Group B comprised a total of 90 laboratory ferret sera collected from a breeding colony in the USA. All ferrets in group B were female, with ages ranging from 13 to 15 weeks. All serum samples were determined to be negative for anti-influenza virus antibodies, and were stored at −80 °C until use.

Detection of IgG and IgM antibodies. Flat-bottom 96-well polystyrene microplates (Immulon 2; Dynex Technologies) were coated with the purified ferret HEV-LPs (1 μg ml^{-1}, 100 μl per well) and incubated overnight at 4 °C. The unbound V-LPs were removed, and the plates were washed twice with 10 mM PBS containing 0.05 % Tween 20 (PBS-T) and then blocked with 200 μl of 5 % skimmed milk (Difco) dissolved in PBS-T for 1 h at 37 °C. After the plates were washed four times with PBS-T, diluted ferret (100 μl per well) serum samples were added in duplicate. The plates were incubated at 37 °C for 1 h and washed three times as described above. The wells were incubated with 100 μl horseradish peroxidase-labelled goat anti-ferret IgG (H+L) (KPL) (1:2000 dilution) or horseradish peroxidase-conjugated goat anti-ferret IgM (ROCKLAND Antibodies & Assays) (1:10 000 dilution), and diluted with PBS-T containing 1 % skimmed milk. The plates were incubated at 37 °C for 1 h and washed four times with PBS-T. The substrate orthophenylenediamine (100 μl) (Sigma) with H_2O_2 was added to each well. The plates were incubated in a dark room at room temperature for 30 min, and then 50 μl of 2 M H_2SO_4 was added to each well. OD values were measured at 492 nm. The pre-immunized and ferret HEV-LPs-immunized ferret sera were used as negative and positive controls, respectively. A sample was considered to be positive when the absorbance exceeded the cut-off value.

Nested broad-spectrum RT-PCR for detection of ferret HEV RNA. The RNA was extracted using the MagNA Pure LC system with MagNA Pure LC Total Nucleic Acid isolation kit (Roche Applied Science) according to the manufacturer's recommendations. RT was performed with a high-capacity cDNA reverse transcription kit (ABI Applied Biosystems) at 25 °C for 10 min, 37 °C for 120 min 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 10 × RT buffer, 0.8 μl 10 mM deoxynucleoside triphosphates, 8 μl RNA and 5.2 μl distilled water.

A nested broad-spectrum RT-PCR analysis was performed to amplify a portion of the ORF1 genome, based on the method described previously with slight modification (Johne *et al.*, 2010b). Five microlitres of cDNA was used for the first PCR in 50 μl of reaction mixture containing an external forward primer, HEV-cs (5′-TCGCGCATCACMTTYTTCCARAA-3′), and an external reverse primer, HEV-cas (5′-GCCATGTTCCAGACDGTRTTCCA-3′). Each cycle consisted of denaturation at 95 °C for 30 s, primer annealing at 52 °C for 45 s and an extension reaction at 72 °C for 60 s, followed by final extension at 72 °C for 7 min. Two microlitres of the first PCR product were used for nested PCR with an internal forward primer, HEV-csn (5′-TGTGCTCTGTTTGGCCCNTGGTTYCDG-3′), and an internal reverse primer, HEV-casn (5′-CCAGGCTCACCRGARTGYTTCTTCCA-3′). Each cycle consisted of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 45 s and an extension reaction at 72 °C for 60 s, followed by final extension at 72 °C for 7 min. The nested PCR products were separated by electrophoresis on 2 % agarose gels.

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High prevalence of rat hepatitis E virus in wild rats in China

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ABSTRACT

Serum samples from a total of 713 wild rats captured in Zhanjiang city in China from December 2011 to September 2012 were investigated for the prevalence of rat hepatitis E virus (HEV) by exploring rat HEV-specific antibodies and RNA. By an ELISA based on recombinant rat HEV-like particles (HEV-LPs), 23.3% (166/713) of the rats were positive for anti-HEV IgG, and 8.3% (59/713) were positive for anti-HEV IgM. The IgG-positive rates in *Rattus norvegicus*, *Bandicota indica*, *Rattus flavipectus*, *Rattus rattoides losea*, and *Rattus rattus hainanus*, were 27.8% (64/230), 23.0% (40/174), 19.9% (34/171), 21.5% (26/121), and 11.8% (2/17), while the IgM-positive rates were 8.3% (19/230), 6.9% (12/174), 8.2% (14/171), 10.7% (13/121), and 5.9% (1/17), respectively. The IgG-positive rate of the rats captured in rural areas, 24.1% (84/348), was higher than that in the central area of Zhanjiang city, 15.1% (32/212). The highest IgG-positive rates, as high as 45.3% (39/86), were detected in wild rats trapped in the garbage dump. Twelve of the 59 IgM-positive serum samples were positive for HEV RNA, which was detected in all of the wild rat species except *R. rattus hainanus*. A phylogenetic analysis of the partial genome of rat HEV ORF1 indicated that all of the 12 HEV strains belong to rat HEV, and no other genotype HEV were detected. The rat HEV from Zhangjiang city could be classified into three separated clusters, suggesting that the infection due to rat HEV with a variety of genome entities occurs extensively among wild rats in China.

1. Introduction

Hepatitis E virus (HEV) is a single-stranded positive-sense RNA virus that belongs to the genus *Hepevirus* in the family *Hepeviridae* (Meng et al., 2012). It is a causative agent of hepatitis E, a viral disease that manifests acute hepatitis (Emerson and Purcell, 2003). Hepatitis E is transmitted not only by the fecal–oral route (Balayan et al., 1983), but also in a zoonotic fashion (Meng, 2010). The HEV genome is approximately 7.2 kb, containing a 5′ non-coding region followed by three overlapping open reading frames (ORFs) and a 3′ non-coding region followed by a poly A tail. ORF1 at the 5′ end of the genome encodes several nonstructural proteins, and ORF2 encodes an immuno-dominant capsid protein (Jameel, 1999). ORF3, which partially overlaps with ORF2, encodes a cytoskeleton-associated phosphoprotein with multiple functions (Korkaya et al., 2001; Zafrullah et al., 1997).

To date, four genotypes (G1, 2, 3 and 4) of HEV have been isolated from humans, and many new HEV strains or HEV-like viruses have been detected from various animals including monkeys, pigs, wild boars, wild deer, mongooses, rabbits, wild rats, ferrets, bats and chickens (Drexler et al., 2012; Johne et al., 2010a; Li et al., 2005; Meng et al., 1997;

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Nakamura et al., 2006; Raj et al., 2012; Tei et al., 2003; Yamamoto et al., 2012; Zhao et al., 2009).

Rat HEV was first detected from Norway rats in Germany (Johne et al., 2010a). Since then, rat HEV strains have been detected from wild rats in the USA, Vietnam and Indonesia (Li et al., 2011; Mulyanto et al., 2013; Purcell et al., 2011), suggesting that rat HEV infection is widely distributed in wild rats around the world. The full-length genomes of rat HEV from Germany and Vietnam have been determined (Johne et al., 2012, 2010a, 2010b; Li et al., 2013a), and partial sequences of rat HEV detected from USA and Indonesia have been reported (Mulyanto et al., 2013; Purcell et al., 2011). The nucleotide sequence identities between Germany rat HEV strains was 81.5–98.6% (Johne et al., 2012), and the full genome of the Vietnam rat HEV strain (V-105, GenBank accession no. JX120573) shared only 76.8–76.9% nucleotide sequence identity with Germany rat HEV strains R63 and R68, suggesting that the genome of rat HEV is genetically diverse (Li et al., 2013a). Furthermore, the detection of a genotype 3 HEV from various species of wild rats in the USA has recently been reported (Lack et al., 2012), suggesting that rats could be a host for mammalian HEVs. However, another report indicated that G1, G2 and G3 HEV failed to infect Sprague-Dawley rats (*Rattus norvegicus*) (Purcell et al., 2011). Our recent study also indicated that G1, G3 and G4 HEV failed to infect Wistar rats (Li et al., 2013b). At this time, the susceptibility and infectivity of human HEV in rats still remains controversial.

To obtain more detailed information about the infection and circulation of HEV in wild rats, we collected 713 sera from wild rats in Zhanjiang city, Guangdong Province, China, and analyzed the anti-rat HEV IgG and IgM antibodies and HEV RNA. We found that at least four species of wild rats in China were infected with genetically diverse strains of rat HEV.

2. Materials and methods

2.1. Serum samples from wild rats

A total of 713 (408 males and 305 females) wild rats were trapped in Zhanjiang, a city in Guangdong Province, China (Fig. 1), between December 2011 and September 2012. The rats were caught using rat traps placed in sugarcane fields or around residences, garbage dumps and pig farms. The trapped rats were immediately conveyed to the laboratory to take blood samples by femoral artery puncture. The serum was separated and stored at −80 °C until use.

