genome also explain: (i) the susceptibility of patients infected with HCV to the development of persistent infection; (ii) the difficulty in developing a prophylactic vaccine against HCV; and (iii) low response of some patients with HCV to antiviral drug therapy [Farci et al., 2002; Chambers et al., 2005]. In addition to genomic mutations and quasispecies, genetic recombination seems to play a significant role in the evolution of RNA viruses. Recombination has been reported in other members of the Flaviviridae family (pestiviruses, flaviviruses, hepaciviruses, and Japanese encephalitis viruses) and a natural intergenotypic recombinant (2k/1b) of HCV was identified for the first time in Saint Petersburg, Russia [Kalinina et al., 2002]. A few further cases of chronic hepatitis C with recombinant HCV have been reported from other countries [Kalinina et al., 2002; Colina et al., 2004; Kageyama et al., 2006; Gao et al., 2007; Morel et al., 2011].

However, little is known about recombinant HCV in Japan, where about two million people are infected with HCV. HCV serotypes and genotypes are checked in Japanese patients with HCV to provide insights into disease pathogenesis and predict the prognosis of antiviral therapy. HCV recombination was not reported in Japan until 2010, which may indicate that HCV recombination is either absent or a rare event in Japan. Regular clinical practice suggested that this may actually reflect an inherent limitation of the HCV characterization procedure in Japan. The HCV serotype is defined using NS4-based immune assays that are not suitable for the identification of HCV recombinants. Likewise, HCV genotyping is based on the assessment of nucleotide sequences in a single subgenomic region and it also fails to consider HCV recombination. Full HCV genome sequencing or the assessment of HCV genotypes in multiple regions are not conducted in regular clinical practice.

Hoshino et al. [2010] have reported a novel 2b/1b HCV recombination in a Japanese patient infected with HCV, which provided credible evidence of the discrepancies in HCV serotyping and HCV genotyping in regular clinical practice. Immune assay indicated that the patient was infected with HCV from serogroup 1. This was supported by HCV genotyping using primers for the NS5B region of HCV. However, when primers for the HCV core region were used to assess the genotype, the patient was found to have HCV genotype 2b.

On the basis of this preliminary observation, an extended study was conducted with serogrouping and genotyping of HCV from 104 patients with chronic hepatitis C. HCV recombinants were detected in two patients with chronic hepatitis C. Sera were available from these patients at different time points during the last 14–18 years. Both received antiviral therapy and one recently developed hepatocellular carcinoma (HCC). Analyses of HCV recombination in eight HCV isolates from these two index patients have provided important insights into HCV virology in Japan.

MATERIALS AND METHODS

This study enrolled 104 patients with chronic hepatitis C who presented at Delta Clinic, Tokorozawa, Saitama, Japan. The diagnosis of chronic hepatitis C was made on the basis of the clinical, biochemical, and virological profiles of patients. All expressed HCV RNA in their sera. None had a concurrent infection with hepatitis B virus or human immune-deficiency virus-1. Two patients with HCV recombinants were marked as index patients. The clinical profiles of the two patients with intergenotypic HCV recombinants are shown in Figure 1. Informed consent was obtained from patients after explaining the nature and purpose of the study.

