence identities of 99.5% with BaliA03-H23 (AB839705) of Indonesian origin.

Three HAV strains, HA13-2173 from patient A2, HA13-2175 from patient A8 and HA13-2176 from patient A9, were classified into subgenotype IIIA. Of note, HA13-2173 shared the highest identity of 98.1% with the Indian IIIA strains, consistent with the patient's history of travel to India. HA13-2175 and HA13-2176 were 100% identical to each other and shared 98.7% similarity with a Korean strain (JQ655151). However, patients A8 and A9 had no history of travel to South Korea, where outbreaks of HAV, with an increasing prevalence of subgenotype IIIA over time, have been reported since 2007.[45,46]

Phylogenetic analysis of HEV strains

Stored serum samples were available from all 23 AH-E patients, and 23 HEV isolates were recovered from these patients in the present study (Table 3). Phylogenetic trees were constructed based on the common 412-nt ORF2 sequence of representative human and animal HEV isolates of Japanese or non-Japanese origin,

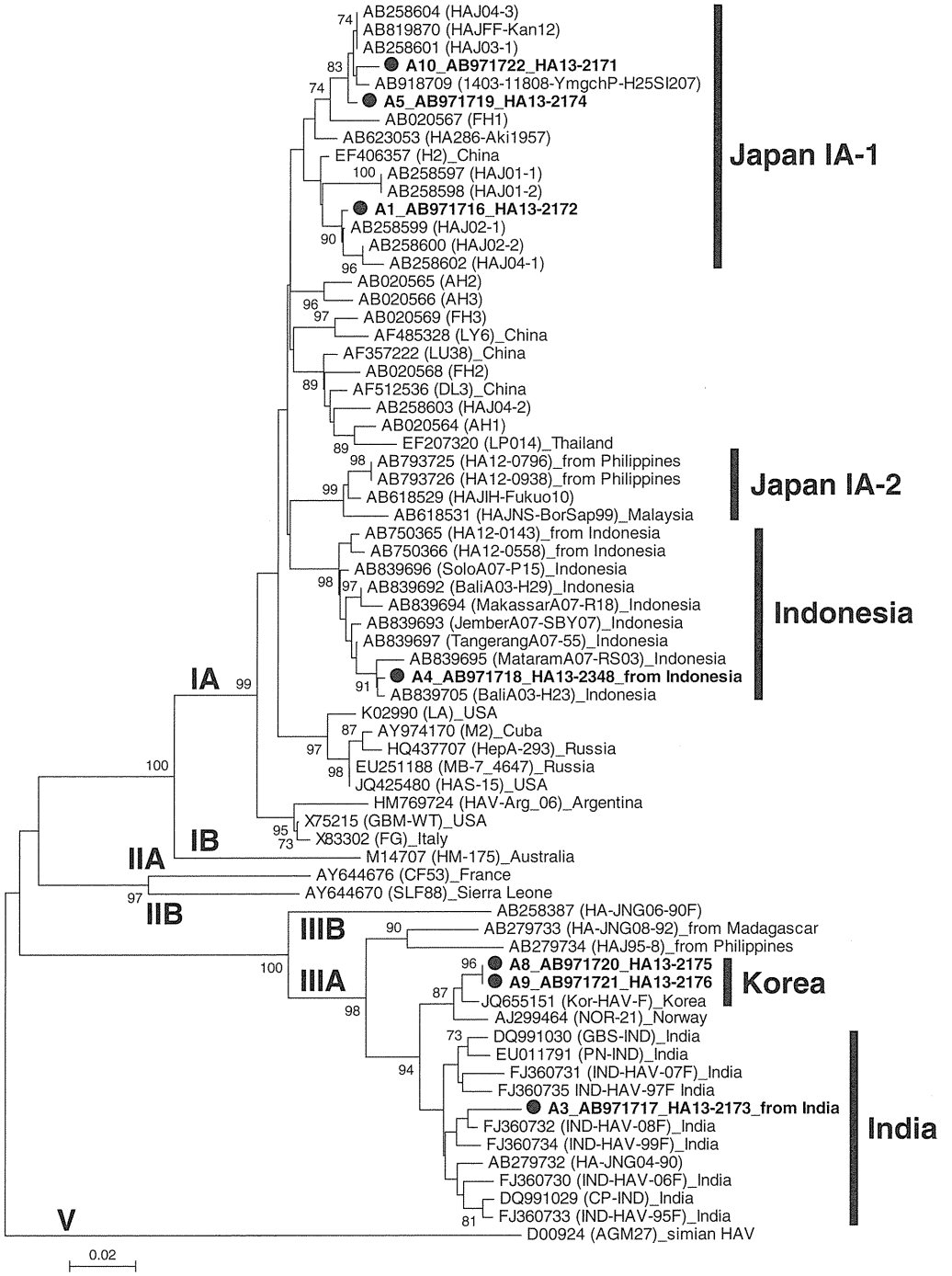

Figure 3 Phylogenetic tree constructed by the neighbor-joining method using AGM27 (D00924) as an outgroup, based on the partial nucleotide (nt) sequences of the VP1–2B region (481 nt). In addition to the seven isolates obtained in the present study, which are indicated with the patient numbers, accession numbers and the isolate names (see Table 2), shown in bold and marked with closed circles, representative HAV isolates of genotypes/subgenotypes IA, IB, IIA, IIB, IIIA and IIIB, whose entire genomic sequences were already known, were included for comparison. The IA isolates of IA-1 and IA-2 sublineages identified during the mini-epidemics in 2010 in Japan[43] and those recovered in Indonesia, as well as the IIIA isolates from Korea and India are indicated with vertical bars for visual clarity. The bootstrap values (>70%) are indicated for the nodes as a percentage of the data obtained from 1000 resamplings. The scale bar is in units of nucleotide substitutions per site.