The areas of the capture are shown in Fig. 1. The wild rats were trapped in Chikan district, the central district of Zhanjiang city, and in seven villages (Chiling, Chaofa, Beigou, Huangwai, Nanpan, Houyang and Xiejiawai) as well as one pig farm and one garbage dump in Mazhang district, which is adjacent to Chikan. All but 7 of the trapped rats were adults. The species of rats were identified as 230 *Rattus* (*R.*) *norvegicus*, 174 *Bandicota indica*, 171 *R. flavipectus*, 121 *R. rattoides losea*, and 17 *R. rattus hainanus* by their morphological characteristics as shown in Table 1.

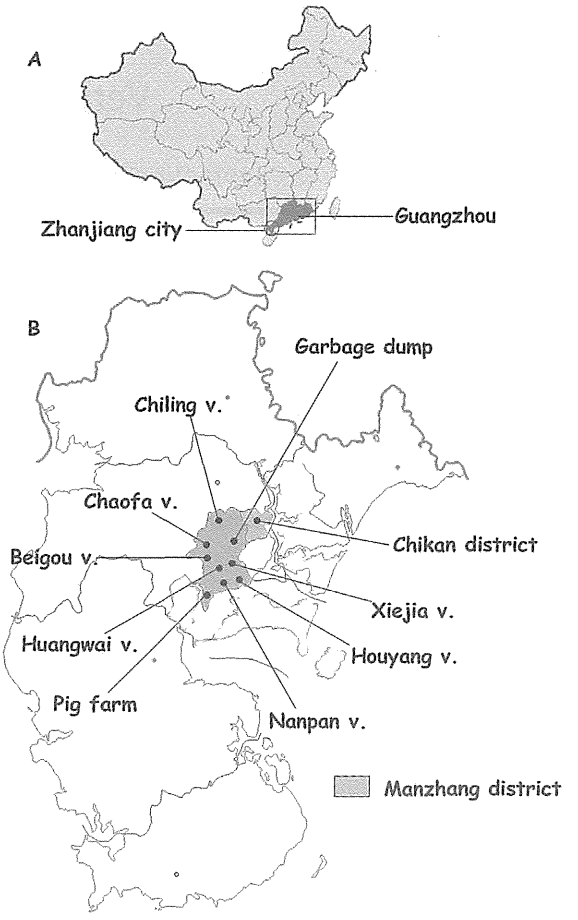

Fig. 1. A map of China (A). Guangdong Province and Zhanjiang city are indicated. A map of Zhanjiang city, Guangdong Province, China, and locations of trapped wild rats (B). The two districts where the rats were trapped, Chikan and Mazhang district, are indicated by gray and light gray, respectively. The locations of seven villages, one pig farm, and one garbage dump are also indicated (●).

2.2. Detection of IgG and IgM antibodies

Anti-HEV IgG and IgM antibodies were detected by enzyme-linked immunosorbent assays (ELISAs) by using of rat HEV-LPs as antigen as described previously (Li et al., 2011).

2.3. Nested broad-spectrum RT-PCR

RNA extraction was performed using the MagNA Pure LC system with MagNA Pure LC Total Nucleic Acid isolation kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's recommendations. Reverse transcription (RT) was performed at 42 °C for 50 min followed by 70 °C for 15 min in a 20 μl reaction mixture containing 1 μl of Superscript™ II RNase H⁻ reverse transcriptase (Invitrogen Inc., Carlsbad, CA), 1 μl of the oligo(dT) primer, 1 μl of RNaseOUT™, 2 μl of 0.1 M dithiothreitol, 4 μl of 5× RT buffer, 1 μl of 10 mM deoxynucleoside triphosphates, 5 μ1 of RNA, and 5 μ1 of distilled water.

Table 1
IgG positive rates in wild rats in Zhanjiang city.

Geographic region	IgG% (positive/tested)					Total
	R. norvegicus	*R. rattoides losea*	*R. flavipectus*	*B. indica*	*R. rattus hainanus*	
Total	27.8% (64/230)	21.5% (26/121)	19.9% (34/171)	23.0% (40/174)	11.8% (2/17)	23.3% (166/713)
Chikan district	13.8% (4/29)	16.4% (12/73)	21.4% (9/42)	8.9% (5/56)	16.7% (2/12)	15.1% (32/212)
Mazhang district	29.9% (60/201)	29.2% (14/48)	19.4% (25/129)	29.7% (35/118)	0% (0/5)	26.7% (134/501)
Huangwai v.	n.a.	0% (0/5)	12.5% (1/8)	16.1% (5/31)	0% (0/1)	13.3% (6/45)
Houyang v.	20% (5/25)	37.5% (3/8)	26.3% (5/19)	61.5% (8/13)	0% (0/1)	31.8% (21/66)
Nanpan v.	0% (0/1)	60% (3/5)	10.5% (2/19)	33.3% (5/15)	n.a.	25.0% (10/40)
Chiling v.	16.7% (1/6)	25% (3/12)	9.1% (1/11)	29.7% (11/37)	0% (0/1)	23.9% (16/67)
Xiejia v.	37.5% (3/8)	31.3% (5/16)	27.7% (13/47)	20% (3/15)	0% (0/1)	27.6% (24/87)
Beigou v.	0% (0/2)	0% (0/1)	0% (0/8)	0% (0/1)	n.a.	0% (0/12)
Chaofa v.	14.3% (1/7)	0% (0/1)	18.8% (3/16)	50% (3/6)	0% (0/1)	22.6% (7/31)
Pig farm	16.7% (11/66)	n.a.	0% (0/1)	n.a.	n.a.	16.4% (11/67)
Garbage dump	45.3% (39/86)	n.a.	n.a.	n.a.	n.a.	45.3% (39/86)

n.a.: sample was not available.

A nested broad-spectrum RT-PCR analysis was performed to amplify a portion of the ORF1 genome, based on the method described previously (Johne et al., 2010b; Li et al., 2011) which are capable of detecting different HEV types including mammalian and avian HEVs. The length of the nested RT-PCR products was 331–334 nucleotides (nt). The nucleotide sequencing of the PCR products was carried out with the primers HEV-csn, HEV-casn, using an ABI 3130 Genetic analyzer automated sequencer (Applied Biosystems, Foster City, CA) and a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) according to the manufacturers' instructions. Sequence analysis was performed using the Genetyx ver.11.0.4 software program (Genetyx Corp., Tokyo, Japan), and multiple alignments were generated using the CLUSTAL W software program version 1.8.1 (Thompson et al., 1994). A phylogenetic tree was constructed by the neighbor-joining method.

2.4. Statistical analysis

Comparisons of the rate of positivity between different areas and between rat species were performed with the unpaired *t*-test. The statistical analysis was performed using SPSS version 16.

3. Results

3.1. Prevalence of IgG and IgM antibodies in wild rats

The rat serum samples were tested for the presence of anti-HEV IgG and IgM antibodies, with the result that 23.3% (166/713) and 8.3% (59/713) of the samples were positive for IgG and IgM antibody, respectively (Fig. 2A, Total). Among the animals trapped in Zhanjiang city, 212 were from Chikan district, and 348 were from 7 villages in the Mazhang district (Fig. 1 and Table 1). The positive rates of anti-rat HEV IgG in wild rats trapped in Chikan district was 15.1% (32/212), which was lower than that in Mazhang district, 24.1% (84/348) ($P < 0.05$). The positive rates of rat HEV IgG among the 7 villages differed substantially, ranging from 0% and 31.8% (Table 1). We also trapped 86 wild rats in a garbage dump and 67 wild rats at a pig farm in Mazhang district. The positive rate was as high as 45.3% (39/86) in the garbage dump, while the rate at the pig farm was 16.7% (11/67), much lower than the mean positive rate (23.3%) (Fig. 2A). The IgG-positive

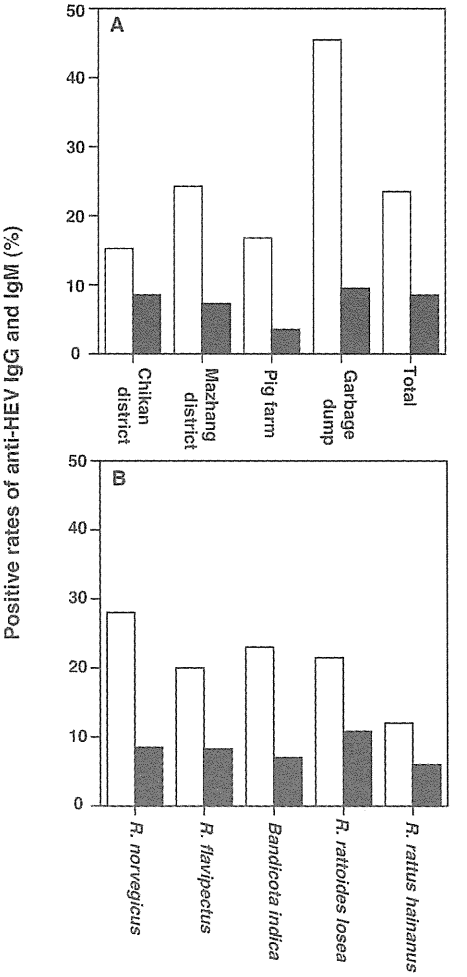

Fig. 2. Positive rates of anti-HEV IgG and IgM antibodies in wild rats. The positive rates by different areas (A), and those by different rat species (B) are shown. White bars, IgG; black bars, IgM.

