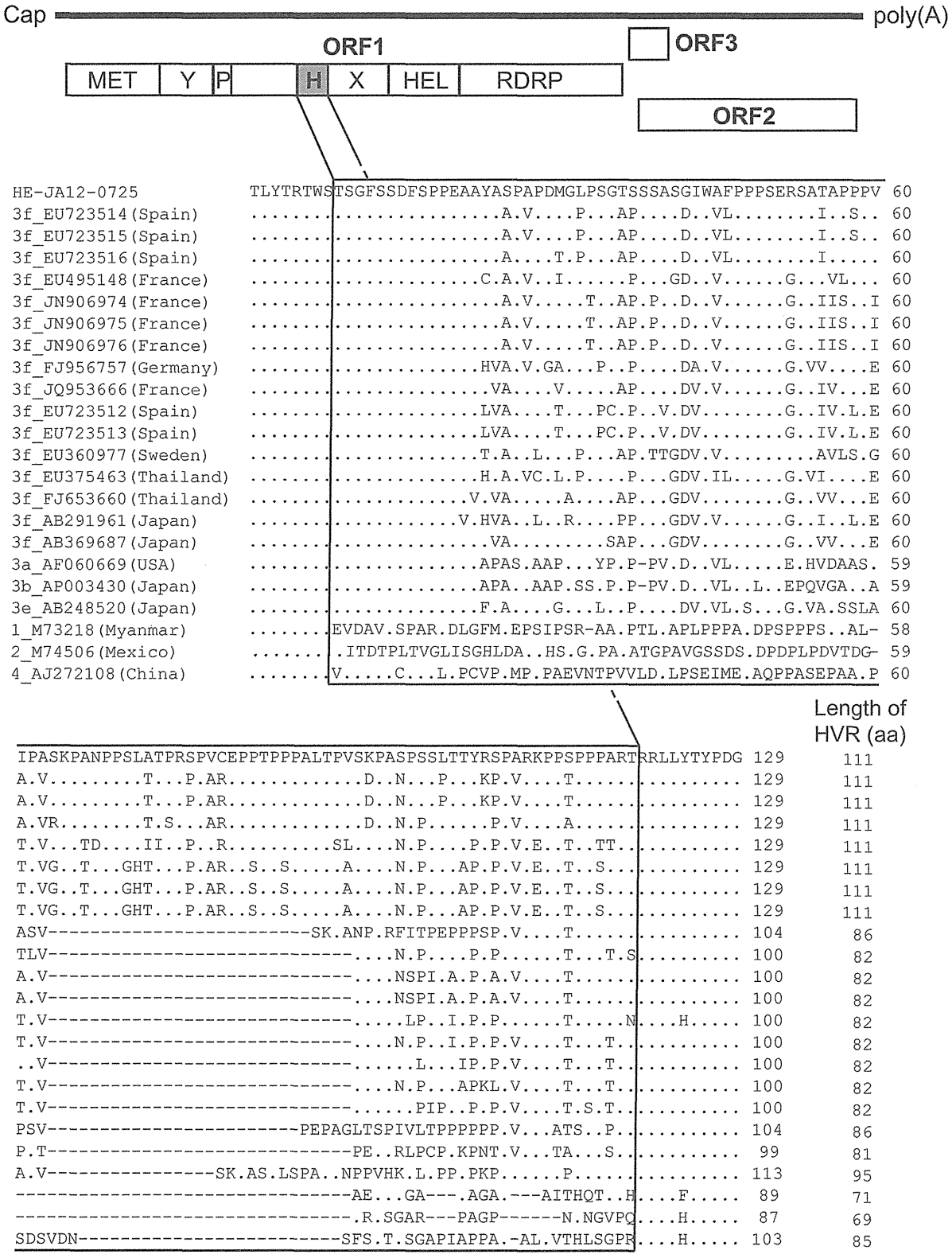

Fig. 1. The characteristics of the hypervariable region and its surrounding amino acid sequence within ORF1 of the subgenotype 3f HEV strains. The genomic organization of HEV is shown at the top, with three ORFs. MET, methyltransferase; Y, Y domain; P, papain-like protease; H, hypervariable region (HVR); X, X domain; HEL, RNA helicase; RDRP, RNA-dependent RNA polymerase. The amino acid sequences of 17 3f strains, including the HE-JA12-0725 strain obtained in the present study, and one representative strain each of subgenotypes 3a, 3b, and 3e and genotypes 1, 2, and 4 are aligned and their HVR sequences are boxed. Dots indicate amino acids that are identical to the top sequence, and bars denote deletions of amino acids.

strain obtained in the present study was located separately from the Thai cluster, and segregated into the Spanish swine 3f cluster, supported by a high bootstrap value (100%). These results suggest the circulation of 3f strains classifiable into two distinct clusters, Spanish-type and Thai-type, in Japan.

As indicated in Supplementary Table S3 and Figure 1, this speculation was supported by the analysis of the HVR sequence within ORF1. Based on the relatively low similarity (up to 92.7% over the entire genome) to Spanish swine HEV strains as the most homologous strains, it is assumed that the HE-JA12-0725 strain obtained in the present study may have been brought from Europe to Japan in the distant past and then become diversified, as has been documented for another European-type subgenotype within genotype 3 (subgenotype 3e) in Japan, which is presumed to have entered Japan from European countries through the importation of large-race pigs in the 1960s [Nakano et al., 2013].

In addition, the present case demonstrated a severe form of acute hepatitis, with histological findings compatible with AIH. The detailed clinical condition of patients infected with 3f strains is still unknown, although cases of fulminant hepatitis E infected with 3f HEV strains have been reported in Europe [Mateos et al., 2010; Doudier et al., 2014]. In this regard, this study will be useful for understanding the detailed background of the clinical presentation of the disease, including the disease severity, and therefore deserves special consideration. In future studies, the autochthony and disease severity of subgenotype 3f strains should be evaluated.

In conclusion, this study reported the results of a full genome analysis of a European-type subgenotype 3f strain obtained from a patient with autochthonous acute hepatitis E in Japan. The case would be meaningful in view of epidemics and the origin of HEV, for facilitating the development of further HEV research. Further studies including more patients and accurate HEV subgrouping are needed to determine if the HEV genotypic patterns are associated with the disease severity.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Supplementary Table S1. The Laboratory Data on Admission

Hematology		Electrolytes and renal function	
WBC	5000/μl	BUN	14 mg/dl
Neutrophils	68%	Creatinine	0.61 mg/dl
Lymphocytes	21.4%	Na	137 mEq/L
Monocytes	9.2%	K	3.7 mEq/L
Eosinophils	0.8%	Cl	104 mEq/L
Basophils	0.6%		
RBC	467 x 10^4/μl	Blood coagulation	
Hemoglobin	14.0 g/dl	Prothrombin time (%)	69
Hematocrit	42.40%		
Platelets	10.5 x 10^4/μl	Viral markers	
		IgM anti-HAV	(-)
Blood Chemistry		HBsAg	(-)
TP	7.4 g/dl	IgM anti-HBc	(-)
Albumin	3.5 g/dl	Anti-HBc	(+)
T-bil	2.3 mg/dl	HBV DNA	(-)
D-bil	1.3 mg/dl	Anti-HCV	(-)
AST	1141 IU/L	HCV RNA	(-)
ALT	920 IU/L	IgM anti-CMV	0.42 (-)
LDH	532 IU/L	IgG anti-CMV	11.8 (+)
ALP	576 IU/L	IgM anti-EBV VCA	< 10 (-)
γ-GT	235 IU/L	IgG anti-EBV VCA	320 (+)
ChE	236 IU/L	EBNA	80 (+)
T. chol	144 mg/dl	IgG anti-HEV[a]	(+)
Triglycerides	83 mg/dl	IgM anti-HEV[a]	(+)
IgG	2205 mg/dl	IgA anti-HEV[a]	(+)
IgM	178 mg/dl	HEV RNA[a]	(+)
ANA	40 (+)		
AMA	< 5 (-)		
CRP	2.05 mg/dl		

γ-GT, γ-glutamyltransferase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANA, antinuclear antibodies; AMA, antimitochondrial antibodies; AST, aspartate aminotransferase; BUN, blood urea nitrogen; ChE, cholinesterase; CMV, cytomegalovirus; D .bil, direct bilirubin; EBV, Epstein-Barr virus; HBsAg, hepatitis B surface antigen; HCV Ab, hepatitis C virus antibody; IgM HAV Ab, immunoglobulin M-hepatitis A antibody; LDH, lactate dehydrogenase; RBC, red blood cell count; T. bil, total bilirubin; T. chol, total cholesterol; WBC, white blood cell count.
[a]HEV-related markers were measured on the next day of the hospitalization.