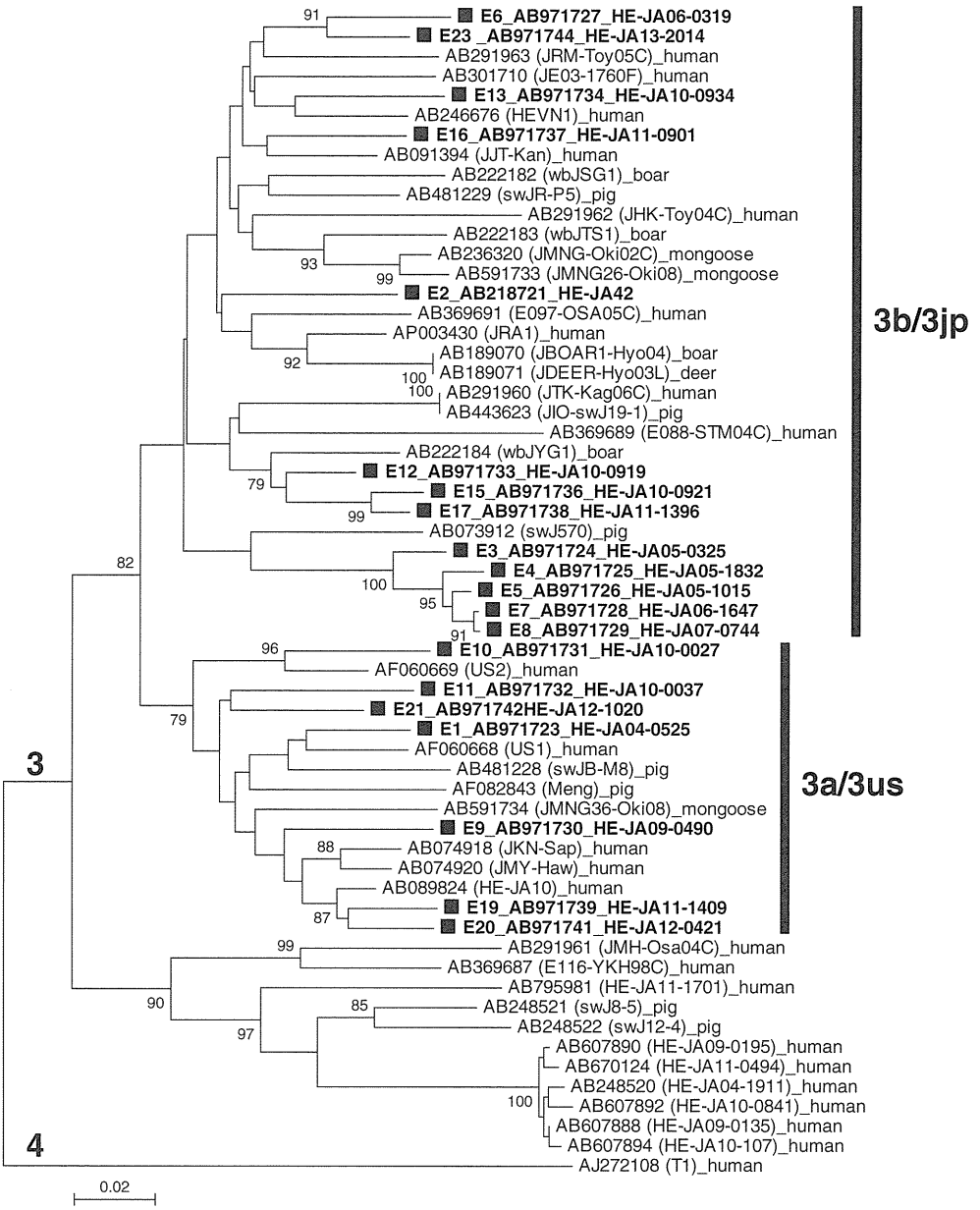

Figure 4 Neighbor-joining tree of the 412-nucleotide ORF2 sequences of the 20 genotype 3 hepatitis E virus isolates obtained in this study (see Table 3), along with 38 reference sequences of genotype 3 and the outgroup isolate of genotype 4 (AJ272108). The reference sequences are shown with accession numbers, followed by the isolate name and the name of animal species. The two major subgenotypes 3b (3jp) and 3a (3us), within the Japan-indigenous genotype 3[3,30] are indicated by vertical bars for visual clarity. The 20 genotype 3 strains isolated in this study are shown in bold and marked with closed boxes. The bootstrap values (>70%) are indicated for the nodes as a percentage of the data obtained from 1000 resamplings. The scale bar is in units of nucleotide substitutions per site.

including those obtained in the present study, focused on genotype 3 (Fig. 4) or genotype 4 (Fig. 5). As illustrated in Figure 4, among the 20 genotype 3 isolates obtained in the present study, 13 HEV isolates formed a cluster of 3b/3jp, but differed from each other by 0.2–13.6%. The remaining seven genotype 3 isolates were segregated into a cluster of 3a/3us, but a pairwise comparison revealed a nucleotide sequence difference of

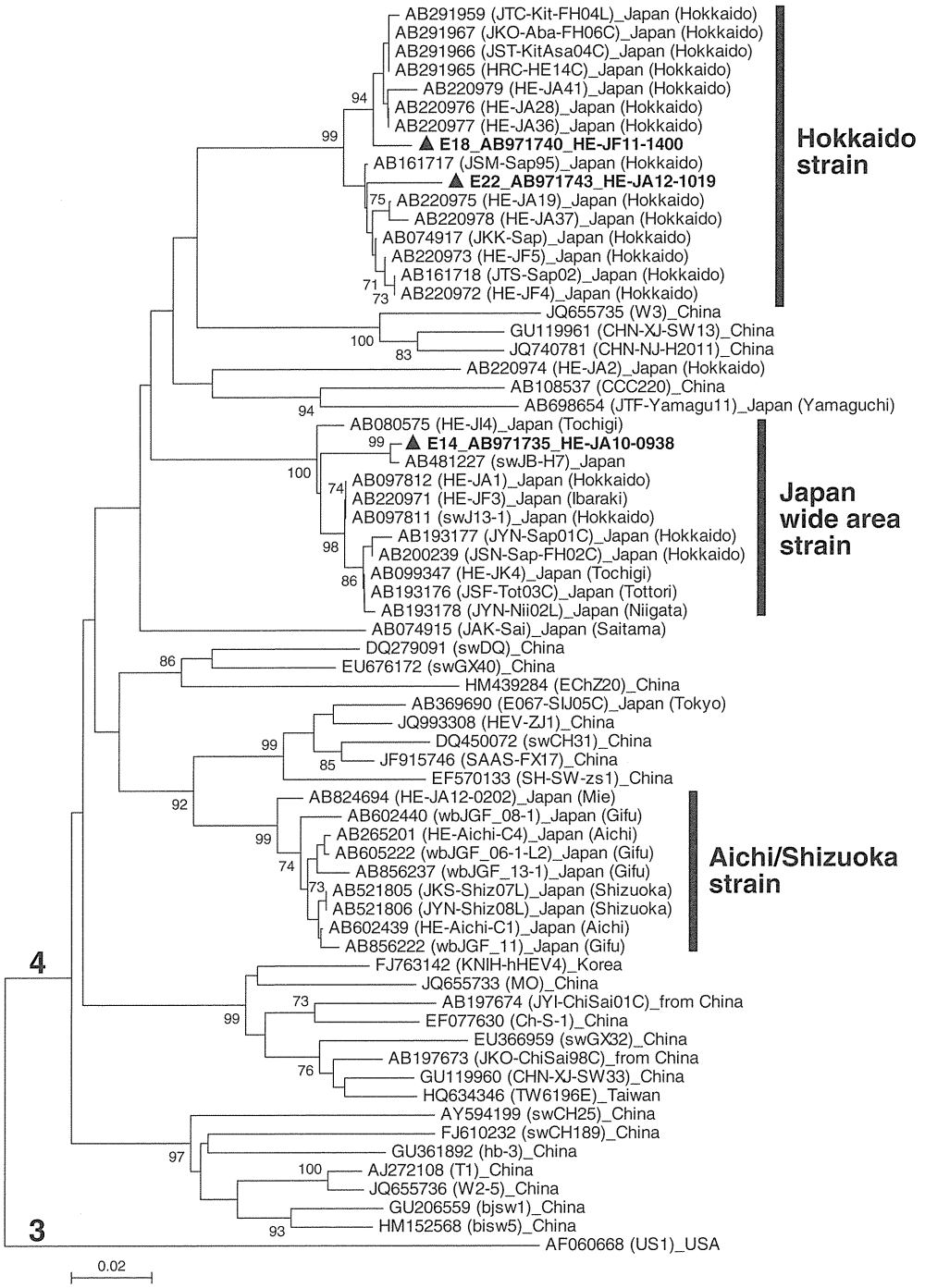

Figure 5 Neighbor-joining tree of the 412-nucleotide ORF2 sequences of the three genotype 4 hepatitis E virus isolates obtained in this study (see Table 3) with 63 reference sequences of genotype 4 and the outgroup isolate of genotype 3 (AF060668). The reference sequences are shown with accession numbers, followed by the isolate name and the name of the country where it was isolated. For the strains isolated in Japan, the name of the prefecture is indicated in parentheses. Three representative strains distributed in Hokkaido and Aichi/Shizuoka and widely across Japan[47,48] are indicated by vertical bars for visual clarity. The three genotype 4 strains isolated in this study are shown in bold and are marked with closed triangles. The bootstrap values (>70%) are indicated for the nodes as a percentage of the data obtained from 1000 resamplings. The scale bar is in units of nucleotide substitutions per site.