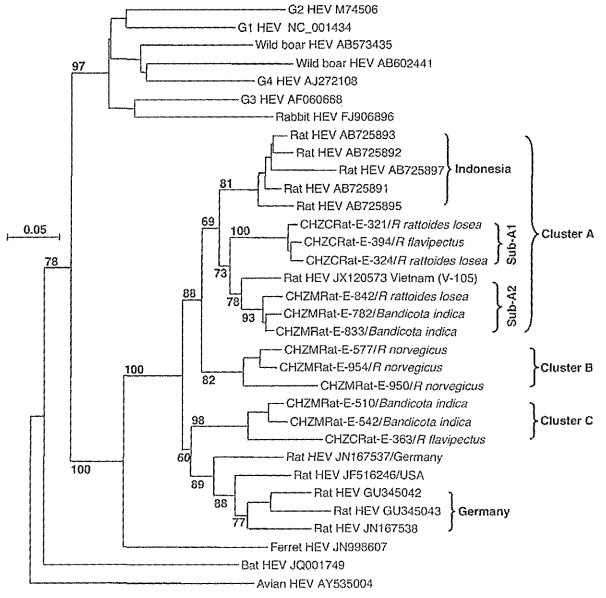

Fig. 3. Phylogenetic analysis of rat HEV strains based on the partial nucleotide sequence of ORF1 (281 nt) using avian HEV as an outgroup. Bootstrap values were determined based on 1000 re-samplings of the data sets.

rates by sex were 23.5% (80/341) in males and 23.1% (86/372) in female rats, with no significant difference between them ($P > 0.05$).

The IgG-positive rates in *R. norvegicus*, *B. indica*, *R. rattoides losea*, *R. flavipectus*, and *R. rattus hainanus* were 27.8%, 23.0%, 21.5%, 19.9%, and 11.8%, while the IgM-positive rates were 8.3%, 6.9%, 10.7%, 8.2%, and 5.9%, respectively (Fig. 2B and Table 1); there was no difference in antibody rates among wild rat species ($P > 0.05$). *R. norvegicus*, and *R. flavipectus* are commensal rodents, while *B. indica*, *R. rattoides losea* and *R. rattus hainanus* are wild rodents. The IgG-positive rates were 24.4% (98/401) in commensal rodents and 21.8% (68/312) in wild rodents, with no significant difference between them ($P > 0.05$) (Table 1). These results suggest that rat HEV infection occurs constantly among wild rats in China.

3.2. Detection of the rat HEV genome in wild rats

A total of 59 IgM-positive serum samples were selected to detect rat HEV RNA by a nested broad-spectrum RT-PCR (Johne et al., 2010b), and 12 samples were found positive for HEV. Except primer sequences, the remaining 281 nt corresponding to nt 4107–4387 in the C-terminal ORF1of the rat HEV genome (GU345042) were sequenced. A phylogenetic analysis indicated that all of the 12 HEV strains belonged to rat HEV, and were clearly separated into three clusters A, B, and C, with cluster A further divided into two sub-clusters, Sub-A1 and Sub-A2 (Fig. 3). In each cluster, the rat HEV strains were closely related each other, with nucleotide sequence identities in A1, A2, B and C of 97.5–97.8%, 97.8–98.1%, 94.9–96.5%, and 94.6–98.7%, respectively. Three strains in the Sub-A1 and three strains in the Sub-A2 are closely related to a Vietnamese rat HEV strain V-105, with identities of 88.9–94.3% and 93.4–94.3%, respectively. Sub-A1 and Sub-A2 also differed from Indonesian strains which forms other sub-cluster in Cluster A. All the Chinese rat HEV strains shared identities of 78.9–89.9% with the Indonesian strain (AB72893). Cluster B and C shared identities of 86.1–87.0% and 82.3–83.9% with V-105, respectively. Strains in cluster A, B, and C shared 74.8–78.7%, 76.4–78.7%, and 78.7–79.9% identity with the German strain GU345042 and shared 76.7–78.6%, 75.6–78.3% and 80.3–81.4% with other Germany strain JN176538, respectively.

As shown in Table 2 and Fig. 3, 6 rat HEV RNA samples in cluster A were amplified with sera from *R. rattoides losea*, *R. flavipectus* and *B. indica* (Fig. 3). All of the three sequences in cluster B were from *R. norvegicus*, and the cluster C sequences were from *R. flavipectus* and *B. indica*, indicating that there was no clear relationship between cluster and rat species. Interestingly, all of the three sequences in cluster A1 were detected in the Chikan district, and 2 out of the 3 sequences in sub-A2 were detected in Chiling village; furthermore, 2 out of the 3 sequences in cluster C were detected in Huangwai village, suggesting that the same virus strains might circulate in limited areas.

4. Discussion

Zhanjiang city is located at the southernmost tip of China, and many species of wild rats live in this area. *R. norvegicus* and *R. flavipectus* are the major species of commensal rodents, whereas *B. indica* and *R. rattoides losea* predominate among the wild rodents in this area. Almost all wild rats trapped in this study belonged to these major species. Monitoring of the major species of wild rats might reflect the overall trend of rat HEV infection in wild rats. In this study we examined a total of 713 wild rat serum samples, and found that the HEV IgG-positive rates were 23.3%, similar to the rates recently reported for Vietnam, Germany and Indonesia (Johne et al., 2012; Li et al., 2011; Mulyanto et al., 2013). Furthermore, anti-rat HEV IgG antibody was detected not only in *R. norvegicus* rats but also in other species of wild rats, indicating that wild rats in China are commonly infected with rat HEV.

The wild rats were trapped in two districts of Zhanjiang city, the central Chikan district and the suburban Mazhang district. The public sanitation environment of Chikan district is considerably better than that of the Mazhang district. When the rat HEV IgG-positive rate in these two areas was compared, we found the positive rate to be higher in the suburbs than in the central city, especially in the garbage dump (Table 1). Human HEV is generally transmitted by the fecal–oral route, and our recent study (Li et al., 2013b) confirmed that rat HEV is also transmitted by that route. It was also reported that the seroprevalence of rat HEV is well correlated with sanitary conditions in Indonesia (Mulyanto et al., 2013). These observations suggest that the environmental hygiene influences rat HEV infection.

A notable finding in this study was that the rat HEV sequences detected in Zhanjiang city were diverse. The 12 rat HEV strains were separated into three clusters with nucleotides identities of 79.4–97.8%. In addition, we found

Table 2
Twelve wild rats positive for RT-PCR.

Date of sampling	Sex	Trapped place	Species of rat	OD values		Name of rat HEV	Cluster
				IgG	IgM		
2012.01.12	F	Chikan district	*R. rattoides losea*	0.923	0.421	CHZCRat-E-321	A1
2012.01.12	F	Chikan district	*R. rattoides losea*	0.593	0.624	CHZCRat-E-324	A1
2012.02.16	F	Chikan district	*R. flavipectus*	1.977	0.979	CHZCRat-E-363	C
2012.02.17	M	Chikan district	*R. flavipectus*	0.107	0.268	CHZCRat-E-394	A1
2012.04.15	M	Huangwai v.	*B. indica*	1.562	0.461	CHZCRat-E-510	C
2012.04.15	F	Huangwai v.	*B. indica*	2.498	0.675	CHZCRat-E-542	C
2012.04.18	F	Pig farm	*R. norvegicus*	2.886	0.707	CHZCRat-E-577	B
2012.05.14	M	Napan v.	*B. indica*	0.125	0.676	CHZCRat-E-782	A2
2012.06.16	M	Chiling v.	*B. indica*	1.931	2.028	CHZCRat-E-833	A2
2012.06.16	M	Chiling v.	*R. rattoides losea*	2.337	0.416	CHZCRat-E-842	A2
2012.08.12	M	Garbage dump	*R. norvegicus*	0.288	0.244	CHZCRat-E-950	B
2012.08.13	F	Garbage dump	*R. norvegicus*	0.722	0.558	CHZCRat-E-954	B

rat HEVs in the same cluster in different rat species, and the same rat species occupying different rat HEV clusters, indicating that rat HEV infection does not occur in a species-specific manner. The genome diversity of rat HEV has also been found in rat HEV that detected in Germany, and it would be of interest to examine whether rat HEV infection in other countries is different from that in China and Germany. Although we amplified and compared the 281 nt sequences in this study, the genetic analysis with the full genome, rather than a partial sequence might give us more information about rat HEV.