Supplementary Table S2. Laboratory Parameters, Anti-HEV Antibody Levels and HEV RNA in Periodic Serum Samples Obtained From a Patient With Autochthonous Severe Acute Hepatitis E.

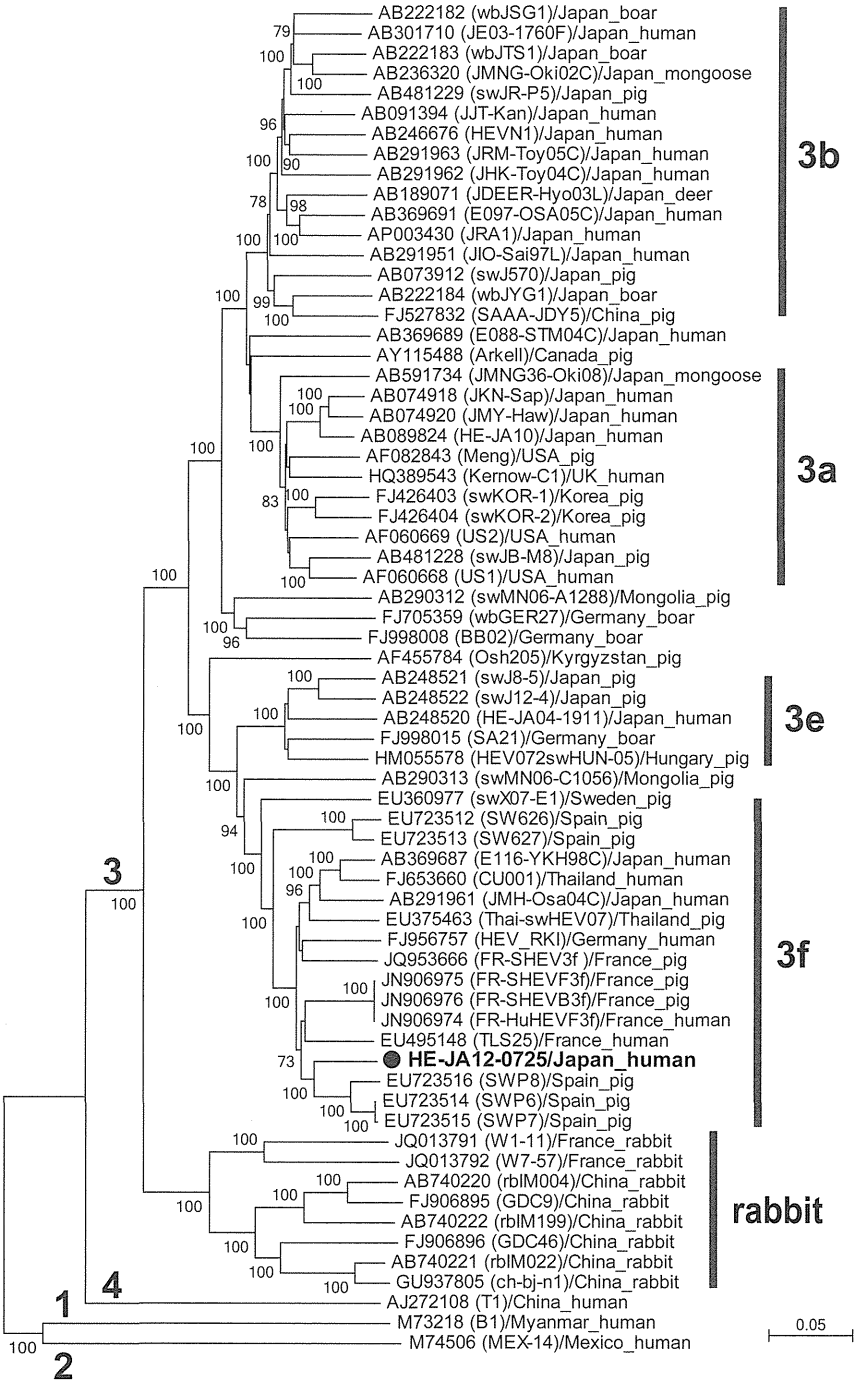
Days after admission	ALT (IU/L)	AST (IU/L)	Total bilirubin (mg/dL)	Prothrombin time (%)	Anti-HEV (absorbance at 450 nm)[a]			HEV RNA
					IgG-class	IgM-class	IgA-class	
0	920	1,141	2.3	69				
1	1,118	1,617	2.9	58	0.837 (+)	2.751 (+)	>3.000 (+)	+
2	1,071	1,237	3.5	61				
4	925	1,055	5.2	51	1.121 (+)	2.818 (+)	>3.000 (+)	+
5	715	971	5.9	39				
7	523	304	5.7	52				
9	300	76	3.7	51				
12	224	80	5.2	55				
15	166	63	5.9	68				
19	145	57	3.9	79	1.525 (+)	2.969 (+)	>3.000 (+)	+
22	120	52	3.6	81				
25	127	45	3.8	83				
28	112	47	3.0	86				
32	90	39	2.2	81				
40	82	40	1.7	90	2.210 (+)	2.855 (+)	2.769 (+)	-
47	68	50	1.4	87	2.242 (+)	2.719 (+)	2.406 (+)	-
61	55	48	1.1	95	2.360 (+)	2.810 (+)	1.789 (+)	-

[a]Cut-off values for anti-HEV IgG, IgM and IgA were 0.175, 0.440 and 0.642, respectively.

Supplementary Table S3. Comparison of the HE-JA12-0725 Isolate Obtained in the Present Study With the Reported Genotype 3f and Other Representative Genotypes 1-4 Strains Within Three ORFs at the Nucleotide and Amino Acid Levels

Isolate name	Genotype or subgenotype	Host	GenBank accession no.	Country of origin	ORF1[a]				ORF2	ORF3
					Nucleotide		Amino acid		Identity (%) in nucleotides (amino acids)	Identity (%) in nucleotides (amino acids)
					Length (nt)	Identity (%)	Length (aa)	Identity (%)		
SWP6	3f	Pig	EU723514	Spain	**5199**	92.2	**1733**	97.7	93.9 (98.7)	96.4 (99.1)
SWP7	3f	Pig	EU723515	Spain	**5199**	92.0	**1733**	97.5	93.8 (98.7)	96.4 (99.1)
SWP8	3f	Pig	EU723516	Spain	**5199**	92.2	**1733**	97.6	93.9 (98.6)	96.1 (98.2)
TLS25	3f	Human	EU495148	France	**5199**	90.5	**1733**	96.9	93.0 (98.3)	97.3 (99.1)
FR-HuHEVF3f	3f	Human	JN906974	France	**5199**	90.5	**1733**	97.1	93.1 (98.0)	95.5 (98.2)
FR-SHEVF3f	3f	Pig	JN906975	France	**5199**	90.5	**1733**	97.1	93.1 (98.0)	95.5 (98.2)
FR-SHEVB3f	3f	Pig	JN906976	France	**5199**	90.5	**1733**	97.1	93.1 (98.0)	95.5 (98.2)
HEV_RK1	3f	Human	FJ956757	Germany	5124	88.0	1708	95.6	92.3 (97.8)	96.4 (96.4)
FR-SHEV3f	3f	Pig	JQ953666	France	5112	88.7	1704	95.9	91.5 (97.8)	96.1 (99.1)
SW626	3f	Pig	EU723512	Spain	5112	85.8	1704	95.3	89.3 (97.8)	95.2 (98.2)
SW627	3f	Pig	EU723513	Spain	5112	85.8	1704	95.3	94.0 (98.6)	95.8 (100)
swX07-E1	3f	Pig	EU360977	Sweden	5112	87.0	1704	96.6	89.1 (97.4)	94.3 (95.5)
Thai-swHEV07	3f	Pig	EU375463	Thailand	5112	87.9	1704	95.2	93.1 (98.0)	95.2 (97.3)
CU001	3f	Pig	FJ653660	Thailand	5112	88.2	1704	95.9	92.5 (98.3)	96.4 (99.1)
E116-YKH98C	3f	Human	AB369687	Japan	5112	88.7	1704	95.7	93.0 (98.3)	96.1 (99.1)
JMH-Osa04C	3f	Human	AB291961	Japan	5112	88.3	1704	95.7	92.6 (97.7)	96.1 (100)
HEV-US2	3a	Human	AF060669	USA	5124	78.6	1708	92.3	80.1 (92.1)	94.1 (95.6)
JRA-1	3b	Human	AP003430	Japan	5109	79.0	1703	92.5	78.8 (90.7)	94.1 (95.5)
HE-JA04-1911-	3e	Human	AB248520	Japan	5151	82.7	1717	95.0	85.6 (96.5)	95.2 (99.1)
B1	1	Human	M73218	Myanmar	4978	69.8	1659	85.6	84.5 (96.8)	84.7 (81.4)
MEX-14	2	Human	M74506	Mexico	5073	70.7	1691	80.3	86.9 (97.7)	84.7 (79.6)
T1	4	Human	AJ272108	China	5121	72.7	1707	83.6	81.1 (93.0)	84.9 (78.3)

[a]The lengths of ORF1 at the nucleotide and amino acid levels that are identical to those of the HB-JA12-0725 isolate are indicated in bold.