4.1–10.4% among them, suggesting the circulation of a heterogeneous population of genotype 3 HEV strains in the studied area.

The genotype 4 HEV strains circulating in Japan can be provisionally divided into three lineages, including the Hokkaido strains distributed in Sapporo, Kitami, Abashiri and Hakodate,[47] represented by JKK-Sap (AB074917), the Aichi/Shizuoka strains distributed widely in four prefectures (Aichi, Shizuoka, Gifu and Mie),[48] represented by HE-Aichi-C1 (AB602439) and the "Japan-wide area" strains distributed widely across Japan (Fig. 5). Two of the three genotype 4 HEV strains obtained in the present study were grouped into the Hokkaido strain cluster, supported by a high bootstrap value of 99%, but shared nucleotide sequence identities of only 96.1–98.7% with the reported Hokkaido strains. The remaining genotype 4 strain (HE-JA10-0938 from patient E14) was segregated into the "Japan-wide area" strain cluster, but was only 96.3–99.5% identical to the reported "Japan-wide area" strains. These results indicate that polyphyletic HEV strains are circulating in the northern part of Honshu Island.

DISCUSSION

HEPATITIS A VIRUS and HEV are common causes of waterborne disease (found in both drinking and recreational water) in humans, and are known as enterically transmitted hepatitis viruses, which are usually transmitted via the fecal–oral routes. However, indigenous (autochthonous) HEV infection via the intake of meat/viscera from domestic pigs and wild animals such as boar and deer has been increasingly reported in industrialized countries including Japan.[13,14,17,18] In addition, the HEV genotype, in particular, genotype 4, has been recognized to be closely associated with the severity of ALI, leading to ALF in Japan.[7,49,50] Because the majority of individuals who contract HEV infection are likely to be asymptomatic and show a subclinical course, it is estimated that, at most, 1% of patients with clinical symptoms related to acute HEV infection would visit hospitals.[7,22] Since a serological test (anti-HEV IgA enzyme immunoassay) for the diagnosis of AH-E, which is covered by the government insurance program, was made available for routine examination in October 2010 in Japan, the reports of AH-E have been gradually increasing. However, the number of patients with AH-E registered in the public health center of each prefecture in Japan has remained lower than estimated. Therefore, the changing profiles of the prevalence of AH-E patients and the distribution of viral genotypes in domestic areas of Japan are still unclear.

The present study revealed that HEV was more frequently implicated in the occurrence of AH than HAV (17.0% vs 7.4%) during the period 2004–2013 in the northern part of Honshu, including four prefectures (Iwate, Aomori, Akita and Miyagi), located in North Tohoku district, where the prevalence of anti-HEV IgG in healthy adults is known to be high.[22] Although the prevalence of AH-A and AH-E differed by year and the annual number of patients with AH-E tended to increase after 2009, there were no appreciable differences in the prevalence of AH-A and AH-E between the two groups divided by (2004–2008 and 2009–2013). This phenomenon may be due to an increasing awareness of AH-E and recent activities by the registration system, which have helped enlighten both physicians and the general population about the disease. Although the distribution of the HAV genotype (IA and IIIA) was unchanged, and the HEV genotype 3 was predominant throughout the observation period, subgenotype IIIA HAV, similar to the Korean strain of the same subgenotype, and genotype 4 HEV were found in this area after 2010.

Recent reports indicated that outbreaks of HAV, with the subgenotype shifting from IA to IIIA, occurred from 2007 in South Korea, a neighboring country.[45,46] The identification of subgenotype IIIA HAV homologous to Korean IIIA HAV from two patients (A8 and A9) in the present study, who had never been to South Korea, suggests that Korean IIIA HAV has been indigenized in Japan, and is most likely distributed widely in this country. Considering the fact that there has been a recent decrease in the proportion of people who have immunity against HAV in Japan[51] and a proposed association of subgenotype IIIA HAV and ALF,[52] particular attention should be paid to the circulation of IIIA HAV in Japan.

Hepatitis E virus infection is generally a self-limited transient infection, and HEV is eliminated by the immune response of the host. Therefore, AH-E does not require antiviral therapy, although some patients may require treatment of symptoms. However, our previous studies revealed that 0.8% (1/128) of patients infected with genotype 3 HEV and 8.1% (6/74) of patients with genotype 4 HEV contracted fulminant hepatitis E (ALF with HE).[7] In fact, in this study, one of the three patients with genotype 4 HEV developed LOHF and died of hepatic failure, while two of the 20 patients with genotype 3 HEV contracted ALF without HE, but recovered

without the aid of any antiviral treatment. Notably, since Gerolami *et al.*[53] reported that ribavirin was effective for the treatment of severe AH-E, it may be necessary to consider early treatment with antiviral drugs, such as ribavirin, in patients with severe AH, with a lower PT and high predictive value for HE.

Recently, patients with chronic HEV infection with solid-organ transplantation receiving immunosuppressive treatment have been the focus of many studies.[54,55] The age of the patients, their history of surgical treatment for cancer and some disease complications, such as diabetes mellitus, were likely associated with their immunosuppressive status. In this study, we were able to confirm that the HEV RNA in serum samples became undetectable within 1–5 months after the disease onset in nine (E1, E7–E9, E11, E16 and E20–E22) out of the 21 AH-E patients who recovered from the disease, while it was unknown whether HEV was really eliminated from the host in the remaining 12 patients. Because the results of liver function tests were normalized in all 12 patients, it seems unlikely that they progressed to chronicity. Of note, HEV viremia persisted for 4 months after admission in patient E9, probably due to the administration of a steroid based on the initial diagnosis of drug-induced liver injury. However, chronic hepatitis E in immunocompetent patients also has been recently reported.[56,57] Therefore, HEV viremia should be examined in patients with persistently elevated levels of liver enzymes, preferably in all AH-E patients, and it should be clearly demonstrated that HEV has been cleared from the infected patients in order to rule out the progression to a chronic state. This should be performed at least until it becomes evident what percentage of immunocompetent patients with clinical or subclinical HEV infection will develop chronic disease.