An early report showed that human HEV is transmissible to Wistar rats (Maneerat et al., 1996), and a portion of the G3 HEV genome has been detected recently in a variety of wild-caught rat species. However, it has been reported that G1, G2 and G3 HEV do not infect laboratory rats (Purcell et al., 2011). Furthermore, in our recent study (Li et al., 2013b) G1, G3 and G4 HEV failed to infect laboratory Wistar rats. Thus the role of rats in mammalian HEV infections remains controversial.

The nested broad-spectrum RT-PCR used in this study was capable of detecting both rat HEV and other mammalian HEVs (Johne et al., 2010b); however, all of the 12 HEV strains detected in Zhanjiang city were identified as rat HEV, and no mammalian HEV genome was detected in the wild rats, even in the 67 wild rats trapped at a pig farm. These results strongly suggested that the wild rats are susceptible exclusively to rat HEV.

In conclusion, the present study revealed that rat HEV infection is spread widely among wild rats in Zhanjiang, China. The rat HEV Chinese strains are genetically different from the prototypic German strains, and rat HEV infection is not restricted to any particular rat species. More serological as well as genetic studies are needed to clarify the epidemiology of rat HEV in nature.

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Special Report

Etiology and prognosis of fulminant hepatitis and late-onset hepatic failure in Japan: Summary of the annual nationwide survey between 2004 and 2009

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Aim: To summarize the annual nationwide survey on fulminant hepatitis (FH) and late-onset hepatic failure (LOHF) between 2004 and 2009 in Japan.

Methods: The annual survey was performed in a two-step questionnaire process to detail the clinical profile and prognosis of patients in special hospitals.

Results: Four hundred and sixty ($n = 227$ acute type; $n = 233$ subacute type) patients had FH and 28 patients had LOHF. The mean age of patients with FH and LOHF were 51.1 ± 17.0 and 58.0 ± 14.4 years, respectively. The causes of FH were hepatitis A virus in 3.0%, hepatitis B virus (HBV) in 40.2%, other viruses in 2.0%, autoimmune hepatitis in 8.3%, drug allergy-induced in 14.6% and indeterminate etiology in 29.6% of patients. HBV reactivation due to immunosuppressive therapy was observed in 6.8% of FH patients. The short-term survival rates of patients without liver transplantation (LT) were 48.7% and 24.2% for the acute and subacute type, respectively, and 13.0% for LOHF. The prognosis was poor in patients with HBV reactivation. The implementation rate for LT in FH patients was equivalent to that in the previous survey. The short-term survival rates of total patients, including LT patients, were 54.2% and 40.8% for the acute and subacute type, respectively, and 28.6% for LOHF.

Conclusion: The demographic features and etiology of FH patients has gradually changed. HBV reactivation due to immunosuppressive therapy is problematic. Despite advances in therapeutic approaches, the prognosis of patients without LT has not improved.

Key words: acute liver failure, fulminant hepatitis, Japan, liver transplantation, viral hepatitis

INTRODUCTION

IN JAPAN, FULMINANT hepatitis (FH) is defined as having hepatitis, when grade II or worse hepatic encephalopathy develops within 8 weeks of the onset of disease symptoms, with a prothrombin time of 40% or less.[1,2] FH is further classified into two subtypes, acute and subacute types, in which encephalopathy occurs within 10 days and later than 11 days, respectively, of the onset of the disease symptoms. Patients showing a prothrombin time of 40% or less, with hepatic encephalopathy developing between 8 and 24 weeks of disease onset are classified as having late-onset hepatic failure (LOHF).[3] Etiologies with hepatitis present in the histology, such as viral infection, autoimmune hepatitis and drug allergy-induced liver injury are defined as causes of

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FH and LOHF. In contrast, acute liver failure due to other causes with the absence of hepatitis in the histology, such as drug toxicity, circulatory disturbance and metabolic disease, are excluded as causes of FH and LOHF. Recently, a novel diagnostic criteria for acute liver failure in Japan was established by the Intractable Hepato-Biliary Disease Study Group.[4,5] These criteria included other causes with liver damage without the absence of hepatitis in the histology in addition to the present criteria.

Among viral infection, hepatitis B virus (HBV) is a major cause of FH in Japan.[6,7] HBV infection is classified into transient HBV infection type and acute exacerbation in an HBV inactive carrier. With advances in cytotoxic chemotherapy and immunosuppressive therapy, reactivation of hepatitis B is becoming a clinical problem.[8] Moreover, recent introduction of rituximab plus steroid combination therapy for non-Hodgkin's lymphoma has been associated with HBV reactivation in transiently infected patients, namely, de novo hepatitis. However, the prevalence of HBV reactivation in patients with FH and LOHF is unknown.

Advances in therapeutic strategies for FH and LOHF have improved the prognosis. Since 1988, living-donor liver transplantation (LT) has been adopted in patients who are beyond the supportive care of a critical unit.[6] Recently, artificial liver support with high-flow or on-line hemodiafiltration (HDF) has been used. Since 2006, a nucleoside analog, entecavir, has been used as a substitute for lamivudine, as an antiviral agent for HBV. However, it is unknown whether these new treatments improve the prognosis of FH.

The Intractable Hepato-Biliary Diseases Study Group has annually performed a nationwide survey of patients with FH and LOHF since 1983.[6] Since 2000, approximately 600 hospitals have been enrolled in the survey. This report summarizes the results of the survey between 2004 and 2009 to addresses the trends in the etiology and prognosis of patients with FH and LOHF and compares them with the previous survey.[7]

METHODS

THE NATIONWIDE SURVEY was performed annually. The number of hospitals for survey has changed in each year. Maximum (608) was in 2007 and minimum (544) was in 2006, with active members of the Japan Society of Hepatology and the Japanese Society of Gastroenterology between 2005 and 2010. The survey was performed in a two-step questionnaire process to detail the clinical profile and prognosis of patients who were diagnosed as FH or LOHF in the previous year. The recovery rate of the first and second questionnaire was 39–59% and 60–100%, respectively. Patients who met the diagnostic criteria for FH or LOHF were entered into the survey. Patients under 1 year of age, those with alcoholic hepatitis, those with chronic liver diseases and those with acute liver failure with no histological features of hepatitis were excluded from the analysis.

According to criteria described in previous reports,[7,9] the etiology of FH and LOHF was classified into five categories: (i) viral infection; (ii) autoimmune hepatitis; (iii) drug allergy-induced liver injury; (iv) indeterminate etiology despite sufficient examinations; and (v) unclassified due to insufficient examinations. Patients with viral infection consisted of those with hepatitis A virus (HAV), HBV, hepatitis C virus (HCV), hepatitis E virus (HEV) and other viruses. The patients with HBV infection were classified into three subgroups according to serum markers of HBV, hepatitis B core antibody (HBcAb) and immunoglobulin (Ig)M-HBcAb: (i) transient HBV infection; (ii) acute exacerbation in HBV carriers; and (iii) indeterminate infection patterns. In the present study, we classified acute exacerbation in HBV carriers into three subgroups according to the new criteria:[4,5] (i) inactive carriers, without drug exposure; (ii) reactivation in inactive carriers by immunosuppressant and/or anticancer drugs; and (iii) reactivation in transiently infected patients by immunosuppressant and/or anticancer drugs (i.e. de novo hepatitis). Because not every patient was examined for serological markers of transient HBV infection before the onset of FH and LOHF (with HBcAb and/or hepatitis B surface antigen [HBsAg] in serum), we defined HBV reactivation as that occurring in transiently infected patients when they developed HBV-related hepatitis due to immunosuppressive therapy or cytotoxic chemotherapy with reappearance of HBsAg in the serum and did not conform to the criteria of transient HBV infection.

The statistical significance of differences between groups was assessed using Student's *t*-test, Fisher's exact test or Kruskal–Wallis one-way ANOVA. Data are shown as mean ± standard deviation. The study was conducted with the approval of the Ethical Committee of Kagoshima University Graduate School of Medical and Dental Sciences.