Supplementary Fig. S1

A neighbor-joining tree of the entire genomic sequence of the hepatitis E virus (HEV) isolate (HE-JA12-0725) obtained in this study with 66 reference sequences of genotypes 1-4. Each reference sequence is shown with the accession number, followed by the isolate name in parenthesis, the name of the country where it was isolated and the animal species from which it was isolated. The four genotypes (1, 2, 3 and 4) and the subgenotypes indigenous to Japan (3b, 3a and 3e), in addition to the 3f subgenotype and genotype 3 observed in rabbits, are shown. The 3f strain isolated in this study is shown in bold and marked with a closed circle. The bootstrap values (>70%) are indicated for the nodes as a percentage of the data obtained from 1,000 resamplings. The scale bar is in units of nucleotide substitutions per site.

false-positive results or asymptomatic infections. However, our findings of elevated levels of subtype H7N9 antibody among 6.7% of contacts during this epidemic in China offer evidence that human-to-human transmission of H7N9 virus may occur among contacts of infected persons.

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Hepatitis E Epidemic, Biratnagar, Nepal, 2014

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To the Editor: We report a recent epidemic of hepatitis E in Biratnagar, Nepal. During the third week of April 2014, a total of 11 patients with acute jaundice came to hospitals in Biratnagar. IgM against hepatitis E virus (HEV) was detected in serum samples from all 11 patients. During the next 7 weeks, 1,861 patients with acute jaundice came to the outpatient departments of 2 of 5 large hospitals in Biratnagar; 123 patients were admitted to these 2 hospitals.

Registries at these 2 hospitals indicated that 2 patients with acute jaundice came to these hospitals on April 14. On April 28; May 5, 12, 19, and 26; and June 2, 9, 16, 23, and 30, the number of patients with acute jaundice who came to these 2 hospitals were 42, 67, 58, 69, 48, 21, 5, 3, 1, and 0, respectively. Registries showed that this increased frequency of acute jaundice lasted until the end of May 2014, when it began to decrease and reached near zero by the first week of July. In addition, unusually large numbers of patients with acute jaundice came to 25 smaller private health care facilities in Biratnagar during April–May 2014.

The Private and Boarding Schools' Organization of Nepal closed 80 schools in Biratnagar and surrounding areas during the second week of May 2014 because of risk for disease transmission (*1*). The Biratnagar Zonal Health Authority and National Health Authority of Nepal issued special alerts by mass media regarding jaundice after the third week of April and advised using boiled water for consumption (*2*).

Registries of major hospitals, smaller health clinics, and private physicians indicated that ≈7,000 patients were

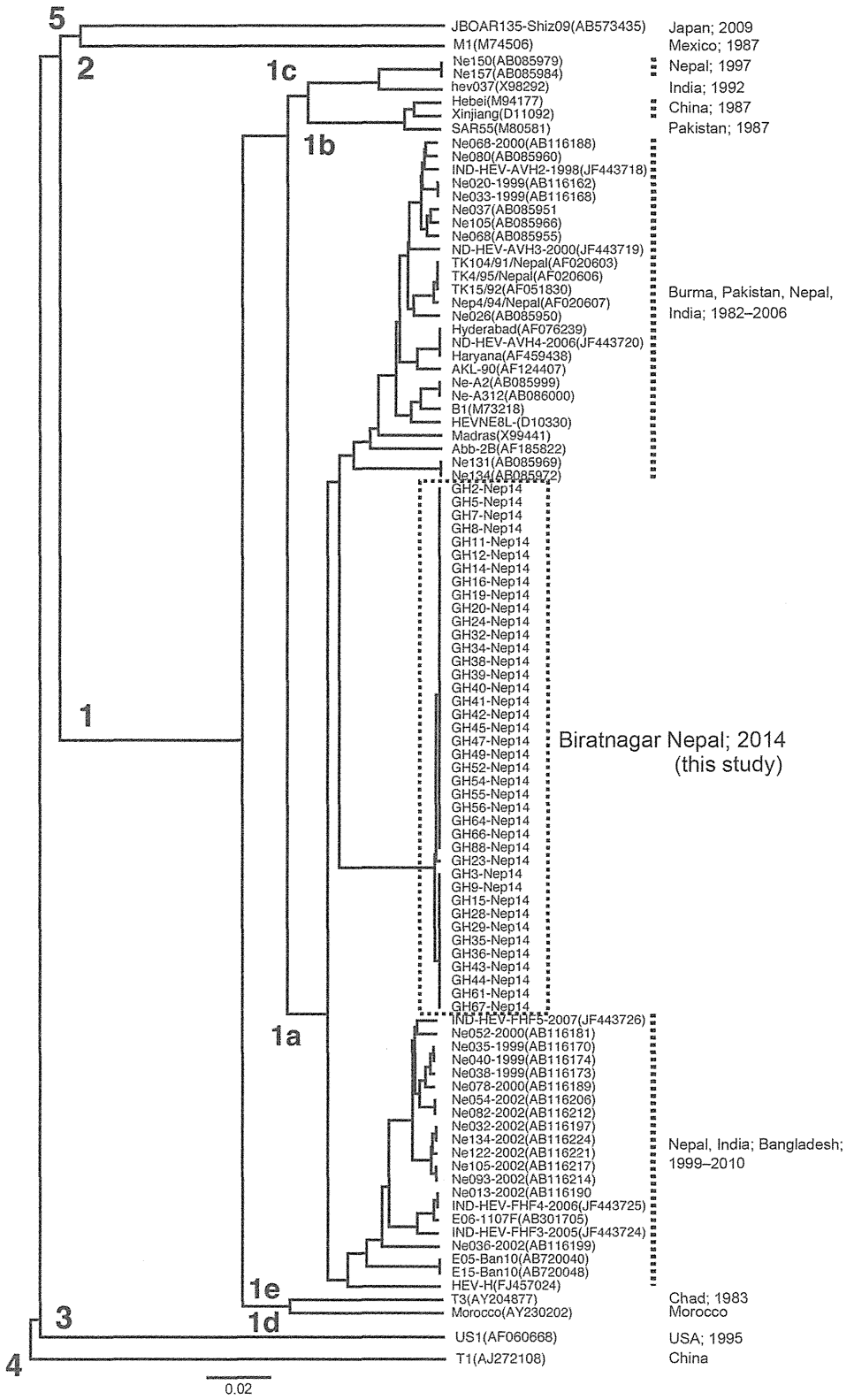

Figure. Phylogenetic analysis (UPGMA) of hepatitis E virus (HEV) sequences from the epidemic in Biratnagar, Nepal, 2014, on the basis of 412 nt within open reading frame 2 (DDBJ/EMBL/GenBank accession nos. AB98608–AB986107). All 40 HEV isolates from epidemic in Biratnagar segregated into a single, new cluster within genotype 1a. Values along branches indicate genotypes. Scale bar indicates nucleotide substitutions per site.

affected during this epidemic. Fourteen patients died; these deaths occurred in Kathmandu, the capital of Nepal, or in different cities in India after these patients were transferred there for better treatment. Fifty pregnant women had acute jaundice, but none of these women died.

The epidemic was presumed to be caused by consumption of contaminated water (*3*). In February and March 2014, water and sewerage pipelines were damaged in different areas of Biratnagar during construction and repair of roads. A survey conducted by the Department of Community Medicine, B.P. Koirala Institute of Health (Dharan, Nepal), found high levels of coliform bacteria in water supplies from different areas in Biratnagar during the epidemic. Tap water also looked cloudy and visibly contaminated (*3*).