Our registration system for a prospective cohort study of ALI in the northern part of Honshu, Japan, included four prefectures (Iwate, Aomori, Akita and Miyagi) located in North Tohoku district. However, seven of the 10 AH-A patients and 20 of the 23 AH-E patients evaluated in this study were registered in Iwate prefecture: the remaining three AH-A patients were from Aomori ($n = 2$) and Miyagi ($n = 1$) prefectures and the remaining three AH-E patients were from Akita prefecture. In the present study, most of the ALI cases were enrolled from Iwate, accounting for 85.9% and 82.6% of the total cases with viral and non-viral infections, respectively (Table 1). In non-Iwate prefectures, both HAV and HEV infections were also noted in the 2004–2008 and 2009–2013 groups. Although the prevalence of AH-A and AH-E patients would be expected to differ by prefecture, due to the small number of patients enrolled in non-Iwate areas, analysis of the prevalence of diseases and chronological trend in each prefecture was preliminary. It is very likely that patients with mild disease status (with the PT value of >80%), particularly those with a definitive diagnosis of a recognized virus infection, might not have been reported in the present registration system. Therefore, continued activities to elucidate the precise prevalence of AH with HAV or HEV infection, including the virus genotypes, and to clarify the routes of infection of these viruses in each prefecture in the northern part of Honshu will be necessary in future studies. In addition, it will be necessary to establish a new comprehensive and uniform questionnaire for AH patients to achieve higher quality of registration system.

In conclusion, in the northern part of Honshu (mainly in Iwate), Japan, where anti-HEV IgG is known to be highly prevalent,[22] HEV (not HAV) was found to be the dominant cause of AH, following HBV, during the period 2004–2013. The recent emergence of genotype 4 HEV in this area, which is associated with the development of a severe form AH-E or fulminant hepatitis E, suggests the need for continued careful surveys of clinical and subclinical HEV infection. Most importantly, because the majority of AH-A and AH-E cases have unknown sources and routes of infection in this area, the clarification of the source(s) and transmission route(s) of HAV and HEV infections is urgently required to prevent unexpected expansions and outbreaks of these viral infections.

ACKNOWLEDGMENTS

WE EXPRESS OUR deep gratitude to the many physicians who participated in this prospective cohort study of acute liver injury in the northern part of Honshu, Japan. We also thank Miss Kouko Motodate for the preparation of serum samples. This study was supported in part by a grant from the Ministry of Health, Labor and Welfare of Japan (H24-Hepatitis-General-002).

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Journal of General Virology (2014), **95**, 2223–2232 DOI 10.1099/vir.0.068429-0

Consensus proposals for classification of the family *Hepeviridae*

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The family *Hepeviridae* consists of positive-stranded RNA viruses that infect a wide range of mammalian species, as well as chickens and trout. A subset of these viruses infects humans and can cause a self-limiting acute hepatitis that may become chronic in immunosuppressed individuals. Current published descriptions of the taxonomical divisions within the family *Hepeviridae* are contradictory in relation to the assignment of species and genotypes. Through analysis of existing sequence information, we propose a taxonomic scheme in which the family is divided into the genera *Orthohepevirus* (all mammalian and avian hepatitis E virus (HEV) isolates) and *Piscihepevirus* (cutthroat trout virus). Species within the genus *Orthohepevirus* are designated *Orthohepevirus* A (isolates from human, pig, wild boar, deer, mongoose, rabbit and camel), *Orthohepevirus* B (isolates from chicken), *Orthohepevirus* C (isolates from rat, greater bandicoot, Asian musk shrew, ferret and mink) and *Orthohepevirus* D (isolates from bat). Proposals are also made for the designation of genotypes within the human and rat HEVs. This hierarchical system is congruent with hepevirus phylogeny, and the three classification levels (genus, species and genotype) are consistent with, and reflect discontinuities in the ranges of pairwise distances between amino acid sequences. Adoption of this system would include the avoidance of host names in taxonomic identifiers and provide a logical framework for the assignment of novel variants.

Received 18 June 2014
Accepted 30 June 2014

INTRODUCTION

Hepatitis E virus (HEV) is the cause of a self-limiting hepatitis with mortality rates of <2 % for immune competent individuals. However, higher mortality rates (10–30 %) are observed amongst pregnant women, while infection may become chronic in immunocompromised individuals. HEV was first recognized in the 1980s and its nucleotide sequence published in the 1990s (Huang *et al.*, 1992; Reyes *et al.*, 1990; Tam *et al.*, 1991; Tsarev *et al.*, 1992). Since then, HEV variants have been detected in a variety of human populations and in potential zoonotic sources such as pig, wild boar, deer, rabbit and mongoose. The variants that infect humans have been classified into four genotypes. Genotypes 1 and 2 are transmitted faecal–orally between humans, and genotypes 3 and 4 may be transmitted to humans zoonotically from infected pigs, deer and wild boar. These genotypes have been subdivided further into numerous subtypes (Lu *et al.*, 2006),

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although the underlying criteria are controversial (Okamoto, 2007; Smith *et al.*, 2013). A more distantly related virus detected in chickens also has been divided into genotypes (Bilic *et al.*, 2009; Hsu & Tsai, 2014; Huang *et al.*, 2004).

This relatively simple taxonomical landscape was disturbed recently by the discovery of viruses that are related to human HEV but infect rabbits (Zhao *et al.*, 2009) and wild boar (Takahashi *et al.*, 2011). Also, more divergent, HEV-like viruses have been described in rats (Johne *et al.*, 2010a), ferrets (Raj *et al.*, 2012) and bats (Drexler *et al.*, 2012), and an even more divergent virus has been isolated from cutthroat trout (Batts *et al.*, 2011). This last virus has a genome organization similar to that of HEVs, but shares very low levels of nucleotide and amino acid sequence identity. Finally, a virus detected in sewage has a partial genome sequence suggesting that it may represent a further, highly divergent member of the family *Hepeviridae* (Ng *et al.*, 2012).

Several recent papers have attempted to summarize this diversity, but have unfortunately reached different conclusions, resulting in the use of multiple, contradictory definitions in the literature. For example, some authors have argued that avian HEV, bat HEV and cutthroat trout virus should be considered as belonging to different genera (Meng, 2013), while others have suggested that avian HEV, bat HEV and rat HEV should be considered as species within a single genus, with cutthroat trout virus as the sole member of a distinct genus (Smith *et al.*, 2013). Both schemes are out-of-date in light of the recent publication of sequences from divergent variants isolated from moose (Lin *et al.*, 2014), mink (Krog *et al.*, 2013), fox (Bodewes *et al.*, 2013), ferret (Li *et al.*, 2014), wild boar (Takahashi *et al.*, 2014) and camel (Woo *et al.*, 2014).

The purpose of this paper is to present a consensus taxonomic framework that provides an agreed basis for the classification of currently described HEV variants, taking into account phylogenetic relationships, the extent of sequence identity and host range. This framework will facilitate the future classification of novel members of this family by providing researchers with straightforward guidelines for assigning new HEV variants. It also helps to clarify the zoonotic threat of particular HEV variants, so that, for example, rat HEV, which is a variant that has not so far been detected in humans, is clearly distinguished from variants of human HEV genotype 3 that are also found in rats (Lack *et al.*, 2012). We propose the use of a common reference sequence and numbering system to simplify comparisons between different studies.

Our model for this consensus approach is that of *Hepatitus C virus* (HCV), in which the adoption of a consensus classification system (Simmonds *et al.*, 2005) and numbering system with respect to a reference sequence (Kuiken *et al.*, 2006) have stabilized the terminology used in HCV research and assisted researchers in providing unique and rational attribution of genotypes and subtypes. The utility of this framework is illustrated by the fact that an update to the HCV classification system almost 10 years later (Smith *et al.*, 2014) was required simply to accommodate the large number of subsequently assigned subtypes, whereas only minor changes to the consensus rules for genotype and subtype assignment were necessary.