RESULTS

Demographic features and survival rates

FROM 2004–2009, 582 PATIENTS were enrolled in the survey. Ninety-four patients were excluded from

the survey according to the exclusion criteria. Consequently, 460 patients ($n = 227$ acute type; $n = 233$ subacute type) were classified as having FH and 28 as having LOHF (Table 1). The incidence of the acute and subacute types of FH was similar and the incidence of LOHF was one-sixteenth of FH. The male : female ratio was higher for the acute type and lower for the subacute type of FH and LOHF. The mean age of patients was significantly higher for the subacute type of FH and LOHF than that for the acute type of FH. Almost half of the patients with FH and LOHF had complications which preceded the onset of acute liver failure. Furthermore, approximately 60% of patients with FH had received daily medication. This tendency for receiving medication was more obvious in patients with the subacute type of FH and LOHF.

The survival rates of patients without LT were 48.7% for the acute type and 24.2% for the subacute type of FH, and 13.0% for LOHF. The survival rates of the subacute type of FH and LOHF was worse than that of the acute type. The prognosis of both the acute type and the subacute type of FH appeared to be equivalent annually. The survival rates of patients with LT were 79.6% for FH and 100% for LOHF, with no difference in these rates among the disease types.

Clinical profile

Symptoms, imaging findings and complications are shown in Table 2. Since 2006, diagnostic criteria of systemic inflammatory response syndrome (SIRS) for fever, tachycardia and tachypnea have been adopted in the survey.[10] Icterus, flapping tremor, ascites, hepatic fetor, tachycardia, tachypnea and pretibial edema were frequently found. The frequency of patients with ascites and pretibial edema was significantly greater in the subacute type of FH and LOHF than in the acute type of FH. In contrast, fever appeared more frequently in patients with the acute type of FH. The frequency of liver atrophy was greater in the subacute type of FH, and even higher in LOHF, than in the acute type of FH.

With regard to complications, disseminated intravascular coagulation, renal failure and bacterial infection were found in more than 30% of patients with FH and LOHF. Brain edema was less frequent in the subacute type than in the acute type of FH.

Causes of FH and LOHF

The cause of FH was identified as viral infection in 46.1% of the patients (Table 3). The frequencies of viral infection were highest for the acute type of FH. HAV infection was found in 3% of patients with FH. HBV infection was found in 40.2% of patients with FH and 32.1% of patients with LOHF. Transient HBV infection was more frequent in the acute type than in the subacute type of FH, whereas the frequency of acute exacerbation in HBV carriers was greater in the subacute type than in the acute type of FH. HBV reactivation in inactive carriers and in transiently infected patients were found in 3.3% and 3.5% of patients with FH, respectively. With regard to underlying diseases in patients with HBV reactivation, non-Hodgkin's lymphoma/mucosa-associated lymphoid tissue lymphoma was most prevalent in 50% of inactive carriers and in 76% of those with transiently infected patients. Among patients with HBV

Table 1 Demographic features and survival rates of patients with fulminant hepatitis (FH) and late-onset hepatic failure (LOHF)

	FH			LOHF ($n = 28$)
	Total ($n = 460$)	Acute type ($n = 227$)	Subacute type ($n = 233$)	
Male/female	227/233	127/100	100/133**	9/19*
Age (years; mean ± SD)	51.1 ± 17.0	48.8 ± 16.9	53.4 ± 16.7**	58.0 ± 14.4**
HBV carrier (%)	13.1 (52/397)	10.5 (19/181)	15.3 (33/216)	22.2 (6/27)
Complications preceding acute liver failure (%)†	46.4 (208/448)	40.0 (88/220)	52.6 (120/228)**	50.0 (14/28)
History of medication (%)	59.9 (260/434)	51.2 (108/211)	68.2 (152/223)**	71.4 (20/28)*
Survival rates				
All patients	47.4 (218/460)	54.2 (123/227)	40.8 (95/233)**	28.6 (8/28)*
No LT	37.5 (132/352)	48.7 (93/191)	24.2 (39/161)**	13.0 (3/23)**
LT	79.6 (86/108)	83.3 (30/36)	77.8 (56/72)	100 (5/5)

*$P < 0.05$, **$P < 0.01$ vs acute type.
†Diseases such as metabolic syndrome, malignancy and psychiatric disorders.
Data in parenthesis indicate patient numbers.
HBV, hepatitis B virus; LT, liver transplantation; SD, standard deviation.

Table 2 Symptoms, imaging findings and complications of patients with fulminant hepatitis (FH) and late-onset hepatic failure (LOHF)

	FH			LOHF ($n = 28$)
	Total ($n = 460$)	Acute type ($n = 227$)	Subacute type ($n = 233$)	
(a) Symptoms at diagnosis				
Fever†	13.0 (42/322)	17.5 (28/160)	8.6 (14/162)*	0 (0/23)*
Icterus	96.8 (427/441)	95.0 (208/219)	98.6 (219/222)*	96.4 (27/28)
Ascites	57.2 (237/414)	45.2 (88/204)	71.0 (149/210)**	81.5 (22/27)**
Convulsion	5.2 (22/422)	6.7 (14/210)	3.8 (8/212)	0 (0/27)
Tachycardia‡	36.7 (117/319)	39.5 (62/157)	34.0 (55/162)	47.8 (11/23)
Tachypnea§	34.5 (87/252)	39.1 (52/133)	29.4 (35/119)	31.6 (6/19)
Flapping tremor	79.0 (309/391)	75.8 (144/190)	82.1 (165/201)	80.8 (21/26)
Hepatic fetor	46.6 (146/313)	49.0 (73/149)	44.5 (73/164)	42.1 (8/19)
Pretibial edema	35.5 (127/358)	24.1 (42/174)	46.2 (85/184)**	75.0 (15/20)**,***
(b) Imaging findings				
Liver atrophy¶	58.8 (255/434)	45.6 (98/215)	71.7 (157/219)**	92.6 (25/27)**,***
(c) Complications				
Infection	34.8 (149/428)	32.9 (68/207)	36.7 (81/221)	51.9 (14/27)
Brain edema	18.5 (71/384)	24.1 (46/191)	13.0 (25/193)**	22.7 (5/22)
Gastrointestinal bleeding	13.2 (59/446)	11.0 (24/219)	15.4 (35/227)	20.0 (5/25)
Renal failure	38.9 (177/455)	40.9 (92/225)	37.0 (85/230)	39.3 (11/28)
DIC	34.6 (150/433)	35.7 (76/213)	33.6 (74/220)	53.8 (14/26)
Congestive heart failure	7.3 (31/427)	8.9 (19/214)	5.6 (12/213)	12.0 (3/25)

*$P < 0.05$, **$P < 0.01$ vs acute type, ***$P < 0.05$ vs subacute type.
†Temperature: >38°C or <36°C.
‡Heart rate: >90 beats/min.
§Respiratory rate: >20 breaths/min or $PaCO_2$: <32 Torr.
† ‡ § Cases between 2005 and 2009.
¶Liver atrophy detected by ultrasound and/or computed tomography imaging.
Data in parentheses indicate patient numbers.
DIC, disseminated intravascular coagulation.

reactivation, rituximab plus steroid combination chemotherapy was administrated to 35% of patients in inactive carriers and to 59% of those with transiently infected patients. HCV and HEV infection were less frequently found. In the survey, Epstein–Barr virus, herpes simplex virus and human herpes virus type-6 were found as other causes of viral hepatitis.

Autoimmune hepatitis was frequently observed in patients with the subacute type of FH and LOHF. Drug allergy-induced liver injury was observed in approximately 10–20% of patients irrespective of disease types. Anti-tuberculosis agents, non-steroidal anti-inflammatory drugs, anticancer agents, drugs for metabolic syndrome, and various herbal and natural remedies were the probable causative agents for this liver injury in the survey. Notably, the etiology was indeterminate in approximately 40% of patients with the subacute type of FH.