To obtain more information about the epidemic, the incidence of acute jaundice was determined for 656 prisoners and 75 security personnel at the Biratnagar Jail. The study protocol was approved by the Liver Foundation Nepal. Informed consent was not obtained because identity of patient samples remained anonymous. Acute jaundice was detected among 30 (4.6%) prisoners and 4 (5.3%) security personnel. The same source of consumable water was used by the general population, inmates, and security personnel in Biratnagar.

To identify the causative agent of this epidemic, serum samples from 48 patients were obtained at Koshi Zonal Hospital, the largest government hospital in this zone. Hepatitis A virus RNA and IgM against hepatitis B virus core antigen was not detected in the 48 serum samples. Conversely, IgG, IgM, and IgA against HEV were detected in 47 (97.9%), 45 (94%), and 45 (94%) serum samples, respectively, and HEV RNA was detected in 42 (87.5%) of 48 serum samples, which indicated that the epidemic was caused by HEV.

A partial 412-nt sequence from open reading frame 2 corresponding to nt 5944–6355 of the HEV B1 genome (*4*) was obtained as reported (*5*). We obtained 40 HEV isolates from the 42 samples and sequenced partial 412-nt segments. All 40 HEV sequences from the epidemic in Biratnagar segregated into a cluster within genotype 1a (Figure). These sequences showed 99.8% nt identity with each other but only 90.8%–95.4% nt identity with other HEV isolates from Nepal and those from India, Bangladesh, Pakistan, and China.

Compared with previous HEV epidemics in Nepal (*6*) and other parts of the Indian subcontinent, the local government of Biratnagar and central government of Nepal took steps to contain the reported epidemic. Activities of public and private sectors in Biratnagar ended the epidemic in ≈12 weeks, and no new cases of acute jaundice have been reported in Biratnagar.

Persons in Biratnagar were given information regarding epidemics and ways to contain them. They were instructed by electronic media to use boiled water for consumption. It became clear that additional information regarding about maintaining water and sewerage systems during road construction and repair should also be provided. Because 14 patients died of HEV infection during this epidemic, more preparedness for epidemics of waterborne diseases is required to minimize unnecessary illnesses and deaths.

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Human Parvovirus 4 Infection among Mothers and Children in South Africa

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To the Editor: Human parvovirus 4 (PARV4) is a single-stranded DNA virus in the family *Parvoviridae* (*1*).

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Short Report: Molecular Detection of Hepatitis E Virus in Rivers in the Philippines

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Abstract. To understand the hepatitis E virus (HEV)-pollution status in the environment in the Philippines, a total of 12 water samples were collected from rivers in Manila City for detection of HEV RNA. Three of 12 samples were positive for HEV RNA indicating that HEV is circulating in the Philippines. Phylogenetic analysis classified all of the HEV sequences into genotype 3.

Hepatitis E virus (HEV) is a positive-sense single-stranded RNA virus that belongs to the genus *Hepevirus* in the family *Hepeviridae*.[1–3] The HEV is the causative agent of acute or fulminant hepatitis E, primarily transmitted by the fecal–oral route.[4] The relatively high mortality rate in HEV-infected pregnant women, up to 28%, is unique among hepatitis viruses.[1,5] Hepatitis E is a zoonotic disease, with swine, wild boars, and wild deer serving as the reservoir for human infections.[6–8] Four genotypes of HEV (G1–G4) have been detected in humans and G3 and G4 HEV are responsible for sporadic and autochthonous infections in both humans and other animal species worldwide.[9–12]

The HEV is a public health concern in many Asian and African countries where sanitation conditions are insufficient.[13,14] Large waterborne outbreaks with high attack rates among young adults have been described in regions characterized by poor sanitary conditions in countries such as China, India, Somalia, and Uganda.[15] However, there have been no reports of HEV infection in the Philippines. No information about hepatitis E patients or HEV infection in animals has been reported, and no sequence data have been deposited from this country. There is also no report of the HEV-pollution status of the environmental sewage water. With the hypothesis that environmental water samples may reflect the prevalence of HEV circulation, we examined river water samples to investigate HEV in the environment in one of the most densely populated cities in the world, Manila City, a metropolitan area in the Philippines with over 10 million residents.

A total of 12 water samples were collected from rivers that run through Manila City. Six sampling sites were selected (Figure 1). Sampling sites 1 to 3 were in the Pasig River, sites 4 and 5 were in the Paranaque River, and site 6 was in the Las Pinas River. These rivers receive the wastewater from the residents nearby. Water samples were drawn at all locations during both the dry season (December 23, 2012) and the wet season (July 23, 2013), and were named D1 to D6 and W1 to W6, respectively. The water samples were kept at 4°C during transport.

The concentration and purification of these water samples was carried out as described previously[16]; briefly, 500 mL of water was collected from each sampling site, and centrifuged at 3,000 rpm for 30 min at 4°C. Then, 2.5 mM $MgCl_2$ was added to the supernatant to a final concentration of 0.05 mM. The pH value was adjusted to 3.5. The solution was filtered through a 0.45-μm mixed cellulose ester membrane filter (Merck Millipore, Tokyo, Japan) by a positive-pressure pump. Absorbents on the filter were then eluted with 10 mL of 3% beef extract solution by ultrasonication, three times. The solution was centrifuged at 12,000 rpm for 30 min, and the supernatant was stored at –80°C until RNA extraction.

The RNA was extracted using the MagNA Pure LC Total Nucleic Acid isolation kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's recommendations. Reverse transcription was performed with a high-capacity cDNA reverse transcription kit (ABI Applied Biosystems, Carlsbad, CA) at 25°C for 10 min, 37°C for 120 min, and followed by 85°C for 5 min in a 20-μL reaction mixture containing 1 μL reverse transcriptase, 2 μL of the random primer, 1 μL RNase inhibitor, 2 μL RT buffer, 0.8 μL 10-mM deoxynucleoside triphosphates, 8 μL RNA, and 5.2 μL distilled water. A nested reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed to amplify a portion of the ORF2 genome, based on the method described previously.[17]

By RT-PCR, three samples (W4, W5, and W6) of the 12 water samples were positive for HEV RNA. Excluding the primer sequences, the length of the nested RT-PCR products was 338 nucleotides corresponding to nt 5959-6296 in the ORF2 of the Myanmar strain (D10330). The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and cloned into TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA). Each of 20 clones was sequenced. The clones with the same nucleotide sequence were counted as one strain. Finally, 21 HEV strains were obtained (GenBank accession nos. KF546257–KF546277), of which five strains were isolated from W4, 10 strains from W5, and six strains from W6. Phylogenetic analysis indicated that all 21 strains were G3 HEV. With the exception of strain W5-13, the other 20 strains' sequences belonged to subgenotype 3a,[18] separated into four clusters (cluster 1 to 4) with nucleotides sequence identities of 89.6–99.7% (Figure 2). In cluster 1, the sequences of three strains isolated from W6 were close to that of HEV strain EF530663 (isolated from a patient in Hungary) with nucleotide sequence identities of 92.3% to 92.6%. The nucleotide sequences of all nine of the strains in cluster 2 detected from W5 were close to that of