RESULTS AND DISCUSSION

Genera

Members of the family *Hepeviridae* are positive-stranded RNA viruses with genomes of 6.6 to 7.3 kb. The longest ORF (ORF1) encodes a non-structural protein with several distinct domains: methyltransferase, Y-domain, papain-like protease, polyproline region (also known as the hypervariable region; HVR), macro domain, helicase and RNA-dependent RNA polymerase. ORF1 is followed by ORF2, which encodes the capsid protein, and ORF3, which overlaps with ORF2 and encodes a phosphoprotein that modulates cellular activities.

Despite this conserved genome structure, phylogenetic analysis within the family is complicated by difficulty in aligning the genome sequences of the most divergent variants. In particular, amino acid sequence identities between cutthroat trout virus and other members of the family are only 26–27 % (ORF1), 18–21 % (ORF2) and 13–16 % (ORF3). In contrast, identities between avian, rat and human HEV stand at 42–49 %, 42–55 % and 20–29 %, respectively (Batts *et al.*, 2011). These average figures mask the fact that, in some genomic regions, no credible amino acid sequence alignment is achievable (Drexler *et al.*, 2012; Huang *et al.*, 2004; Johne *et al.*, 2010b; Smith *et al.*, 2013). Hence, phylogenetic comparisons simply based on pairwise (p)-distances between complete genome nucleotide sequences (Fig. 1a) may give a distorted impression of the relationships between variants. In addition, phylogenetic analysis must take into account the fact that substitutions at synonymous sites are saturated even in comparisons between the different genotypes of human HEV (Smith *et al.*, 2013).

To recover more accurately sequence relationships between the most divergent variants in the *Hepeviridae*, we screened for regions of the genome that are clearly homologous using the Motif Scan program (http://myhits.isb-sib.ch). This identified three subgenomic regions in ORF1 comprising the methyltransferase [ORF1 residues 28 (ORF1-28) to ORF1-389 numbered relative to the sequence of the HEV Burma isolate, GenBank accession M73218], helicase (ORF1-971 to ORF1-1185) and RNA-dependent RNA polymerase (ORF1-1249 to ORF1-1671). Maximum-likelihood analysis of alignments of each region reproduced phylogenetic trees with a similar topology but with much shorter terminal branches (Fig. 1b–d) than obtained from complete genome sequences. Branch lengths in these trees likely represent better reconstructions of evolutionary depth and demonstrate that cutthroat trout virus is substantially more divergent from other members of the family

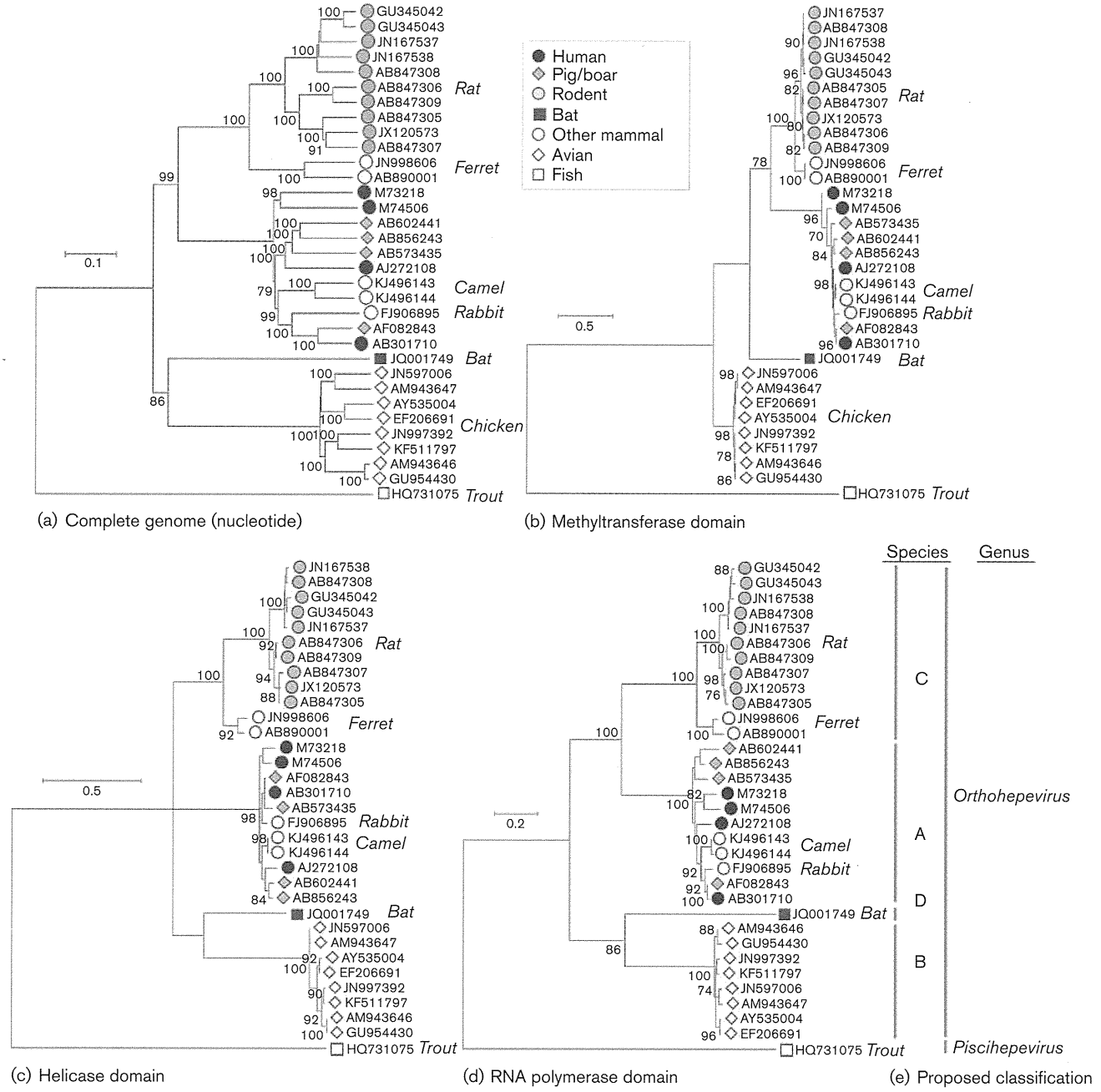


Fig. 1. Phylogenetic analysis of members of the family *Hepeviridae*. (a) Neighbour-joining tree of p-distances among aligned complete genome sequences. (b) Maximum-likelihood tree for amino acid sequences in methyltransferase domain (ORF1-28 to ORF1-389) (c) helicase domain (ORF1-971 to ORF1-1185) (d) RNA-dependent RNA polymerase domain (ORF1-1249 to ORF-1-1671). (e) Proposed classification. Maximum-likelihood trees were computed using the model according to Le & Gascuel with a gamma distribution of evolutionary rates among sites with some invariant sites. Branches supported by >70 % of bootstrap replicates are indicated.