Therapies

For artificial liver support, plasma exchange and HDF were performed in most patients with FH (Table 4). Conventional HDF and continuous HDF (CHDF) were performed in 22.5% and 51.8% of patients with FH, respectively. A more powerful method, high-flow HDF (HF-HDF), high-flow CHDF (HF-CHDF) and on-line HDF were performed in 2.6%, 11.7% and 1.8% of the patients, respectively. The nucleoside analogs lamivudine and entecavir were used in approximately a quarter of patients with FH. Entecavir were used more frequently than lamivudine since 2007. Glucocorticosteroid, mainly as steroid pulse therapy, were administrated in more than 70% of patients with FH and LOHF. Anticoagulation therapy were performed in approximately 40–50% of patients with FH and LOHF. Glucagon/insulin, branched-chain amino acid-rich solution,

Table 3 Causes of fulminant hepatitis (FH) and late-onset hepatic failure (LOHF)

	FH			LOHF ($n = 28$)
	Total ($n = 460$)	Acute type ($n = 227$)	Subacute type ($n = 233$)	
Viral infection	46.1 (212)	62.6 (142)	30.0 (70)	32.1 (9)
HAV	3.0 (14)	5.7 (13)	0.4 (1)	0 (0)
HBV	40.2 (185)	54.2 (123)	26.6 (62)	32.1 (9)
(1) Transient infection	19.6 (90)	35.2 (80)	4.3 (10)	3.6 (1)
(2) Acute exacerbation in HBV carrier	14.1 (65)	7.9 (18)	20.2 (47)	25.0 (7)
(i) Inactive carrier, without drug exposure	7.4 (34)	6.2 (14)	8.6 (20)	3.6 (1)
(ii) Reactivation in inactive carrier†	3.3 (15)	1.8 (4)	4.7 (11)	17.9 (5)
(iii) Reactivation in transiently infected patient‡	3.5 (16)	0 (0)	6.9 (16)	3.6 (1)
(3) Indeterminate infection patterns	6.5 (30)	11.0 (25)	2.1 (5)	3.6 (1)
HCV	1.1 (5)	0.9 (2)	1.3 (3)	0 (0)
HEV	0.9 (4)	0.9 (2)	0.9 (2)	0 (0)
Other viruses	0.9 (4)	0.9 (2)	0.9 (2)	0 (0)
Autoimmune hepatitis	8.3 (38)	2.2 (5)	14.2 (33)	32.1 (9)
Drug allergy-induced liver injury	14.6 (67)	13.7 (31)	15.5 (36)	17.9 (5)
Indeterminate§	29.6 (136)	19.4 (44)	39.5 (92)	17.9 (5)
Unclassified¶	1.5 (7)	2.2 (5)	0.9 (2)	0 (0)

†Reactivation in inactive carrier by immunosuppressant and/or anticancer drugs.
‡Reactivation in transiently infected patients by immunosuppressant and/or anticancer drugs (de novo hepatitis).
§Indeterminate etiology despite sufficient examinations.
¶Unclassified due to insufficient examinations.
Data in parentheses indicate patient numbers.
HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus.

cyclosporin A and prostaglandin E_1 therapy were administrated less frequently compared with the previous survey.

Liver transplantation was performed in 23.5% and 17.9% of patients with FH and LOHF, respectively. Two patients received deceased-donor LT and 111 patients received living-donor LT. The frequency of LT was significantly greater in the subacute type than in the acute type of FH.

Prognosis

The prognosis of patients with FH and LOHF differed depending on the etiology (Table 5). Prognosis was good in patients with HAV infection. The prognosis was fair in patients with transient HBV infection. In contrast, the prognosis was poor in acute exacerbation in HBV carriers. The prognosis was extremely poor in patients with HBV reactivation, either from inactive carriers or transiently infected patients. Patients with the subacute type of FH and LOHF caused by autoimmune hepatitis, drug allergy-induced liver injury and indeterminate etiology also showed a poor prognosis.

The clinical features of the patients appeared to be associated with the prognosis. In the acute type of FH with no LT, the frequency of patients with SIRS (tachycardia or tachypnea) was greater in patients who died than in surviving patients ($P < 0.05$). Liver atrophy on ultrasound and/or computed tomography imaging was an important factor in predicting the prognosis of FH and LOHF with no LT. The frequencies were 25.0% and 64.5% in patients with the acute type ($P < 0.01$) and 55.6% and 78.1% in those with the subacute type of FH in surviving patients and those who died, respectively ($P < 0.05$).

Prognosis also appeared to be affected by complications. Any of the complications significantly decreased survival rate (data not shown). Furthermore, the number of these complications affected the prognosis. The survival rate of patients with the acute type of FH was greater than 80% in those with no complications, while it was less than 30% in those with two or more complications. The survival rate of patients with the subacute type of FH was decreased in proportion to the number of complications.

Table 4 Therapies for patients with fulminant hepatitis (FH) and late-onset hepatic failure (LOHF)

	FH			LOHF ($n = 28$)
	Total ($n = 460$)	Acute type ($n = 227$)	Subacute type ($n = 233$)	
Plasma exchange	90.9 (418/460)	92.5 (210/227)	89.3 (208/233)	71.4 (20/28)**,***
Hemodiafiltration	75.0 (342/456)	75.1 (169/225)	74.9 (173/231)	57.1 (16/28)
Glucocorticosteroids	72.4 (333/460)	68.3 (155/227)	76.4 (178/233)	89.3 (25/28)*
Glucagon/insulin	14.6 (67/459)	13.7 (31/227)	14.7 (34/232)	17.9 (5/28)
BCAA-rich solution	19.1 (87/456)	14.3 (32/223)	23.6 (55/233)*	39.3 (11/28)**
Prostaglandin E_1	7.0 (32/458)	6.7 (15/225)	7.3 (17/233)	3.6 (1/28)
Cyclosporin A	10.0 (46/460)	7.0 (16/227)	12.9 (30/233)*	10.7 (3/28)
Interferon	14.1 (65/460)	15.4 (35/227)	12.9 (30/233)	10.7 (3/28)
Nucleoside analog	38.9 (179/460)	50.9 (115/226)	27.5 (64/233)**	32.1 (9/28)
Lamivudine	25.5 (116/455)	40.0 (76/224)	30.4 (40/231)	12.5 (6/28)
Entecavir†	22.4 (70/312)	27.7 (41/148)	17.7 (29/164)	33.3 (5/15)
Anticoagulation therapy‡	47.2 (216/458)	43.2 (98/227)	51.1 (118/231)	39.3 (11/28)
Liver transplantation	23.5 (108/460)	15.9 (36/227)	30.9 (72/233)	17.9 (5/28)

*$P < 0.05$, **$P < 0.01$ vs acute type, ***$P < 0.05$ vs subacute type.
†Cases between 2006 and 2009.
‡Drugs such as antithrombin III concentrate and protease inhibitor compounds, gabexate mesylate and nafamostat mesilate.
Data in parentheses indicate patient numbers.
BCAA, branched-chain amino acid.

DISCUSSION

IN THIS SURVEY, 488 patients were enrolled over 6 years. In the previous 6-year survey, 697 patients (634 for FH and 64 for LOHF) were enrolled.[7] The incidence ratio of LOHF to FH was decreased from 9:1 to 16:1. In national epidemiology research, the annual incidence of FH was estimated at 1050 cases in 1996 and 429 cases in 2004.[11] Therefore, the incidence of FH and LOHF could be decreasing longitudinally. In this survey, the mean age of patients with FH and LOHF was older than that in the previous survey. More patients with complications received daily medication compared with the previous survey. Changes in demographic features of the patients may affect the etiology and prognosis of FH. A relationship between daily dose of oral medication and idiosyncratic drug-induced liver injury has been reported.[12] Additionally, older age is considered a poor prognostic factor in acute liver failure and may be considered a relative contraindication for LT.[13,14]

The current study showed that HBV still remains a major cause of FH and LOHF. Notably, almost half of acute exacerbations in HBV carriers occurred in patients with HBV reactivation owing to immunosuppressive or cytotoxic therapy. Approximately 80% of patients with transiently infected patients had received rituximab plus steroid combination therapy for non-Hodgkin's lymphoma. This combination therapy has been identified as a risk factor for HBV reactivation in HBsAg positive/negative patients with non-Hodgkin's lymphoma.[15,16] Our survey revealed that careful attention is necessary for transiently infected patients, as well as for inactive HBV carriers using intensive immunosuppressive agents.

The frequency of HAV infection in patients with FH was decreased compared with the previous survey. This result is compatible with no occurrence of outbreak of acute hepatitis A during this period. In Japan, zoonotic transmission from pigs, wild boar and deer, either foodborne or otherwise, is the cause of HEV infection.[17,18] In the currently studied survey, two-thirds of the patients were from endemic areas (Hokkaido Island and the northern part of mainland Honshu) in Japan.

The other principal finding in this survey was that the etiology was indeterminate in approximately 40% of patients with FH. One of the reasons for this result may be the failure of diagnosis for autoimmune hepatitis or drug-induced liver injury. Although the diagnosis of autoimmune hepatitis relies on the presence of serum autoantibodies, with higher IgG levels (>2 g/dL), acute-onset autoimmune hepatitis does not always show typical clinical features.[19–21] Additionally, the sensitivity of the drug-induced lymphocyte stimulation test for diagnosis is not completely reliable.