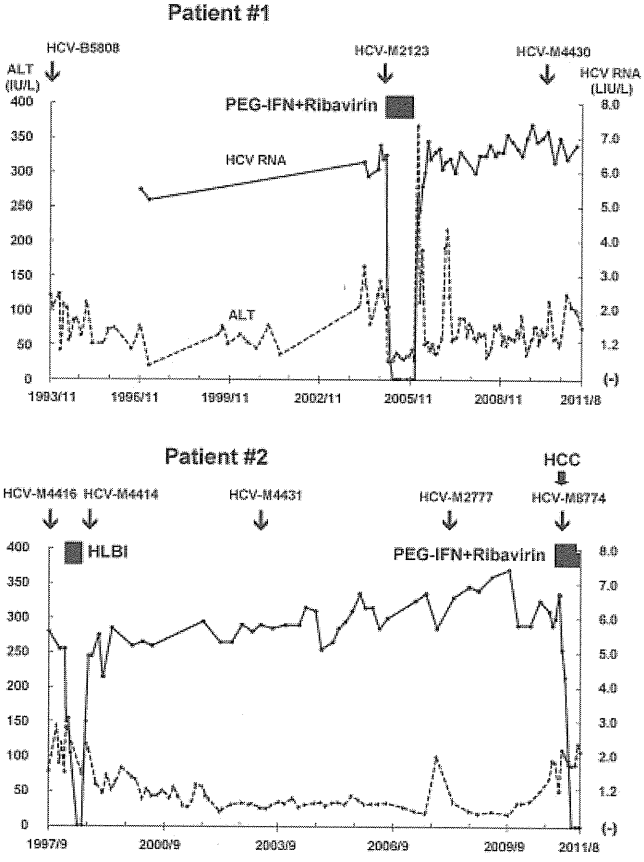

Fig. 1. Clinical profiles of two index patients with recombinant hepatitis C virus (HCV) 2b/1b. Both patients received antiviral therapy, but a sustained response was not achieved in either patients. Three sera samples were available from Patient #1, as shown by arrows; (1) during first appearance at the hospital in 1993 (MCV-B5808), (2) before therapy with peg-interferon (IFN) and ribavirin in 2005 (HCV-M2123), and (3) in 2010 (HCV-M4430). Five sera samples were collected from Patient #2; (1) before treatment with human lymphoblastoid interferon (HBLI) in 1997 (HCV-M4416), (2) after interferon therapy in 1998 (HCV-M4414), (3) in 2003 (HCV-MM4431), (4) before treatment with peg-IFN and ribavirin in 2007 (HCV-M2777), and (5) in 2011, after development of hepatocellular carcinoma (HCV-M8774). Treatment with antiviral drugs is shown as a black bar. HCC in Patient #2 indicates the time of diagnosis of hepatocellular carcinoma.

Serotyping of HCV

The serotypes of HCV isolates from all patients with chronic hepatitis C were determined using a commercial ELISA with NS4-based immunoassays, according to the manufacturer's instructions (Immucheck F-HCV Gr. Sysmex Corporation, Kobe, Japan).

RNA Extraction, cDNA Synthesis, Amplification, and Sequencing

Isolation of RNA, genotyping of HCV, and sequencing of the HCV genome were conducted according to previously described methods, but with some modifications [Takahashi et al., 2009]. Nucleic acids were extracted from serum using a QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany). One of the primers were specific for 2b/1b HCV recombinant (Primer C, Table I). HCV-RNA genomes were reverse transcribed and cDNA was amplified using PCR with primers specific for nine overlapping regions of the HCV genome (Table I). Reverse transcription and first-round PCR were conducted using a PrimeScript II High Fidelity One Step RT-PCR Kit (Takara Bio Inc, Shiga, Japan), while second-round PCR was conducted with PrimeSTAR GXL DNA Polymerase (Takara Bio Inc). The 5′-terminal sequences were amplified using a SMARTer RACE cDNA Amplification Kit (Clontech Laboratories Inc., Mountain View, CA).

Final products were sequenced using a 3100 DNA Sequencer with a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Genetic analyses of HCV sequences were conducted using the neighbor-joining method with the program Genetyx-Mac Version 13 (Genetyx Corporation, Tokyo, Japan).

RESULTS

HCV serotypes and HCV genotypes were in complete agreement in 101 of the 104 chronic hepatitis C patients used in this cohort. However, a discrepancy between the HCV serogroup and HCV genotype was detected in three patients. One of them belonged to HCV serogroup 1 with HCV genotype 2, but HCV recombination was not detected in this patient. On the other hand, two patients with chronic hepatitis C