Hepeviridae than indicated by nucleotide sequence comparisons. Similarly, p-distances amongst these subgenomic amino acid sequences form non-overlapping distributions, with the greatest distances observed in comparisons including cutthroat trout virus (Fig. 2). In addition, cutthroat trout virus has an aberrant genome organization (ORF3 is displaced towards the middle of ORF2) and a distinct host range (fish rather than mammals or birds). For these reasons, we recommend that cutthroat trout virus should be assigned to the new genus *Piscihepevirus* (Table 1), as proposed previously (Meng, 2013), with the single species *Piscihepevirus A*. We prefer these genus and species names

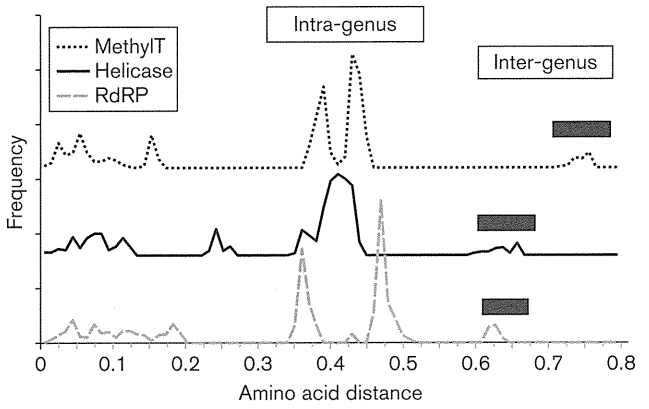


Fig. 2. Frequency distribution of distances among the sequences of members of the family *Hepeviridae*. Plots show the frequency of amino acid sequence p-distances among amino acid sequences of the methyltransferase (ORF1-28 to ORF1-389, dotted line), helicase (ORF1-971 to ORF1-1185, solid line) and RdRP (ORF1-1249 to ORF1-1671, dashed line) domains. The methyltransferase and helicase distance frequencies are shifted by 160 and 80 respectively in order to improve legibility. Filled bars indicate the range of inter-generic distances.

to *Cutrovirus* (Batts *et al.*, 2011), since the virus appears to be confined to fish, having also been detected in five other species of trout (Batts *et al.*, 2011).

We propose that the remaining HEV variants should be considered as members of the genus *Orthohepevirus* (Table 1), extending the usage of a previous proposal (Meng, 2013). This change of genus name from *Hepevirus* is preferred because it makes it clear that not all members of the *Hepeviridae* belong to the same genus.

Analysis of part of the RdRP domain from an incomplete virus genome sequence (Hepelivirus) obtained from raw sewage suggests that this may represent an additional genus within the family *Hepeviridae* (Ng *et al.*, 2012). However, a complete genome sequence would be required to confirm the taxonomic position of this virus, for which the host range remains unknown.

Species

The next obvious level of sequence diversity amongst members of the genus *Orthohepevirus* is that which separates variants originally isolated from different host species (Fig. 3). For example, all isolates from chickens belong to one clade, as do those from rats and ferrets, those from humans, deer, mongooses, pigs, wild boar and camels, while a single complete genome sequence isolated from a bat forms a fourth group. These four groupings are observed regardless, whether the analysis is performed on complete genome nucleotide sequences or subgenomic amino acid sequences (Figs 1 and 3). We propose that these groupings correspond

Table 1. Proposed classification of the family *Hepeviridae*

Family	Genus	Species	Prototype isolate	GenBank accession	Predominant host species	Genotype	Reference strain	Reference accession
Hepeviridae	*Orthohepevirus*	*Orthohepevirus* A	Burma	M73218	Human			
			Burma	M73218	Human			
			Burma	M73218	Human	HEV-1	Burma	M73218
					Human	HEV-2	Mexico	M74506
					Human, pig, rabbit, deer, mongoose	HEV-3	Meng	M74506
					Human, pig	HEV-4	T1	AJ272108
					Wild boar	HEV-5	JBOAR135-Shiz09	AB573435
					Wild boar	HEV-6	wbJOY_06	AB602441
					Camel	HEV-7	DcHEV-178C	KJ496143
		Orthohepevirus B	F93-5077	AY535004	Chicken			
		Orthohepevirus C	R63	GU345042	Rat	HEV-C1	R63	GU345042
					Ferret	HEV-C2	FRHEV4	JN998606
		Orthohepevirus D	BatHEV/BS7/GE/2009	JQ001749	Bat			
	Piscihepevirus		Heenan Lake	HQ731075	Trout			
		Piscihepevirus A	Heenan Lake	HQ731075	Trout			

to four species and that they be designated *Orthohepevirus* A to *Orthohepevirus* D (Table 1).

Subgenomic sequences of variants isolated from other mammalian species have recently become available but phylogenetic analyses are complicated because they overlap with only relatively short regions. The most complete sequence (5081 nt) is derived from a variant isolated from a moose (Lin *et al.*, 2014). Phylogenetic analysis of the amino acid sequence of this isolate for part of ORF1 and all of ORF2 (Fig. 3) shows that it is distinct from all other variants, although it shares a branch with species *Orthohepevirus* A in the same way that the ferret variants group with rat-derived species *Orthohepevirus* C. Similar analysis of virus sequences isolated from mink (Krog *et al.*, 2013) shows that these group with sequences isolated from ferrets. A sequence isolated from a fox (Bodewes *et al.*, 2013) appears to be more distinct (Fig. 3d) and could represent an additional species. However, this last analysis was based on a relatively short subgenomic region (ORF1-1420 to ORF1-1505) in which the grouping of species *Orthohepevirus* C isolates from rat and ferret, and of the moose variant with *Orthohepevirus* A variants from human, pig and wild boar, were less distinct than for comparisons based on longer coding regions (Fig. 3). We recommend that assignment of the moose and fox variants to particular *Orthohepevirus* species should not be made until comparisons based upon complete genome sequences are available.

An advantage of using species names of the form *Orthohepevirus* A, etc., rather than names based on avian HEV, rat HEV or bat HEV, is that they will not be compromised by current or future discoveries on the extent of host range. For example, variants of species *Orthohepevirus* C have been isolated from *Rattus* (Li *et al.*, 2013) and *Bandicota* species (the greater bandicoot rat) (Li *et al.*, 2013), which are members of the order Rodentia, but also from Asian musk shrews (Guan *et al.*, 2013), which are members of the order Soricomorpha, and from ferrets (Li *et al.*, 2014; Raj *et al.*, 2012) and mink (Krog *et al.*, 2013), which are members of the order Carnivora. Wider screening for members of this species, and also for members of species *Orthohepevirus* B and *Orthohepevirus* D, may reveal wider host ranges than those recognized at present.