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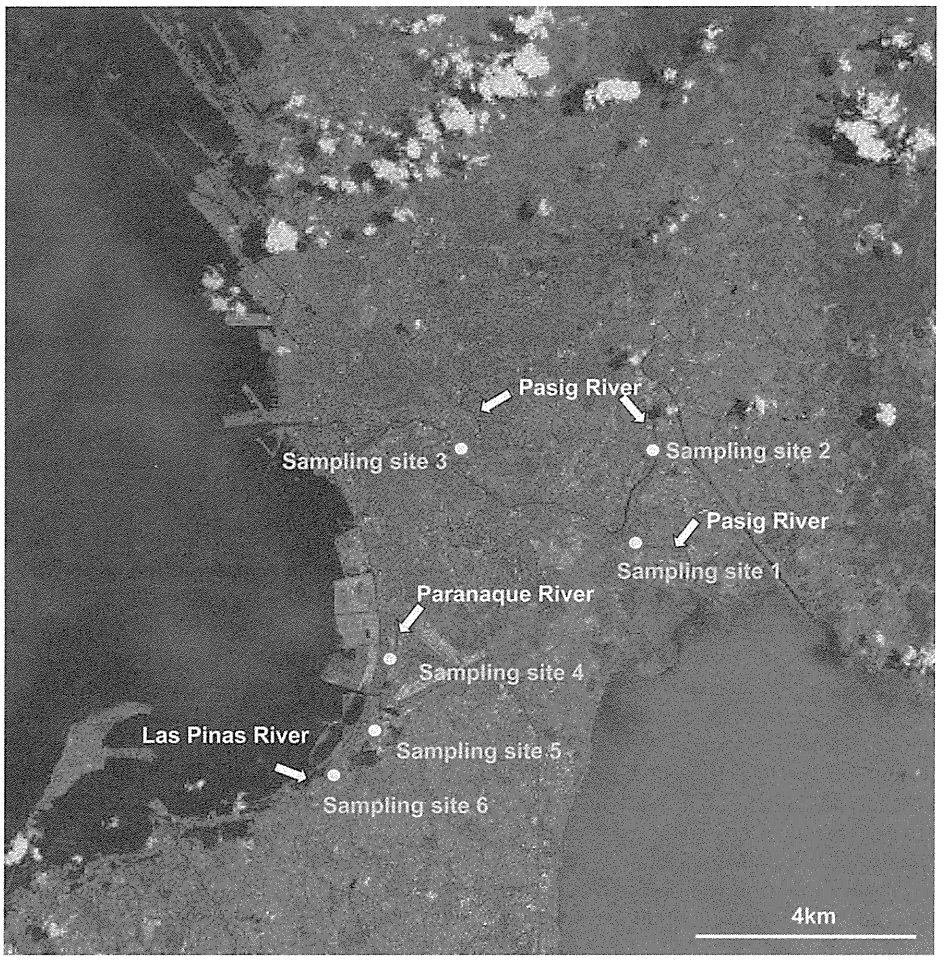

FIGURE 1. Map of Manila City and the sampling sites. Circles indicate the sampling sites. Each river is labeled with an arrow.

a Japan swine HEV strain (AB094215) with identities of 91.1–92.6%. Cluster 3 contained six strains three from W4 and three from W6. Their sequences were close to that of AB671098, isolated from a Japanese donor, with nucleotide sequence identities of 93.5–94.4%. Cluster 4 comprised two strains from W4, with sequences close to the Japan strain AB 807429 (identities of 91.7–92.0%). The strain W5-13 does not belong to any known sub-genotype and shares identities of 84.0–84.3%, 90.2–91.7%, 85.5–88.2%, and 83.7–84.0%, with the Philippines HEV strains in clusters 1 to 4, respectively. The strain W5-13 thus constitutes a new sub-genotype of G3 HEV.

A basic local alignment tool (BLAST) analysis showed that the nucleotide sequence identities between these HEV strains detected in the Philippines and other HEV strains that have been published in GenBank were lower than 94.4%, indicating that area-specific HEV strains are circulating in the Philippines. All 21 of the HEV strains we detected in the river water were collected during the wet season, suggesting that the wet season presents a higher risk of individuals in the area contracting HEV infections.

The results of this study beg the question, what is the source of HEV detected in the Manila City rivers? Because no epidemiological information about HEV in the Philippines is currently available, for human patients, animal outbreaks, or genetic sequences, it is difficult to speculate about the sources of HEV. However, because the HEV is primarily transmitted by the fecal–oral route, HEV might be present in rivers containing human or animal stool. In this study, all of the HEV strains were detected from sampling sits 4–6, located in the Paranaque River and the Las Pinas River. None of the water samples from the Pasig River (sampling sites 1–3) were found to be HEV RNA positive. The Paranaque River and the Las Pinas River are considerably smaller than the Pasig River, and flow through a residential area having high population density. The degree of wastewater pollution is higher for sampling sites 4–6 than for sampling sites 1–3. All of the HEV detected in the river water samples belonged to G3. Genotype 3 HEV can be isolated not only from infected humans but is known to be zoonotic and has also been isolated from domestic swine and wild boars, wild deer, mongoose, and rabbits.[6,7,9,11,19,20] The rivers were probably contaminated with HEV by human or animal excrement, or both.

In conclusion, we have detected and here reported HEV in the Philippines for the first time, and showed that G3 HEV in particular is circulating in the rivers of Manila City. To fully elucidate and address the HEV infection situation in the Philippines, it will be necessary to collect and analyze hepatitis patients' information and investigate the prevalence of HEV infection in swine and wild animals in these areas.

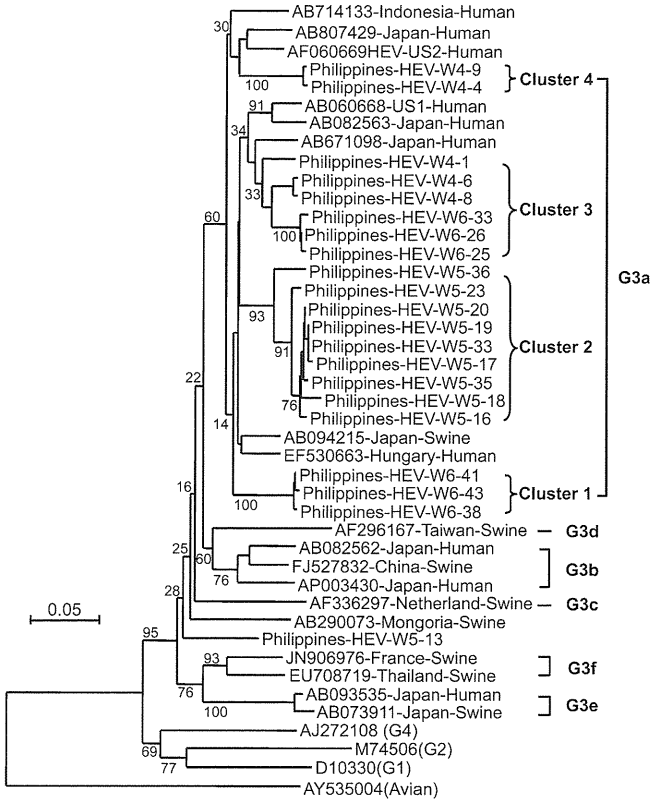

FIGURE 2. Phylogenetic analysis of HEV isolated from river water samples in Manila City, the Philippines. Nucleic acid sequence alignment was performed using ClustalX 1.81 (www.clustal.org). The genetic distance was calculated by Kimura's two-parameter method. A phylogenetic tree with 1,000 bootstrap replicates was generated by the neighbor-joining method based on the partial genome (338 nt) of HEV ORF2 of the genotypes 1–4 and avian HEV isolates. The scale bar indicates nucleotide substitutions per site.

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Short Communication

Ferret Hepatitis E Virus Infection in Japan

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SUMMARY: We examined 85 fecal samples from pet ferrets in 10 animal hospitals in Japan for the detection of ferret hepatitis E virus (HEV) RNA. We found that 6 (7.1%) of the samples were positive for ferret HEV RNA. Phylogenetic analysis based on the partial ORF1 indicated that these ferret HEV strains were clearly separated from the Netherlands strains and were divided into 2 distinct clusters. These results suggest that ferret HEV is genetically diverse, and since ferrets are not indigenous to Japan, ferret HEV has been introduced into Japan through importation.

Hepatitis E virus (HEV) is a single-stranded positive-sense RNA virus that belongs to the genus *Hepevirus* in the family *Hepeviridae* and is the causative agent of hepatitis E (1,2). Four genotypes of HEV (G1-G4) have been detected in humans. Moreover, G3 and G4 HEV have also been isolated from swine, wild boar, and deer and are responsible for zoonotic infections (3-5). In addition to these 4 genotypes, novel HEV or HEV-like viruses have been detected in various animals, including monkeys, rabbits, rats, ferrets, chicken, mink, moose, red fox, and bats (6-13). However, whether the HEV infection is transmitted from these animals to humans is yet unclear.

Ferret HEV was first detected in ferrets in the Netherlands (9). The genome structure of ferret HEV is similar to that of other HEVs and contains 3 open reading frames (ORF1-3). ORF1, ORF2, and ORF3 encode a nonstructural protein of 1589 or 1596 amino acids (aa), a capsid protein of 654 aa, and a functionally unknown protein of 108 aa, respectively (9,14). An earlier study from our group revealed that the expression of the ferret HEV ORF2 protein allowed their assembly into virus-like particles (V-LPs). Enzyme-linked immunosorbent assays (ELISAs) employed for the detection of anti-ferret HEV IgG and IgM antibodies using ferret HEV-LPs as the antigen revealed that the IgG- and IgM-positive rates were 23.3% and 24.4%, respectively, among ferrets in the U.S. (15). The ferret HEV RNA detected in U.S. ferrets was genetically distinct from that of the Netherlands strains (15). Moreover, the antibody against ferret HEV-LPs failed to neutralize G3 HEV, suggesting that the serotypes of these 2 HEVs are different (15). However, the pathogenicity and epidemiology of ferret HEV remain unclear.