TABLE I. HCV-Specific Oligonucleotide Primers Used in This Study

Primer—specificity	Stage—polarity	Nucleotide sequence (5′–3′)	Nt position[a]
Primer set A—2b	1st—sense	CCATGAATCACTCCCCTGTG	30–49
	2nd—sense	GAACTACTGTCTTCACGCAG	52–71
	1st—antisense	ARGCTGTCATTGCARTTGAG	1,620–1,639
	2nd—antisense	GCGGTCCGGTTTATGTGCCA	1,599–1,618
Primer set B—2b	1st—sense	GCATGGCATGGGACATGATG	1,291–1,310
	2nd—sense	CCAACTCTYACCATGATCCT	1,323–1,342
	1st—antisense	AGGAGARTCTTGTATGCTGG	2,850–2,869
	2nd—antisense	GTAAGAGTRAAGATGGATAT	2,829–2,848
Primer set C—2b/1b	1st—sense	TGGATGCTCATYATACTGGG	2,559–2,578
	2nd—sense	GAGGCRGCGCTTGAGAAGCT	2,586–2,605
	1st—antisense	GTAGCGGTGGCGAGYACGAC	4,383–4,402
	2nd—antisense	CCAGCCGTCTCCGCTTGGTCC	4,352–4,372
Primer set D—1b	1st—sense	GGCGCCTAYGACATCATAAT	4,275–4,294
	2nd—sense	TGTGATGARTGCCACTCAAC	4,296–4,315
	1st—antisense	GGTGGTGAGCGGGCTGGTGA	5,743–5,762
	2nd—antisense	CGCTGATGAARTTCCACATG	5,645–5,664
Primer set E—1b	1st—sense	TGGAARTGTCTCATACGGCT	5,175–5,194
	2nd—sense	ATCATGGCATGCATGTCGGC	5,286–5,305
	1st—antisense	AYGCCCGTCACGTAGTGGAA	6,648–6,667
	2nd—antisense	GTGGTGTAYGCGTTGATGGG	6,534–6,553
Primer set F—1b	1st—sense	TGGGACTGGATATGCACGGT	6,294–6,313
	2nd—sense	AAGACCTGGCTCCAGTCCAA	6,327–6,346
	1st—antisense	ACTGTGGACGCCTTCGCCTT	7,824–7,843
	2nd—antisense	TCCTTGAGCACGTCCCGGTA	7,800–7,819
Primer set G—1b	1st—sense	TCCATGCCCCCCCTTGAGGG	7,512–7,531
	2nd—sense	AGCGACGGGTCTTGGTCTAC	7,554–7,573
	1st—antisense	CATGAAGCCACCCTATTGAT	9,054–9,073
	2nd—antisense	TGGAGAGTAACTATGGAGTGA	9,027–9,047
Primer set H—1b	1st—sense	GAGGCTATGACTAGGTACTC	8,631–8,650
	2nd—sense	AGACAGCTAGACACACTCCA	8,803–8,822
	1st—antisense	ATGGCCTATTGGCCTGGAGTG	9,400–9,420
	2nd—antisense	CTATTGGCCTGGAGTGKTTA	9,396–9,415
Primer set I—1b	1st—sense	CTCCATAGTTACTCTCCAGG	9,030–9,049
	2nd—sense	TTGCGAGTCTGGAGACATCGG	9,099–9,119
	1st—antisense	ATGATCTGCAGAGAGGCCAG	X-tail
	2nd—antisense	CACGGACCTTTCACAGCTAG	X-tail

[a]Analyzed according to genome sequence of HCV-M2123(AB558135).

showed HCV recombination of 2b/1b and these patients were regarded as index patients. Their clinical features of two patients with HCV recombinants are shown in Figure 1. Patient #1 was born in 1962 and works as a health care worker. In 1988, she experienced a needle prick accident at the age of 26 and expressed HCV antibody in her sera with elevated levels of serum alanine aminotransferase (ALT). Sera were collected from this patient on three occasions: (i) in 1993, when she first attended hospital; (ii) in 2005, prior to interferon therapy; and (iii) in 2011, to assess the present status of her illness (Fig. 1). Patient #2 is a 72-year-old male with a history of blood transfusion during an operation in 1963. A regular health check in 1996 showed that he was expressing HCV antibody in his sera. Sera were collected from this patient for characterization of HCV on five occasions: (i) in 1997, when first attending the hospital before interferon therapy; (ii) in 1998, after interferon therapy; (iii) in 2003, when HCV RNA was high and ALT remained elevated after antiviral therapy; (iv) in 2010, before starting a second regime of antiviral therapy; and (v) in 2011, when he developed HCC.

Analyses of HCV Sequences of Patient #1 and Patient #2

Serotyping of patient sera using NS4-based immunoassays indicated that both index patients were infected with HCV serogroup 1. Three HCV isolates, that is, HCV-B5808, HCV-M2123, and HCV-M4430, were retrieved from sera collected from index Patient #1 over the last 18 years (1993–2011; Fig. 1). Five HCV isolates, that is, HCV-M4416, HCV-M4414, HCV-M4431, HCV-M2777, and HCV-M8774, were isolated from index Patient #2 over the last 14 years (1997–2011; Fig. 1). To gain better insight, the near-complete genomic structure of all eight HCV isolates (three from Patient #1 and five from Patient #2) was determined by amplifying nine overlapping regions of the HCV genome (Table I). Using these primers, near-complete nucleotide sequences of HCV except the 3′-tail were obtained. The nucleotide (nt) sequence of HCV-B5808 (HCV isolate of Patient #1) obtained length of 9,471 nt, comprising of 5′UTR (nt 1–341), open reading frame (nt 342–9,383 for 3014aa), 3′UTR (nt 9,384–9,423). The rest seven HCV isolates retrieved from patient #1 and patient #2 had nt sequences of 9,321–9,471 nt with a polyprotein of 3,014 amino acids. Figure 2 shows that all eight HCV isolates (DDBL/EMBL/GeneBank accession numbers AB5558135 and AB677527–AB677533) from these two patients were related closely to 2b HCV isolates of 5′UTR-NS2 region sequence of known HCV 2b isolates. However, all HCV isolates from the two index patients were similar to reported 1b HCV isolates based on the NS3-3′UTR region (Fig. 2). This suggested that there might be inter-genotypic recombination of 2b and 1b in these two patients. Both patients received antiviral therapy and one developed HCC. The HCV recombinant 2b/1b persisted in all HCV isolates over a period of 14–18 years.