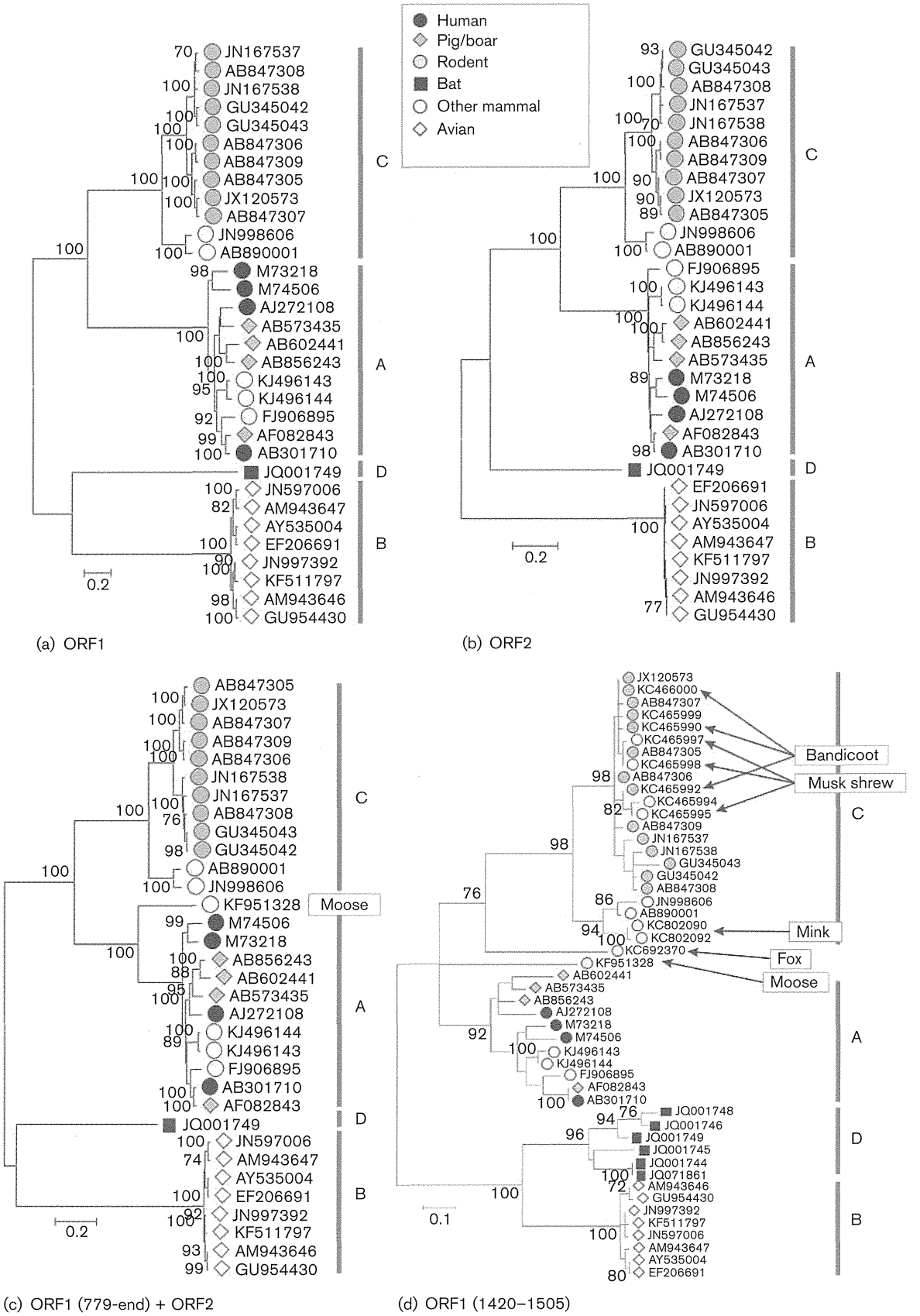
Genotypes

Although the International Committee on Taxonomy of Viruses does not provide official designations for taxonomic entities below the species level, it is useful for researchers to have an agreed designation of genotypes within each species grouping.

***Orthohepevirus* A.** Within species *Orthohepevirus* A, four genotypes are currently described that infect humans (HEV-1, HEV-2, HEV-3 and HEV-4), and assignment of complete genome sequences to these genotypes is generally unambiguous. The only exceptions are recombinant viruses, which have been documented both within and between genotypes (Chen *et al.*, 2012; van Cuyck *et al.*, 2005; Fan, 2009; Wang *et al.*, 2010), although in some cases recombinant viruses may represent laboratory artefacts (Wang *et al.*, 2010). More problematic is the designation of additional genotypes HEV-5 and HEV-6, names which have been variously assigned to avian HEV and rat HEV (here proposed to be classified as members of the species *Orthohepevirus* B and *Orthohepevirus* C, respectively) and variants isolated from wild boar (Oliveira-Filho *et al.*, 2013; Smith *et al.*, 2013; Takahashi *et al.*, 2011), or by implication to variants isolated from rabbits (Geng *et al.*, 2011; Zhao *et al.*, 2009).

Phylogenetic analysis of the nucleotide and amino acid sequences of concatenated ORF1 and ORF2 (excluding the HVR and the hypervariable insertion found in all rabbit HEV isolates) for these variants, including a divergent variant recently described from wild boar (Takahashi *et al.*, 2014), reveals four fully supported branches consisting of (i) HEV-1 and HEV-2, (ii) HEV-3 and variants isolated from rabbits, together with a closely related patient sequence, and (iii) HEV-4 together with all three isolates from wild boar and (iv) isolates from camels (Fig. 4). However, given the precedence of HEV-1 and HEV-2 forming well recognized and phylogenetically distinct genotypes, the least disruptive way of representing these phylogenetic relationships would be to retain the HEV-1, HEV-2, HEV-3 and HEV-4 genotype assignments.

Variants derived from rabbits (and the single related human isolate JQ013793) show a wider range of amino acid sequence distances from each other (maximum value 0.081) than HEV-1, -3 and -4 (maximum values 0.041, 0.053 and 0.053 respectively) while minimum distances between them and HEV-3 variants are lower (0.061) than between rabbit sequences and other HEV genotypes or variants (minimum value 0.108). A previous study discussed whether rabbit viruses might be assigned to genotype 3 as divergent members or form a separate genotype, since nucleotide and amino acid distances were intermediate between those observed within HEV-1, HEV-3 and HEV-4 and distances between genotypes (Smith *et al.*, 2013). Our analysis here of further rabbit-derived HEV variants (JX121233, JQ768461, JX109834, AB740220, AB740221, AB740222 and JX565469) confirms this finding, for example, the pairwise amino acid distances with genotype 3 that overlap those between genotype 3 and 4. However, the most extreme distances between rabbit HEV and HEV-3 involve the isolates FJ906895 and FJ906896. These sequences contain numerous amino acid substitutions clustered at the C-terminus (FJ906895) or N-terminus (FJ906896), which are at sites that are otherwise highly conserved throughout *Orthohepevirus* A sequences, and so are likely to represent sequencing artefacts. Excluding these two sequences from the analysis of pairwise distances, there was no overlap between amino acid distances between rabbit and HEV-3 sequences, and between HEV-3 and HEV-4 (Fig. 5). Retaining these isolates but excluding the terminal regions containing the aberrant sites from the *Orthohepevirus*


Human
Pig/boar
Rodent
Bat
Other mammal
Avian
JN167537
AB847308
JN167538
GU345042
GU345043
AB847306
AB847309
AB847305
JX120573
AB847307
JN998606
AB890001
M73218
M74506
AJ272108
AB573435
AB602441
AB856243
KJ496143
KJ496144
FJ906895
AF082843
AB301710
JQ001749
JN597006
AM943647
AY535004
EF206691
JN997392
KF511797
AM943646
GU954430
0.2
(a) ORF1
GU345042
GU345043
AB847308
JN167537
JN167538
AB847306
AB847309
AB847307
JX120573
AB847305
JN998606
AB890001
FJ906895
KJ496143
KJ496144
AB602441
AB856243
AB573435
M73218
M74506
AJ272108
AF082843
AB301710
JQ001749
EF206691
JN597006
AY535004
AM943647
KF511797
JN997392
AM943646
GU954430
0.2
(b) ORF2
AB847305
JX120573
AB847307
AB847309
AB847306
JN167538
JN167537
AB847308
GU345043
GU345042
AB890001
JN998606
KF951328
M74506
M73218
AB856243
AB602441
AB573435
AJ272108
KJ496144
KJ496143
FJ906895
AB301710
AF082843
JQ001749
JN597006
AM943647
AY535004
EF206691
JN997392
KF511797
AM943646
GU954430
Moose
0.2
(c) ORF1 (779-end) + ORF2
JX120573
KC466000
AB847307
KC465999
KC465990
KC465997
AB847305
KC465998
AB847306
KC465992
KC465994
KC465995
AB847309
JN167537
JN167538
GU345043
GU345042
AB847308
JN998606
AB890001
KC802090
KC802092
KC692370
KF951328
AB602441
AB573435
AB856243
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FJ906895
AF082843
AB301710
JQ001748
JQ001746
JQ001749
JQ001745
JQ001744
JQ071861
AM943646
GU954430
JN997392
KF511797
JN597006
AM943647
AY535004
EF206691
Bandicoot
Musk shrew
Mink
Fox
Moose
0.1
(d) ORF1 (1420–1505)