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In the present study, we collected a total of 85 fecal samples (47 males and 38 females) and 10 serum samples from ferrets in 10 animal hospitals scattered across 5 prefectures in Japan between October 2009 and September 2013. Most of the ferrets were examined for clinical signs of infection such as diarrhea, anorexia, and hypergammaglobulinemia. The age of the ferrets ranged from 4 months to 9 years and 9 months.

The fecal specimens were diluted with 10 mM phosphate-buffered saline to obtain 10% suspensions (16). RNA was extracted from the diluted fecal suspensions using MagNA Pure LC Total Nucleic Acid isolation kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's recommendations. Reverse transcription (RT) was performed as described previously (17). A nested broad-spectrum RT-polymerase chain reaction (PCR) targeting a portion of ORF1 was performed as described with a slight modification (18). The nested PCR was performed with a forward primer, HEV-cs (5′-TCGCGCATCACMTTYTTCCARAA-3′) and an internal reverse primer, HEV-casn (5′-CCAGGCTCACCRGARTGYTTCTTCCA-3′).

Six (7.1%) of the 85 fecal samples were positive for ferret HEV RNA. All of the nested PCR products were purified and sequenced. Other than the primer sequences, the nested PCR product contained 388 nucleotides corresponding to the C-terminal ORF1 (nt 3885-4272) in the ferret HEV genome (JN998607). Phylogenetic analysis indicated that these strains (GenBank accession nos. AB898199-AB898204) clustered with ferret HEV, and that they were clearly separated from the Netherlands strains and classified into the 2 clusters A and B (Fig. 1).

Five (YFS2, YFS27, YFS28, YFS47, and YFS80) of these strains belonged to cluster A and shared 92.1%-93.2% and 82.4%-84.0% nucleotide sequence identities with the U.S. and Netherlands strains, respectively. Strain YFS51 belonged to cluster B and was closely related to the U.S. strains, with 99.3%-99.6% nucleotide sequence identities. In contrast, strain YFS51 shared 83.2%-84% nucleotide sequence identities with the Netherlands strains. The complete genome of ORF2 (GenBank accession no. AB898198) amplified from ferret fecal sample (YFS2) shared 91.6%-91.7% and

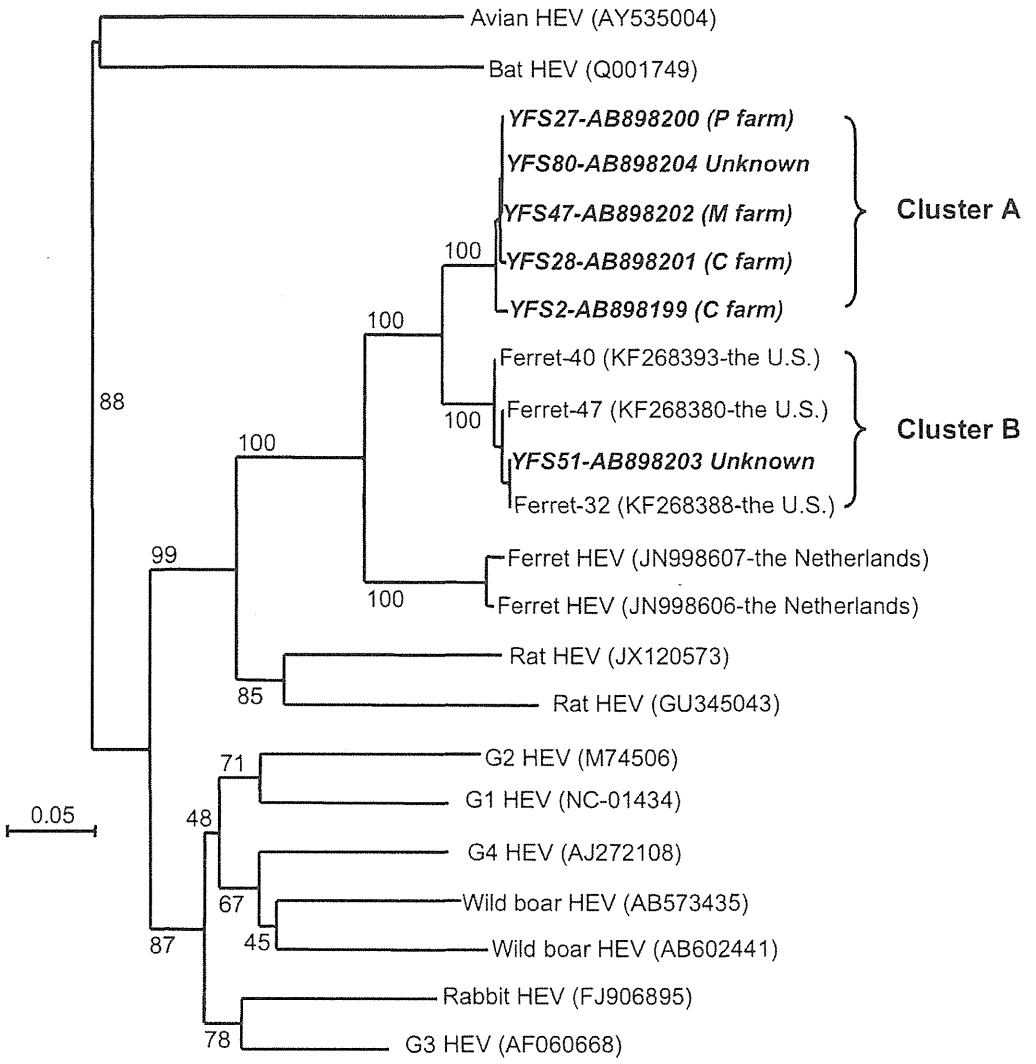

Fig. 1. Phylogenetic analysis of ferret HEV isolates. The nucleic acid sequence alignment was performed using Clustal X 1.81. The genetic distance was calculated by Kimura's two-parameter method. A phylogenetic tree with 1,000 bootstrap replicates was generated by the neighbor-joining method (Njplot 2.3) based on the partial ORF1 genome of HEV isolates. P farm, M farm, and C farm: the name of each ferret farm; unknown: ferrets from unknown origins.

83.5%–83.7% nucleotide sequence identities, and 97.4%–97.6% and 93.9%–94.5% amino acid sequence identities, with the U.S. and Netherlands strains, respectively.

These results suggest that ferret HEV is genetically diverse, consisting of at least 3 clusters. The age of the ferrets that were positive for ferret HEV RNA ranged from 5 months to 6 years, suggesting that ferret HEV infection is likely to occur at a young age. Anti-ferret HEV IgG and IgM antibodies were detected by ELISA, as described previously (15). All 10 tested serum samples were negative for ferret HEV RNA, and the anti-ferret HEV IgG- and IgM-positive rates were 20% (2/10) and 10% (1/10), respectively; this result suggests active ferret HEV transmission and prevalence among pet ferrets.

Ferrets are nonindigenous in Japan and have been imported into the country. In the present study, of the 85 ferrets whose fecal samples were examined, 40, 8, 5, and 32 ferrets were imported from the U.S., New Zealand, Canada, or were of unknown origin, respectively. Of cluster A, strains YFS27 and YFS47 were detected in ferrets from different farms (P and M) in the U.S., and the strains YFS2 and YFS28 from ferrets of the same farm (C) in Canada, suggesting that the circulation of genetically similar ferret HEV strains in these farms.

We found that the YFS2 strain-infected ferret exhibited signs of hepatitis such as anorexia and hepatomegaly. Moreover, the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were found to be 605 IU/L and 149 IU/L, respectively, which is higher than the reference values (ALT, 82–289 IU/L; AST, 28–120 IU/L), indicating hepatocellular injury. These results suggest that ferret HEV infection is likely to be associated with hepatitis in ferrets. In addition to human HEV (G1–G4), HEV-like viruses have been detected in a variety of animals, including mongoose, mink, moose, bats, rats, and chicken (3,8,10,11,13); however, there is no evidence that a particular HEV infection results in hepatitis in host animals, except for chickens

infected with avian HEV and humans infected with G1–G4 HEV. Studies that investigate the pathogenicity of ferret HEV in ferrets would be particularly useful for the development of an animal model of hepatitis E for immunological and pathological studies.