2b/1b Recombinant HCV Found in Eight Isolates Represents a Novel HCV Recombinant

The recombination cross-over point in these HCV isolates was assessed using data from the full genome sequences. The HCV sequences of the NS2 and NS3 regions are in Figure 2. Analysis of the HCV sequences in these regions indicated that the cross-over point was located in NS2 (marked with an arrowhead in Fig. 2). The recombination cross-over point was similar in all eight isolates in the two index patients with chronic hepatitis C. In addition to preliminary report in 2010 of HCV recombinant from Japan [Hoshino et al., 2010], HCV recombinants have been reported from two Asian countries, that is, the Philippines in 2006 [Kageyama et al., 2006] and Japan in 2011 [Yokoyama et al., 2011]. The cross-over points of other recombinant HCV isolates from the Philippines and Japan were located in the NS3 region. Thus, the recombinant HCV isolates found in the current study appear to be novel HCV recombinants in Japan.

Phylogenetic Tree of HCV Recombination in Eight Isolates From Two Index Patients

Figure 3 shows the phylogenetic tree constructed for the full genome sequences of eight HCV isolates from two index patients. Full genome sequencing showed that the 2b/1b HCV recombinant persisted in both patients and all isolates collected at different time points over the last 18 and 14 years from Patient #1 and Patient #2, respectively.

The rate of genetic change in HCV during the course of the chronic infections was comparatively low in these patients. HCV isolate M4430 was isolated from the sera of Patient #1 17 years 6 months after collecting sera containing HCV isolate B5508. HCV isolate M8774 was collected 14 years after isolating M4416 from Patient #2. However, only 0.94×10^{-3} base substitutions per site per year were detected in Patient #1. In the case of Patient #2, the frequency of base substitutions was 0.41×10^{-3} per site per year. This is comparatively lower than the prevalence of HCV genetic drift during natural chronic infections [Okamoto et al., 1992; Ogata et al., 1991].

DISCUSSION

Japan has about 2 million people with HCV infections and most belong to HCV genotype 1. Although several million people throughout Asia are infected with HCV, HCV recombinants have only been identified rarely from Asian countries. A 2b/1b recombinant HCV strain was identified in the Philippines [Kageyama et al., 2006] and Hoshino et al. [2010] subsequently reported the first case of a HCV recombinant from Japan based on the analysis of a partial HCV genomic sequence. Yokoyama et al. [2011]