Fig. 3. Phylogenetic analyses of members of the genus *Orthohepevirus*. Maximum-likelihood tree for amino acid sequences in (a) ORF1 and (b) ORF2, (c) ORF1-779 to the end of ORF2 with the addition of KF951328 from moose, (d) ORF1-1420 to ORF1-1505 with the addition of sequences from mink, fox, greater bandicoot, Asian musk shrew and bat. Maximum-likelihood trees were computed using the model according to Le & Gascuel with a gamma distribution of evolutionary rates among sites, with some invariant sites. Branches supported by >70 % of bootstrap replicates are indicated.

alignment similarly eliminates overlap between these categories (data not shown). Since HEV-3 and the rabbit HEV also share a long branch on phylogenetic analysis, we consider it simplest to provisionally assign the rabbit sequences to genotype HEV-3.

On this basis, amino acid distances of concatenated ORF1 and ORF2 (lacking hypervariable regions) greater than 0.088 could then act as a threshold to demarcate intra- and inter-genotype distances. Using this criterion, the three wild boar isolates would comprise two additional genotypes HEV-5 (AB573435) and HEV-6 (AB602441 and AB856243 differing from each other by 0.076 and from HEV-5 by >0.10), while the variants isolated from camels (differing from all other sequences by >0.095) would become HEV-7.

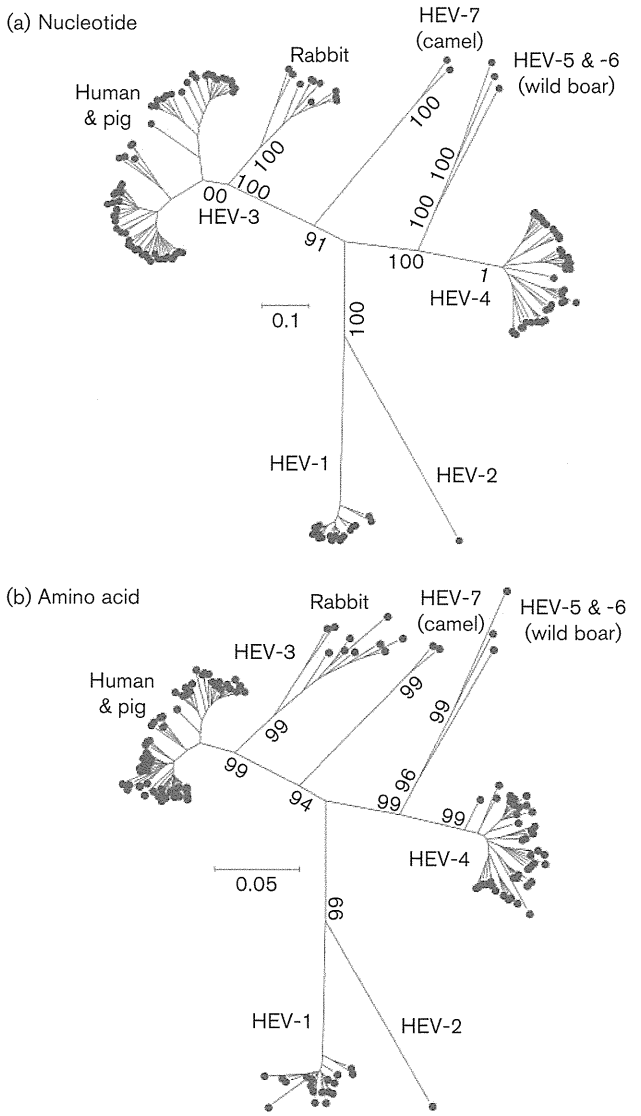

Fig. 4. Phylogenetic analyses of members of *Orthohepevirus* A. Maximum-likelihood trees were produced for 137 concatenated ORF1 and ORF2 regions (excluding the HVR and rabbit HEV-specific insertion) for (a) nucleotide sequences using the general time-reversible model with gamma distribution and invariant sites and (b) amino acid sequences using the Jones–Taylor–Thornton model with frequencies, and gamma distribution with invariant sites. Branches supported by >70 % of bootstrap replicates are indicated.

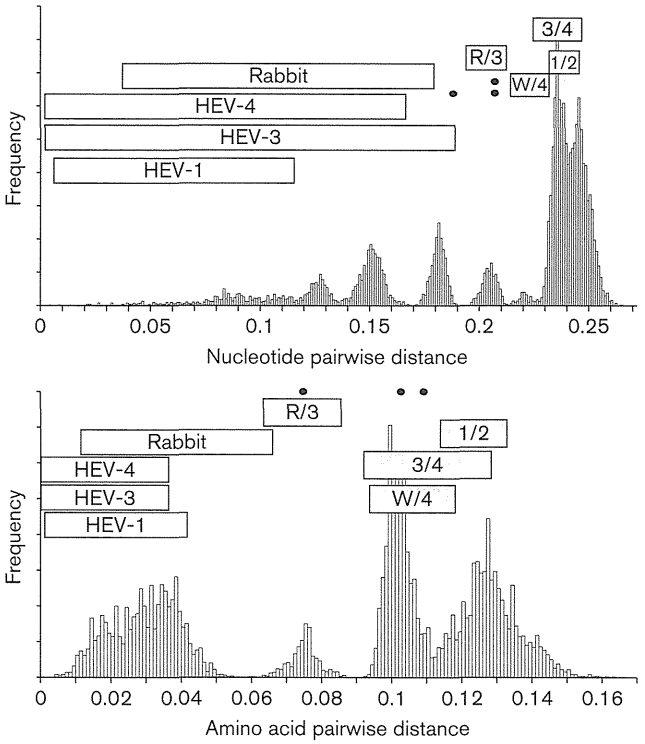

Fig. 5. Frequency distribution of distances among sequences of variants in the genus *Orthohepevirus*. Histograms show the frequency of nucleotide (a) and amino acid sequence p-distances (b) among 135 concatenated ORF1 and ORF2 sequences (excluding the HVR and rabbit HEV-specific HVR insertion). Maximum and minimum intra-genotype ranges of distances within genotypes 1, 3 and 4 and between rabbit sequences are indicated with open bars. Grey-filled bars depict distances between HEV-3 and rabbit variants (R/3) and inter-genotype distances (3/4, HEV-3 and HEV-4; 1/2, HEV-1 and HEV-2; W/4, wild boar and HEV-4). Distances between the three wild boar sequences are indicated with spots.