In conclusion, we detected ferret HEV RNA in pet ferrets in Japan and confirmed the wide distribution of ferret HEV and its likely introduction into Japan through importation. Further studies on the epidemiology, biology, immunology, and pathology of ferret HEV are required to obtain a better understanding of its pathogenicity in Japan.

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Conflict of interest None to declare.

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Complete Genome of Hepatitis E Virus from Laboratory Ferrets

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The complete genome of hepatitis E virus (HEV) from laboratory ferrets imported from the United States was identified. This virus shared only 82.4%–82.5% nt sequence identities with strains from the Netherlands, which indicated that the ferret HEV genome is genetically diverse. Some laboratory ferrets were contaminated with HEV.

Hepatitis E virus (HEV) is a single-stranded positive-sense RNA virus that belongs to the family *Hepeviridae*, genus *Hepevirus*, and is the causative agent of hepatitis E (*1,2*). Because the transmission of HEV from deer, swine, and wild boars to humans is well known, hepatitis E is recognized as a zoonosis. Zoonotic hepatitis E is associated mainly with genotype 3 (G3) and G4 HEV infection (*3,4*). In addition to deer, swine, and wild boars, other animals, including monkeys, rats, ferrets, chickens, and bats, harbor HEV or HEV-like viruses (*5–9*). The genus *Hepevirus* might include 3 additional species (avian HEV, bat HEV, and rat/ferret HEV) (*10*). However, whether HEV from these animals is transmitted to humans is not clear.

HEV has been detected in ferrets (*Mustela putorius*) in the Netherlands (*6*). The ferret HEV genome contains 3 open reading frames (ORFs 1–3). ORF1 encodes a nonstructural protein of 1,596; ORF2 encodes a capsid protein of 654 aa, and ORF3 encodes a functionally unknown phosphoprotein of 108 aa. A putative ORF4 observed in the ferret HEV genome was also found in the rat HEV genome. Nucleotide sequence analyses indicated that the ferret HEV genome shares the highest nucleotide sequence identity (72.3%) with rat HEV. The nucleotide sequence identity between the ferret HEV and G1–4 HEV, rabbit HEV, and avian HEV ranges from 54.5% to 60.5% (*6*). However, the antigenicity, pathogenicity, and epidemiology of ferret HEV remain unclear.

Ferret HEV was also recently detected in the United States in serum (*11*), suggesting that ferret HEV infection is not restricted to the Netherlands and might be distributed in ferrets worldwide. Because ferrets are susceptible to several respiratory viruses, including human and avian influenza viruses, and severe acute respiratory syndrome coronavirus (*12,13*), ferrets have been used as a small-animal model for these viruses. Ferrets are also kept as pets in many countries. Thus, information about ferret HEV epidemiology, distribution, transmission, and pathogenesis is urgently needed.

In this study, we amplified and analyzed the complete genome of the US strains of ferret HEV to confirm whether US strains are new ferret HEV genotypes. Phylogenetic analysis demonstrated that HEVs detected in laboratory ferrets from the United States are genetically different from those detected in the Netherlands, suggesting that the ferret HEV genome is genetically diverse.

The Study

Sixty-three fecal samples were collected from laboratory ferrets (*Mustela putorius furo*) at the National Institute of Infectious Diseases, Tokyo, Japan, on May 24, 2013. These ferrets had been imported from a farm in the United States for influenza research 7 days before sample collection. Fecal specimens were diluted with 10 mmol/L phosphate-buffered saline to prepare a 10% suspension, shaken at 4°C for 1 h, and clarified by centrifugation at 10,000 × *g* for 30 min. The supernatant was passed through a 0.45-μm membrane filter (Millipore, Bedford, MA, USA), and stored at –80°C until use.

RNA was extracted by using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's recommendations. Reverse transcription was performed by using the Superscript II RNase H⁻ reverse transcription procedure (Invitrogen, Carlsbad, CA, USA) and primer TX30SXN as described (*14*). Ferret HEV RNA was detected by using a nested, broad-spectrum reverse transcription PCR (*15*). Forty (63.5%) of 63 fecal specimens were positive for ferret HEV RNA. Sequences were similar to those detected in ferret serum samples in the United States (*11*), which suggested that the laboratory ferrets were infected in the United States and then transported to Japan.

RNA from 2 ferret HEVs was randomly selected, and the full-length genome was amplified by using reverse transcription PCR with primers based on nucleotide sequences derived from strains from the Netherlands and United States (Table 1). Sequence of the 5′-terminal noncoding regions of the genome was determined by using Rapid Amplification of cDNA Ends Kits (Invitrogen) according to the

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Table 1. Oligonucleotides used to amplify ferret hepatitis E viruses

Primer (nucleotide positions)*	Sequence, 5′→3′	Product length, bp†
Forward FF1 (1–21)	GGCAGACCCCTAATGGAGACA	
Reverse FR628 (628–648)	GTTGCGTGCGACATAGGCCTT	626
Forward FF541 (541–561)	AGCAATGTATCGCCATGGCAT	
Reverse FR1535 (1535–1554)	ATCTGCATCAGTCGGGCACA	1,014
Forward FF1518 (1518–1538)	AGGATCTGACAGTAGACCTGT	
Reverse FR2555 (2555–2577)	TGCAATGCCAAATTAGCTGTGT	1,060
Forward FF2401 (2401–2421)	GGCGATGAGTTGTACCTGTTA	
Reverse FR3424 (3432–3445)	GAGCAGCCGGTAACATACTCAA	1,045
Forward FF3336 (3336–3355)	GCACAATTTCTATCTCACCA	
Reverse FR4210 (4210–4230)	ACTCCGAATCAGATGATACA	985
Forward FF4181 (4181–4202)	GGCTGGTGCACCTGAATGGCT	
Reverse FR5800 (5800–5821)	TCAGGCAGACGGCGTATCTTAT	1,641
Forward FF4812 (4812–4831)	ATGGAGCATGTGTACAAGAT	
Reverse TX30SXN	GACTAGTTCTAGATCGCGAGCGGCCGCCC TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT	≈2,050
Reverse FR451‡	ACACCGTGTGAATCCCTCCGT	
Abridged amplification	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG	
Reverse FR279 (279–300)	ATAGATCTAGGATGCGCACCAA	§
Abridged universal amplification	GGCCACGCGTCGACTAGTAC	
Reverse FR191 (191–211)	CGGATGCGACCAAACAACAGA	≈240

*Values in parentheses indicate positions of the primer corresponding to the entire genome of hepatitis E virus (JN998607) isolated from ferret.
†Blank cell indicates that 1 primer pair produced 1 product.
‡Used only for cDNA synthesis.
§PCR product was not detected.

manufacturer's instructions. All PCR products were purified by using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA) and cloned into the TA cloning vector pCR2.1 (Invitrogen). Nucleotide sequencing was conducted by using an ABI 3130 Genetic Analyzer Automated Aequencer (Applied Biosystems, Foster City, CA, USA).

Both ferret HEV genomes consisted of 6,820 nt and a poly (A) tail (GenBank accession nos. AB890001 and AB890374), and nucleotide sequence identity was 99.6%. Genomic structure of strains from the United States was similar to that of 2 strains from the Netherlands. The amino acid alignment of ORF2 indicated that ferret HEV ORF2 has an additional 6 amino acids at the N terminus. However, because the seventh codon is AUG, it is unclear which codon was used for the ORF2 translation initiation.

The ORF1 of strains from the United States encodes 1,589 aa, which is 7 aa shorter than ORF1 of both strains from the Netherlands. In addition, the ferret HEV strains from the United States have 2 aa insertions between amino acid residues 596–597 (Thr) and 631–632 (Ile) and 9 aa deletions in amino acid residues 645–653 (Cys-Leu-Arg-Ser-Ser-Pro-Lys-Pro-Pro), which corresponds to those of strains from the Netherlands. Similar to ferret HEV from the Netherlands, an additional putative 183-aa ORF 4 (nt 30–581) was found in strains from the United States. Analysis of 5 other entire ORF2 sequences (GenBank accession nos. AB890375–AB890379) showed that nucleotide identities among them were 98.9%–99.5%, which indicated that genetically similar ferret HEV strains had circulated at the ferret farm in the United States.