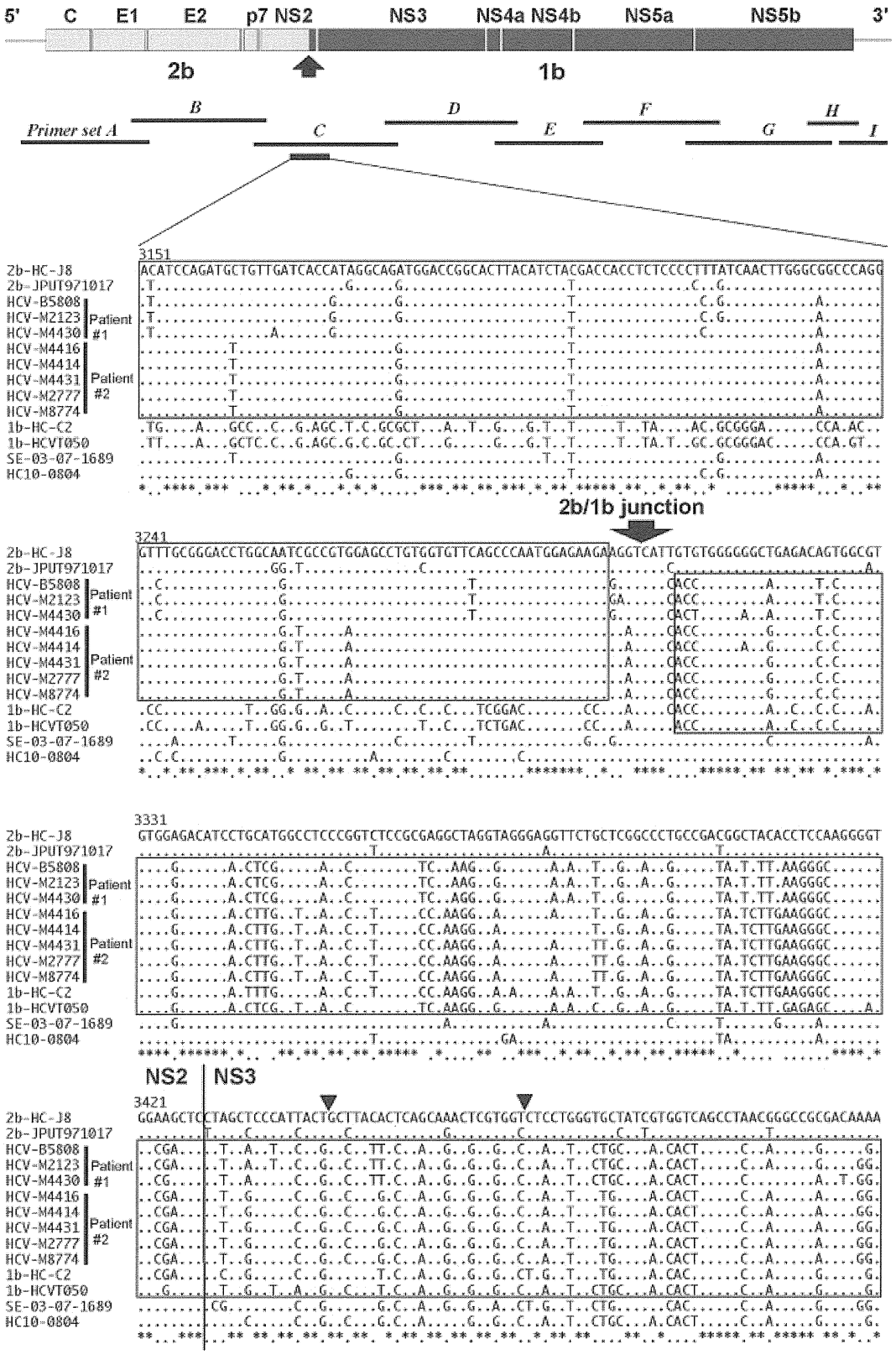

Fig. 2. Alignment of the nucleotide sequences of eight HCV isolates from two index patients with recombinant HCV 2b/1b (three from Patient #1 and five from Patient #2). Four reference sequences (2b-HC-J8, 2b-JPUT971017, 1b-HC-C2 and 1b-HCVT050 and HCV2b/1b) are also shown in this figure. Nucleotide sequences of recombinant HCV strains from the Philippines (SE-03-07-1689) and Japan (HC-10-0804) are shown along with eight clinical isolates from the two patients. The black arrow indicates the possible cross-over point from 2b to 1b in the NS2 region of the eight HCV isolates from the two index patients. The cross-over points in the NS3 region of the HCV recombinants from the Philippines and Japan are shown as black triangles.

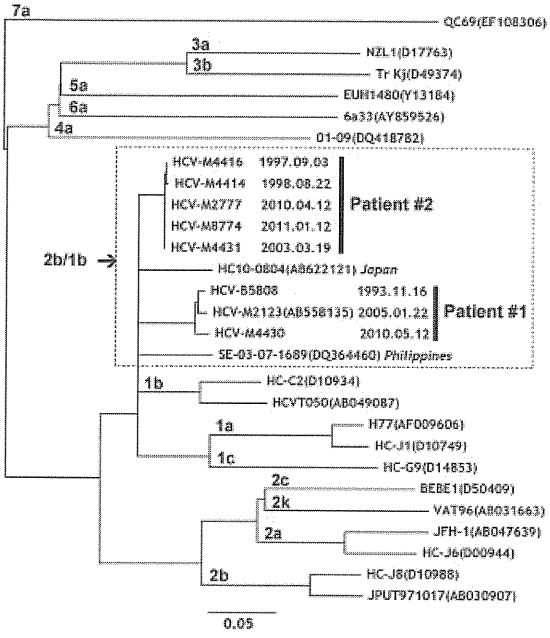

Fig. 3. Rooted neighbor-joining tree showing the phylogenetic relationship among the HCV isolates and prototype strains. All eight isolates from the two index patients (Patient #1 and Patient #2) with chronic hepatitis C are shown in bold. Representative HCV strains of genotype 7a, 3a, 3b, 5a, 6a, 4a, 1b, 1a, 1c, 2c, 2k, 2a, and 2b from different geographical regions are also included. The HCV 2b/1b recombinants reported from the Philippines [Kageyama et al., 2006] and Japan [Yokoyama et al., 2011] are shown for comparison.

reported a child from Japan with a 2b/1b recombinant. It appears that the rate of HCV recombination may be low in Japan, but further study is required.