Table 5 Survival rates and etiology of patients with fulminant hepatitis (FH) and late-onset hepatic failure (LOHF) who did not have liver transplantation

	FH			LOHF ($n = 23$)
	Total ($n = 352$)	Acute type ($n = 191$)	Subacute type ($n = 161$)	
Viral infection	39.8 (70/176)	49.2 (58/118)	20.7 (12/58)**	14.3 (1/7)
HAV	57.1 (8/14)	61.5 (8/13)	0 (0/1)	–
HBV	36.2 (55/152)	46.1 (47/102)	16.0 (8/50)**	14.3 (1/7)
(1) Transient infection	52.6 (40/76)	54.4 (37/68)	37.5 (3/8)	–
(2) Acute exacerbation in HBV carrier	15.1 (8/53)	21.4 (3/14)	12.8 (5/39)	14.3 (1/7)
(i) Inactive carrier, without drug exposure	29.2 (7/24)	27.3 (3/11)	30.8 (4/13)	0 (0/1)
(ii) Reactivation in inactive carrier†	7.7 (1/13)	0 (0/3)	10.0 (1/10)	20.0 (1/5)
(iii) Reactivation in transiently infected patients‡	0 (0/16)	–	0 (0/16)	0 (0/1)
(3) Indeterminate infection patterns	30.4 (7/23)	35.0 (7/20)	0 (0/3)	–
HCV	50.0 (2/4)	100 (1/1)	33.3 (1/3)	–
HEV	75.0 (3/4)	100 (2/2)	50 (1/2)	–
Other viruses	100 (2/2)	–	100 (2/2)	–
Autoimmune hepatitis	32.4 (9/28)	40.0 (2/5)	30.4 (7/23)	12.5 (1/8)
Drug allergy-induced	32.8 (19/58)	43.3 (13/30)	21.4 (6/28)	0 (0/3)
Indeterminate§	37.6 (32/85)	54.5 (18/33)	26.9 (14/52)*	20.0 (1/5)
Unclassified¶	1.5 (7)	40.0 (2/5)	–	–

**$P < 0.01$ vs acute type.
†Reactivation in inactive carrier by immunosuppressant and/or anticancer drugs.
‡Reactivation in transiently infected patients by immunosuppressant and/or anticancer drugs (de novo hepatitis).
§Indeterminate etiology despite sufficient examinations.
¶Unclassified due to insufficient examinations.
Data in parentheses indicate patient numbers.
HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus.

Recently, powerful HDF using large buffer volumes (HF-HDF or HF-CHDF), or on-line HDF has been used. HF-HDF or HF-CHDF has a high recovery rate from a coma.[22–24] On-line HDF has an excellent recovery rate from a coma and is useful as a liver support system.[25] However, only 16% of patients with FH received these powerful HDF in the survey examined in the current study. The frequency of brain edema, gastrointestinal bleeding and congestive heart failure was decreased compared with that in the previous survey. Advances in artificial liver support and management may contribute to prevent these complications. Further evaluation is required to determine whether a new powerful support system can improve the prognosis of FH. The survival rate for FH patients with autoimmune hepatitis improved 17.1% in the previous survey to 32.4% in the 2004–2009 survey. Early commencement of corticosteroids may improve the prognosis. However, the efficacy of these drugs has not been evaluated statistically.

Recently, in patients with acute liver failure due to HBV, entecavir has been used more frequently than lamivudine because of its high potency and extremely low rates of drug resistance.[26] Entecavir beneficially affects the course of acute liver failure as lamivudine.[27,28] Despite the use of entecavir, the prognosis of HBV-infected patients, especially in HBV carriers, has not improved. In the case of HBV reactivation, it is difficult to prevent development of liver failure, even when nucleoside analogs are administrated after the onset of hepatitis. Because these agents require a certain amount of time to decrease HBV DNA in serum, they need to be administrated in the early phase of hepatitis. Guidelines for preventing HBV reactivation recommend the administration of nucleoside analogs before the start of immunosuppressive therapy in inactive carriers and at an early stage of HBV reactivation during or after immunosuppressive therapy in transiently infected patients.[29]

Despite new therapeutic approaches and intensive care, the prognosis of patients without LT with both types of FH and LOHF appeared similar to that in the previous survey. In contrast, the prognosis of patients receiving LT was good in the present survey. Yamashiki

et al. reported that the short-term and long-term outcomes of living-donor LT for acute liver failure were good, irrespective of the etiology and disease types.[30] In the current survey, the implementation rate of receiving LT was almost equivalent to that in the previous survey, irrespective of disease type. Notably, only two patients received deceased-donor LT in the current survey. Recently, patients with FH who received deceased-donor LT have been increasing since the new organ transplant bill passed in 2009. Hepatologists should realize that more donor action to increase deceased-donor LT is necessary to improve the prognosis of patients with FH or LOHF. Determining appropriate judgment to move forward to LT is the most important step. The indications for LT in cases of FH are determined according to the 1996 Guidelines of the Acute Liver Failure Study Group of Japan.[31] To improve the low sensitivity and specificity of assessment in patients with acute and subacute types,[32] new guidelines for using a scoring system have been established by the Intractable Hepato-Biliary Disease Study Group of Japan.[33] This novel scoring system showed sensitivity and specificity of 0.80 and 0.76, respectively, and greater than those in the previous guideline.[33] Recently, new prediction methods using data-mining analysis has been established.[34,35]

In conclusion, the demographic features and etiology of FH and LOHF have been gradually changing. HBV reactivation due to immunosuppressive therapy is a particular problem because of poor prognosis. The subacute types of FH and LOHF have a poor prognosis, irrespective of the etiology. Despite recent advances in therapeutic approaches, the implementation rate for LT and survival rates of patients without LT are similar to those in the previous survey.

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Research Paper

Ultra-Deep Sequencing Analysis of the Hepatitis A Virus 5′-Untranslated Region among Cases of the Same Outbreak from a Single Source

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Abstract

Hepatitis A virus (HAV) is a causative agent of acute viral hepatitis for which an effective vaccine has been developed. Here we describe ultra-deep pyrosequences (UDPSs) of HAV 5′-untranslated region (5′UTR) among cases of the same outbreak, which arose from a single source, associated with a revolving sushi bar. We determined the reference sequence from HAV-derived clone from an attendant by the Sanger method. Sixteen UDPSs from this outbreak and one from another sporadic case were compared with this reference. Nucleotide errors yielded a UDPS error rate of < 1%. This study confirmed that nucleotide substitutions of this region are transition mutations in outbreak cases, that insertion was observed only in non-severe cases, and that these nucleotide substitutions were different from those of the sporadic case. Analysis of UDPSs detected low-prevalence HAV variations in 5′UTR, but no specific mutations associated with severity in these outbreak cases. To our surprise, HAV strains in this outbreak conserved HAV IRES sequence even if we performed analysis of UDPSs. UDPS analysis of HAV 5′UTR gave us no association between the disease severity of hepatitis A and HAV 5′UTR substitutions. It might be more interesting to perform ultra-deep sequencing of full length HAV genome in order to reveal possible unknown genomic determinants associated with disease severity. Further studies will be needed.

Key words: Acute liver failure, HAV, IRES, UDPS, 5′UTR.

INTRODUCTION

Hepatitis A virus (HAV) is a leading cause of acute viral hepatitis and occasionally acute liver failure, a life-threatening disease worldwide, and was first identified almost 40 years ago [1]. HAV is still one of the major causative agents of acute hepatitis despite the availability of an effective vaccine [1]. HAV, a member of the *Picornaviridae* family, has a positive-sense single-stranded RNA genome approximately 7.5 kb in length [2,3]. The genome codes a large open reading frame (ORF), which is flanked by 5′-untranslated region (5′UTR) and 3′UTR. The 5′UTR forms the internal ribosomal entry site (IRES), which

mediates cap-independent translation initiation and is important for HAV replication [4-6].

There have been several reports about the association between the severity of hepatitis A and nucleotide variations in 5'UTR of HAV [7-13]. HAV IRES derived from clinical isolates have shown various activities in *in vitro* cell culture [5,12]. Also complicating this issue is the fact that the definition of acute liver failure differs among different countries [11,14]. So, it is unclear whether the HAV genome sequence affects its virulence or not. Here we report on the ultra-deep pyrosequences (UDPSs) of HAV 5'UTR among cases of the same outbreak, which was derived from a single source, a revolving sushi bar.