Nucleotide and deduced amino acid sequence identities between ferret HEV from the United States and other HEVs are shown in Table 2. The entire genome of strains from the United States shared relatively high nucleotide

Table 2. Nucleotide and deduced amino acid sequence identities between ferret HEV from the United States and other HEVs*

HEV strain (GenBank accession no.)	Full-length genome, %	Ferret HEV (AB890374) Nucleotides, % ORF1	ORF2	ORF3	Amino acids, % ORF1	ORF2	ORF3
Genotype 1 (NC-001434)	53.6	51.4	58.9	50.3	51.4	56.9	22.2
Genotype 2 (M74506)	53.8	51.7	59.3	49.4	56.0	57.2	25.2
Genotype 3 (AF060668)	55.2	53.6	59.4	47.9	54.5	58.1	22.9
Genotype 4 (AJ272108)	53.9	51.5	59.5	49.4	53.5	57.5	28.8
Wild boar HEV (AB573435)	54.4	51.9	60.5	49.1	57.2	57.3	24.3
Wild boar HEV (AB602441)	54.0	51.9	59.1	50.9	57.4	57.0	31.5
Rabbit HEV (FJ906895)	54.8	57.0	60.5	51.2	55.2	57.7	23.3
Rat HEV (GU345042)	61.2	57.0	71.3	63.3	70.1	79.4	40.4
Rat HEV (JX120573)	62.6	58.1	72.8	64.9	74.1	80.0	43.5
Ferret HEV (998606)	82.4	81.4	84.9	85.9	91.5	94.2	73.1
Ferret HEV (998607)	82.4	81.3	85.1	85.9	91.3	94.8	73.1
Ferret HEV (AB890001)	99.6	99.7	99.5	100.0	99.7	99.8	100.0
Avian HEV (AY535004)	50.8	50.5	54.2	47.0	43.1	47.9	24.2

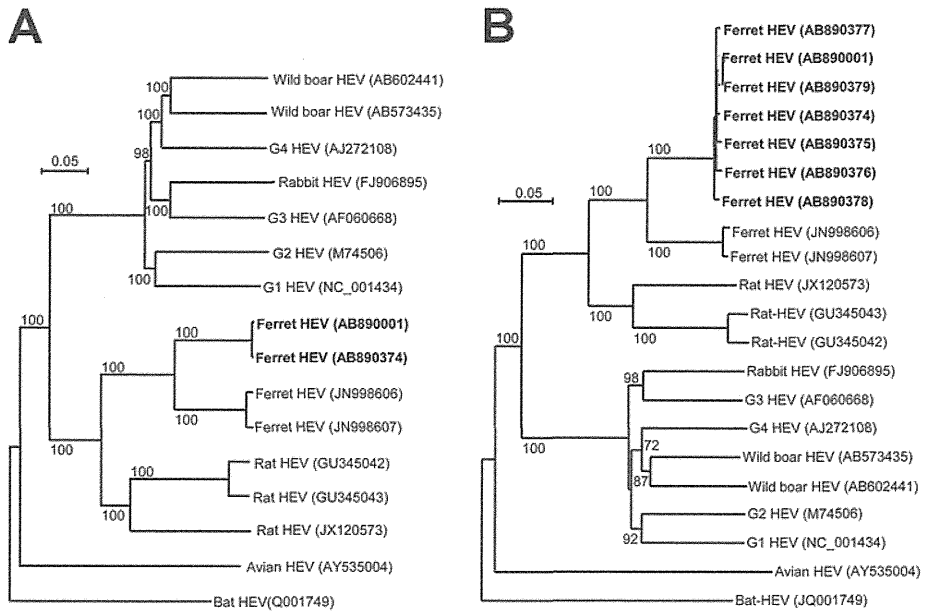

Figure. Phylogenetic relationships among genotypes 1–4 and wild boar, rabbit, rat, avian, bat, and ferret isolates of hepatitis E virus. Nucleic acid sequence alignment was performed by using Clustal X 1.81 (www.clustal.org/clustal2/). Genetic distance was calculated by using Kimura's 2-parameter method. Phylogenetic trees with 1,000 bootstrap replicates were generated by using the neighbor-joining method (Njplot 2.3, http://njplot.sharewarejunction.com/) based on A) the entire genome and B) open reading frame 2. Items in boldface indicate strains isolated in this study. Numbers along branches indicate bootstrap values. Scale bars indicate nucleotide substitutions per site.

sequence identities (82.4%–82.5%) with strains from the Netherlands. We generated phylogenetic trees based on ORF2 or the entire genome. These trees showed that although strains from the United States were closely related to strains from the Netherlands, they formed a new and distinct cluster (Figure). We observed similar phylogenetic clustering when we analyzed nucleotide sequences of ORF1 and ORF3 separately. Although we cannot conclude whether ferret HEV from the United States is a new genotype, these results indicated that there is genetic variety in ferret HEV. Researchers should also bear in mind that some laboratory ferrets are contaminated with ferret HEV.

Conclusions

We amplified the entire genome of 2 ferret HEV strains isolated from laboratory ferrets imported from the United States. Nucleotide sequence comparisons showed that 2 ferret HEV strains from the United States had high (99.6%) identity and shared 98.6%–100% identities with partial sequences of ORF1 that were detected in the United States (*11*), which indicated that genetically similar ferret HEV was circulating in laboratory ferrets.

Although nucleotide sequence identities of the entire genome for strains from the United States and the Netherlands was 82.4%–82.5%, ORF2 showed relatively high amino acid identities (94.2%–94.8%), which suggested that isolated from the United States and the Netherlands share similar antigenicity. Ferret HEV–like particles derived from 1 of the isolates from the Netherlands were cross-reactive with serum from HEV-infected laboratory ferrets in the United States (*11*).

In conclusion, we isolated and identified 2 ferret HEV strains from laboratory ferrets imported from the United States. These strains were genetically distinct from ferret HEV isolates from the Netherlands. Some laboratory ferrets were contaminated with ferret HEV. Further studies are needed to confirm the pathogenicity and zoonotic potential of ferret HEV.

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Construction and characterization of an infectious cDNA clone of rat hepatitis E virus

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Rat hepatitis E virus (HEV) is related to human HEV and has been detected in wild rats worldwide. Here, the complete genome of rat HEV strain R63/DEU/2009 was cloned downstream of the T7 RNA polymerase promoter and capped genomic RNA generated by *in vitro* transcription was injected into nude rats. Rat HEV RNA could be detected in serum and faeces of rats injected intrahepatically, but not in those injected intravenously. Rat HEV RNA-positive faecal suspension was intravenously inoculated into nude rats and Wistar rats leading to rat HEV RNA detection in serum and faeces of nude rats, and to seroconversion in Wistar rats. In addition, rat HEV was isolated in PLC/PRF/5 cells from the rat HEV RNA-positive faecal suspension of nude rats and then passaged. The cell culture supernatant was infectious for nude rats. Genome analysis identified nine point mutations of the cell-culture-passaged virus in comparison with the originally cloned rat HEV genome. The results indicated that infectious rat HEV could be generated from the cDNA clone. As rats are widely used and well-characterized laboratory animals, studies on genetically engineered rat HEV may provide novel insights into organ tropism, replication and excretion kinetics as well as immunological changes induced by hepeviruses.

INTRODUCTION

Rat hepatitis E virus (HEV) was first identified in 2010 in faeces of wild rats from Germany (Johne *et al.*, 2010a). The virus has since been detected in animals from several countries of Europe, Asia and in the USA (Johne *et al.*, 2014a). The host species are mainly rat species (*Rattus norvegicus*, *Rattus rattus* and others), but rat HEV sequences have also been detected in the greater bandicoot (*Bandicota indica*) and the Asian musk shrew (*Suncus murinus*) (Guan *et al.*, 2013; Li *et al.*, 2013a; Johne *et al.*, 2014a).

Analysis of the complete genome sequence indicated that rat HEV is a member of the family *Hepeviridae*, with a distant relationship to the human pathogenic HEV genotypes 1–4 (Johne *et al.*, 2010b). Phylogenetic analysis indicated that rat HEV is most closely related to the recently identified ferret HEV (Raj *et al.*, 2012); both viruses constitute a group clustering between human HEV genotypes 1–4 and hepeviruses from bats and birds (Johne *et al.*, 2014a; Smith *et al.*, 2014). All members of the family *Hepeviridae* have a genome of ssRNA with positive polarity and a length of ~7 kb, which is capped at the 5′ end and polyadenylated at the 3′ end. The rat HEV genome contains ORF1, 2 and 3 encoding a non-structural polyprotein, the capsid protein and a small phosphoprotein, respectively, which are present in all HEV-related viruses. In addition, the rat HEV genome has a small ORF4 overlapping with the 5′ region of ORF1, with unknown function (Johne *et al.*, 2010b; Mulyanto *et al.*, 2014).

Two supplementary tables are available with the online Supplementary Material.