MATERIALS AND METHODS

Patient Samples

This study was approved by the Ethics Committee, Chiba University, Graduate School of Medicine, Chiba (permission number 1160), and conformed to the Declaration of Helsinki. This HAV outbreak in January/February, 2011, was previously reported in detail [15,16]. Briefly, this outbreak was based in a revolving sushi bar located in a central area of Chiba, Japan. Sixteen patients of this outbreak were included, and one sporadic hepatitis A patient from the same area in June, 2010, was also included in the present study (Table 1). All HAV isolates were classified into HAV IA based on VP1/2A region [15,16]. Fourteen of the 16 outbreak patients were admitted to the National Hospital Organization Chiba Medical Center, Chiba, Japan, and the other patients to Chiba University Hospital, Chiba, Japan. Patient no. 12 was a sushi shop attendant and was expected to be one of the sources of this outbreak. Patients no. 1-16 were outbreak cases. Patient no. 17 was a sporadic case unrelated to this outbreak, he was 59 years old with AST 4313 (IU/L), ALT 5693 (IU/L) and nadir prothrombin level 35 (%), and he was admitted to Chiba University Hospital in June 2010. In this outbreak, no patients with hepatic encephalopathy were observed.

Patients with acute hepatitis A showing a plasma prothrombin level of < 40% without developing hepatic encephalopathy were defined as acute hepatitis 'severe form', and 4 'severe form' patients were included in the present study [14]. Patient no. 1-13 and no. 14-17 were acute hepatitis, non-severe form and acute hepatitis, severe form, respectively.

All patients were positive for immunoglobulin M anti-HAV antibody. Acute viral hepatitis B, C, and E were excluded by serological tests. All were negative for anti-HIV. None of the patients had been taking any medication and none had gone abroad, including to Korea [14] or China [17], within 1 month before disease onset. Sera were obtained at admission and stored at -20°C until analysis.

Table 1. Clinical features of 13 patients with non-severe form and 3 patients with severe form hepatitis A in the outbreak of the present study.

	AH (n = 13)	*P*	AH-S (n =3)
Mean age (yr)	39.9 ± 14.4	NS#	40.6 ± 11.9
Gender (M/F)	7/6	NS$	2/1
Laboratory data			
ALT level (IU/L)	3392 ± 1886	0.024##	6940 ± 3550
Total bilirubin (mg/dL)	6.3 ± 3.8	NS#	5.7 ± 3.7
Nadir PT (%)	70.5 ± 19.1	0.031##	29.6 ± 8.3

Abbreviations: PT, prothrombin time. Data were expressed as Mean ± SD. #Statistically not significant (NS) by Student's *t* test. $Statistically not significant by Chi-squared test. ##Significant difference between 13 patients with AH (acute hepatitis, non-severe form) and 3 patients with AH-S (acute hepatitis, severe form) in hepatitis A outbreak by Student's *t* test.

UDPSs of HAV 5'UTR

Nucleic acids were extracted from 140 μL of sera using a QIAamp Viral RNA mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions, and subjected to RT-PCR. For the detection of HAV RNA, two sets of amplification primers were made at the position of 5'UTR based on HAV HM175 (M59810) sequences. Complementary DNA was synthesized with primer 1 (5'-AGTACCTCAGAGGCA AACAC-3') for 1 cycle at 55°C for 30 min and at 85°C for 5 min using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Indianapolis, IN, USA), then amplified with primer 1 and primer 2 (5'-TCTTGGAAGTCCATGGTGAG-3') for 35 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec using a FastStart high fidelity PCR system, dNTPack kit (Roche). Then, the first PCR product was further amplified with primers 3 (5'-CCACATAAGGCCC CAAAGAA-3') and 4 (5'- GGGACTTGATACCTCA CCGC-3') for 35 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec. Amplified products were separated by agarose gel electrophoresis and purified using a High pure PCR clean-up micro kit (Roche). Each amplicon was quantified by Nanodrop Lite spectrophotometer (Thermo Scientific, Madison, WI, USA), and all amplicons from a single viral genome were pooled together at equimolar ratios. Each pool was then quantitated, and approximately 500 ng of each was used in a fragmentation reaction mix, using a GS FLX Titanium Rapid Library Preparation Kit (Roche). Final libraries representing each genome were characterized for average size by using an Agilent High Sensitivity DNA kit on Agilent 2100 Bioanalyzer (Agilent Technologies, Loveland, CO, USA). 4 x 10^7 of molecular DNA libraries were then sub-

jected to emulsion PCR, and enriched DNA beads were loaded onto a picotiter plate and pyrosequenced with a Roche/454 GS Junior sequencer using Titanium chemistry (454 Life Sciences Corp., Branford, CT, USA) [18]. GS Amplicon Variant Analizer Version 2.7 (Roche) was used for read mapping and calculating variant frequencies at each nucleotide position according to the reference sequence.

Reference Plasmid Clone and Sanger Sequencing

We cloned the PCR product from patient no. 12 into the pCR2.1-TOPO vector (Life Technologies, Tokyo, Japan). Sanger sequencing was performed using a BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Life Technologies). Sequences were analyzed using Applied Biosystems 3730xl (Life Technologies).

Statistical Analysis

To obtain the percentage of nucleotide variability in each sample, the total number of nucleotide substitutions was divided by the total number of nucleotides analyzed at each position. Comparison was performed using Fisher's exact test, Chi-squared test, or Student's t-test. All *P-values* were two-tailed, and $P < 0.05$ was considered statistically significant.

RESULTS

Calculation of PCR and Roche/454 GS Junior sequencer error rates

In order to ensure that errors introduced by PCR as well as errors inherent to the Roche/454 pyrosequencing technology were below our minimum variant frequency threshold of 1%, we sequenced the PCR products from 10^3 to 10^4 copies of control plasmid and found no mutations, indicating similar error rates lower than 1%. The average read number was 7753.

UDPS of HAV IRES of acute hepatitis, non-severe form derived from this outbreak

A previous study [9] showed one of the different hot-spots between acute hepatitis and fulminant hepatitis was located in HAV 5'UTR, according to analysis of the complete HAV genome. So we performed UDPS in 13 HAV IRES derived from patients with acute hepatitis, non-severe form, who were involved in this outbreak. The sequences were compared with the reference clonal sequence from patient no. 12, the sushi shop attendant. In these patients, 20 nucleotide substitutions at 19 positions and 3 nucleotide insertions at 3 positions were seen (Table 2), while plasmid control possessed no substitutions. In cases of acute hepatitis, non-severe form, 0-5 nucleotide substitutions and 0-1 nucleotide insertions were seen in each case. In patients no. 5 and no. 13, respectively, 97.8% nucleotide substitution (206C/T) and 18.6% nucleotide substitution (211T/C) were seen, but all other substitutions were lower than 6% at each position.

Table 2. Nucleotide substitutions of HAV IRES from virus with substitutions.

Patient No.	Locations*	Nucleotide Position	Prototype Nucleotide	Nucleotide Substitution
1	Between IIIc' and IIIb'	202	C	T
	IIIa'	220	G	A
	Between IVf and IVi	441	A	G
3	Between IIIa' and IIIe	225	T	C
4	Between IVa' and Va	576	G	A
5	IIIb'	206	C	T
	IIIg	265.5	-	G
8	IIIb'	207	T	C
	IVa	308	A	G
	IVc	335	A	G
	Between IVj' and IVi	482	T	C
	Between IVj' and IVi	484	A	G
10	Between IIIb' and IIIa'	212.5	-	T
	Between IVk and IVk'	466	T	C
	Vb	604	A	G
	Between Vb and Vc	605	T	C
11	IVd	344	T	C
	Vb	597.5	-	A
12	Between IVk and IVk'	471	A	G
	Between IVj' and IVi	484	A	G
	Vc	610	T	C
13	Between IIIb' and IIIa'	211	T	C
	Between IVd and IVe	357	A	G
15	IIIc	157	T	C
	IVf	378	G	A
	IVi	450	G	A
16	IIIf	242	C	T
17	IIIb'	204	A	G
	IIIb'	208	C	T
	Between IIIg and IIIg'	275	A	G
	Between IIIg and IIIg'	276	T	C
	IVf	378	G	A
	Between IVk and IVk'	466	T	C
	Between IVe' and IVd'	527	G	T
	Between IVa' and Va	578	T	C

*Major domains of HAV 5'UTR [19].

UDPS of HAV IRES of acute hepatitis, severe form, derived from this outbreak

We performed UDPS in 3 HAV IRES derived from patients with acute hepatitis, severe form, who were involved in this outbreak. In these patients, 4 nucleotide substitutions at 4 positions and 0 nucleotide insertions were seen (Table 2). In cases of acute hepatitis, severe form, 0-3 nucleotide substitutions were seen in each case. In patient no. 15, 0.49% nucleotide substitution (157C/T), 14.94% nucleotide substitution (378G/A) and 0.37% nucleotide substitution