The current study identified two chronic hepatitis C patients who were infected with recombinant HCV with the 2b/1b genotype. It is noteworthy that the HCV recombinants described in this study are completely different from those reported from the Philippines [Kageyama et al., 2006] and Japan [Yokoyama et al., 2011]. The estimated cross-over points of the recombinant HCV strains from the Philippines and Japan differed from those of the two index patients in the current study (Fig. 2). The recombination cross-over point in previous studies was located in the NS3 region of the HCV genome, whereas it was located in the NS2 region of both patients. Recombinant HCV strains with inter-genotypic junctions in between core and NS4 region have been detected in this study because all reported HCV recombinants have inter-genotypic junctions around NS2-NS3 region of HCV genome and this study was intended to identify inter-genotypic recombinant HCV strains noticed by discrepancies between immunoassays and sequencing. Further studies accomplished with primers capable of detecting inter-genotypic recombinants in other regions of HCV genome may provide further insights about HCV recombinants. Data from the current study and published data suggest that HCV recombinants are a comparatively rare event in HCV evolution, but the nature and properties of HCV recombination merit further studies to develop better insights into HCV virology.

Several points of this study are worthy of mention. This study assessed HCV recombination at different points during HCV infection, that is, during its natural course, before and after antiviral therapy, and after the development of HCC. The analysis of eight HCV isolates retrieved from these two patients at different time points over 14–18 years showed that the 2b/1b HCV recombinants persisted in patients throughout chronic HCV infection, before and after antiviral therapy, and after the development of HCC. The clinical impact of this observation needs to be clarified in future. In addition, the genetic drift of the HCV genome over 14–18 years of chronic HCV infection with recombinant HCV appeared to be low compared to the rate of nucleotide change in normal chronic HCV infections [Ogata et al., 1991; Okamoto et al., 1992].

Another point of profound clinical importance is the discrepancy between HCV immunoassays and HCV genotyping. Also, the surrogacy of HCV genotyping based on the estimation of a single subgenomic HCV region genome appears questionable [Dixit et al., 1995]. The HCV genotype is related to the progression of liver diseases and the response to antiviral therapy in patients with chronic hepatitis C [Shirakawa et al., 2008; Ghany et al., 2009]. This and other studies of HCV recombinants indicate that it would be premature to predict treatment outcome based on current clinical practices that assess either the serogroup or the genotype of only the NS5B or core region. It is tempting to propose that HCV genotype assessments should be conducted for all patients with chronic hepatitis C with genotype 2b who are non-responders to antiviral therapy. In addition to clinical factors, HCV recombination may be another factor determining non-responsiveness to antiviral therapy in patients with chronic hepatitis C.

Both patients with HCV recombinants had a history of possible HCV infection, that is, one patient had a history of needle prick while the second had a blood transfusion. Both patients were natives of Japan and their clinical history provided no clues regarding their infection from outside Japan. The cross over point of HCV recombinant reported by Yokoyama et al. [2011] from Japan also differed from that of HCV isolates shown in this study. Thus, the HCV recombinants described in this study appear to be novel recombinants with novel cross-over points in the NS2 region of the HCV genome.

In conclusion, it appears that different forms of HCV recombination are circulating in Japan. HCV recombination may not be as rare as believed previously. Attention should be given to assessing HCV genotyping using multiple primers. HCV may try to evade host immunity via genotypic recombination, so the real implications of these observations remain to be clarified in the context of vaccine development

and the management of antiviral therapy in non-responders in the future.

REFERENCES

Chambers TJ, Fan X, Droll DA, Hembrador E, Slater T, Nickells MW, Dustin LB, Dibisceglie AM. 2005. Quasispecies heterogeneity within the E1/E2 region as a pretreatment variable during pegylated interferon therapy of chronic hepatitis C virus infection. J Virol 79:3071–3083.

Choo QL, Richman KH, Han JH, Berger K, Lee C, Dong C, Gallegos C, Coit D, Medina-Selby R, Barr PJ, Wiener AJ, Bradley DW, Kuo G, Houghton M. 1991. Genetic organization and diversity of the hepatitis C virus. Proc Natl Acad Sci USA 88:2451–2455.

Chung H, Ueda T, Kudo M. 2010. Changing trends in hepatitis C infection over the past 50 years in Japan. Intervirology 53:39–43.

Colina R, Casane D, Vasquez S, Garcia-Aguirre L, Chunga A, Romero H, Khan B, Cristina J. 2004. Evidence of intratypic recombination in natural populations of hepatitis C virus. J Gen Virol 85:31–37.

Di Bisceglie AM, Hoofnagle JH. 2002. Optimal therapy of hepatitis C. Hepatology 36:S121–S127.

Dixit V, Quan S, Martin P, Larson D, Brezina M, DiNello R, Sra K, Lau JYN, Chien D, Kolberg J, Tagger A, Davis G, Polito A, Gitnick G. 1995. Evaluation of a novel serotyping system for hepatitis C virus: Strong correlation with standard genotyping methodologies. J Clin Microbiol 33:2978–2983.

Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Ogura Y, Izumi N, Marumo F, Sato C. 1996. Mutations in the nonstructural protein 5A gene and response to A natural recombinant hepatitis C virus in Japan interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med 334:77–81.

Farci P, Strazzera R, Alter HJ, Farci S, Degioannis D, Coiana A, Peddis G, Usai F, Serra G, Chessa L, Diaz G, Balestrieri A, Purcell RH. 2002. Early changes in hepatitis C viral quasispecies during interferon therapy predict the therapeutic outcome. Proc Natl Acad Sci USA 99:3081–3086.

Gao F, Nainan OV, Khudyakov Y, Li J, Hong Y, Gonzales AC, Spelbring J, Margolis HS. 2007. Recombinant hepatitis C virus in experimentally infected chimpanzees. J Gen Virol 88:143–147.

Ghany MG, Strader DB, Thomas DL, Seeff LB. 2009. Diagnosis, management, and treatment of hepatitis C: An update. Hepatology 49:1335–1374.

Holmes EC, Worobey M, Rambaut A. 1999. Phylogenetic evidence for recombination in dengue virus. Mol Biol Evol 16:405–409.

Hoshino H, Hino K, Miyakawa Y, Takahashi K, Mishiro S. 2010. A novel 2b/1b recombinant HCV found in a case with discrepancy between serotype and genotype assessed by conventional methods. Kanzo (Japanese) 51:319–321.

Kageyama S, Agdamag DM, Alesna ET, Leanō PS, Heredia AM, Abellanosa-Tac-An IP, Jereza LD, Tanimoto T, Yamamura J, Ichimura H. 2006. A natural inter-genotypic (2b/1b) recombinant of hepatitis C virus in the Philippines. J Med Virol 8:1423–1428.

Kalinina O, Norder H, Mukomolov S, Magnius LO. 2002. A natural intergenotypic recombinant of hepatitis C virus identified in St. Petersburg. J Virol 76:4034–4043.

Moradpour D, Penin F, Rice CM. 2007. Replication of hepatitis C virus. Nat Rev Microbiol 5:453–463.

Morel V, Fournier C, Francois C, Brochot E, Helle F, Duverlie G, Castelain S. 2011. Genetic recombination of the hepatitis C virus: Clinical implications. J Viral Hepat 18:77–83.

Neumann AU, Lam NP, Dahari H, Gretch DR, Wiley TE, Layden TJ, Perelson AS. 1998. Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. Science 282: 103–107.

Ogata N, Alter HJ, Miller RH, Purcell RH. 1991. Nucleotide sequence and mutation rate of H strain of hepatitis C virus. Proc Natl Acad Sci USA 88:3392–3396.

Okamoto H, Kojima M, Okada S, Yoshizawa H, Iizuka H, Tanaka T, Muchmore EE, Peterson DA, Ito Y, Mishiro S. 1992. Genetic drift of hepatitis C virus during a 8.2-year infection in a Chimpanzee: Variability and stability. Virology 190:894–899.

Seeff LB. Natural history of chronic hepatitis C. Hepatology 2002. 36:S35–S46.

Shirakawa H, Matsumoto A, Joshita S, Komatsu M, Tanaka N, Umemura T, Ichijo T, Yoshizawa K, Kiyosawa K, Tanaka E. 2008. Nagano Interferon Treatment Research Group. Pretreatment prediction of virological response to peginterferon plus ribavirin therapy in chronic hepatitis C patients using viral and host factors. Hepatology 48:1753–1760.

Takahashi K, Okamoto H, Abe N, Kawakami M, Matsuda H, Mochida S, Sakugawa H, Suginoshita Y, Watanabe S, Yamamoto K, Miyakawa Y, Mishiro S. 2009. Virulent strain of hepatitis E virus genotype 3, Japan. Emerg Infect Dis 15:704–709.

Yokoyama K, Takahashi M, Nishizawa T, Nagashima S, Jirintai S, Yotsumoto S, Okamoto H, Momoi MY. 2011. Identification and characterization of a natural inter-genotypic (2b/1b) recombinant hepatitis C virus in Japan. Arch Virol 156:1591–1601.

Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Possible Route of Transmission of Highly Pathogenic Avian Influenza Virus Type H5N1 in Family Poultry at Rural Bangladesh

M. S. I. Khan[1, 2]*, S. M. F. Akbar[3], S. T. Hossain[4], M. Mahatab[5], M. M. Hossain[2] and Z. Idrus[1]

[1]Institute of Tropical Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia; [2]Department of Animal Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh; [3]Department of Medical Science, Toshiba General Hospital, Tokyo 140-8522, Japan; [4]Friends In Village Development Bangladesh, Dhaka-1207, Bangladesh; [5]Department of Hepatology, Bangabandhu Sheikh Mujib Medical University, Dhaka-1000, Bangladesh
*Corresponding author: sakirul.khan@gmail.com; sakirul.khan@putra.upm.edu.my

ARTICLE HISTORY

Received: July 20, 2011
Revised: August 17, 2011
Accepted: September 03, 2011

Key words:
Avian influenza (H5N1)
Family poultry
Integrated farming
Risks of infection
Rural area

ABSTRACT

Highly pathogenic avian influenza virus type H5N1 represents one of the major causes of morbidity and mortality of poultry in both developed and developing countries. However, little is known about the transmission of this virus in developing countries that usually raise poultry as family-based farming. The study was conducted at 10 of total 64 administrative districts of Bangladesh that experienced H5N1 virus outbreaks since 2007. Trained field workers visited 30 rural families at each district to check family poultry management system. The collected data were transcribed and coded according to the standardized mutual performance of the field workers. Approximately two-third of farmers (67%) were rearing only chickens and remaining (33%) both chickens and ducks. Most of the farmers provided night shelter to their birds inside their living room (24%) or close proximate (69%). Usually ducks were scavenged in water land (58.6%) or paddy field (18.2%). The majority of owners (93%) also shared the same water land with migratory/wild birds for their daily necessity. The marketing system of poultry was characterized by comprehensive interactions among family poultry and commercial birds for prolonged duration. Unsold or newly bought birds were brought back to farmer's house in almost all instances (97.8%). Findings from this study indicated that interactions of domestic chickens and ducks with their owners (through contaminated agricultural and fisheries tools or clothing) are partially, if not solely, responsible for wide spread transmission of Avian influenza virus type H5N1.

To Cite This Article: Khan MSI, SMF Akbar, ST Hossain, M Mahatab, MM Hossain and Z Idrus, 2012. Possible route of transmission of highly pathogenic avian influenza virus type H5N1 in family poultry at rural Bangladesh. Pak Vet J, 32(1): 112-116.

INTRODUCTION

The highly pathogenic avian influenza (H5N1) virus has been detected in Asia, Europe and Africa that possess health risk to poultry and human. Since 2003, infection with H5N1 virus has caused natural death or killing of over 300 million poultry. Also, 330 human beings have died of this infection (World Bank, 2008; WHO, 2011). In addition, the impact of H5N1 virus on food security and providence of nutrition in developing countries is tremendous because main bulk of their populations depend on eggs and poultry meat as only reliable source of protein (Burgos and Burgos, 2007; Gueye, 2007; Sonaiya, 2007; Das *et al.,* 2008).

Bangladesh, an Asian country with more than 150 million people, has experienced outbreaks of H5N1 virus since 2007 (Biswas *et al,* 2008). Official and conservative estimates indicated that over 1.8 million birds have already died or killed due to H5N1 virus infection (MFL, 2011; OIE, 2011). A single human infection by H5N1 virus has also been reported in Bangladesh (ICDDR'B, 2008; Brooks *et al.,* 2009).

In contrast to developed countries that are mainly rearing industrialized poultry, family poultry represents one of the most important sub-sectors of livestock of Bangladesh. Studies have shown that about 80-90% of rural households keep poultry (Dolberg, 2008; Das *et al.*, 2008), and family poultry is raised by about 90% of total population of Bangladesh (Dolberg, 2008). These poultry

contribute the protein need of the country and also represents a sustainable source of income of villagers.

The infection of H5N1 virus has shown the susceptibility of family poultry population of Bangladesh to destruction and distortion in a short span of time. Although the rural poultry handlers are interested to know about possible steps to block transmission of similar outbreaks of infectious diseases, almost no study has been conducted at Bangladesh to address their queries. It is true that studies have been conducted in mainly developed countries about possible blocking of transmission cycle of infections agents (Sims *et al.*, 2005; Ellis *et al.*, 2006; Khan *et al.*, 2009; Martin *et al.*, 2010; Cecchinato *et al.*, 2011). However, raising of family poultry is endowed with specific features in each developing country on the basis of their socio-economic and cultural heritages.

Therefore, this survey was conducted at 10 of 64 districts of Bangladesh that were mainly affected by H5N1 virus infection since 2007. The salient features of this study were pooled to develop insights about family poultry management systems, particularly those related with transmission of H5N1 virus infection.

MATERIALS AND METHODS

The study was performed based on qualitative and quantitative survey. We employed ten field workers with basic backgrounds of poultry management and conducted observational survey. These persons were trained about our study objectives; (1) nature of poultry rearing at family levels, (2) interactions of poultry with other birds, (3) close observation of poultry marketing and (4) management of unsold birds.

The survey was undertaken at 10 of 64 districts of Bangladesh (Fig.1) based on their demographic variations in terms of H5N1 virus outbreaks. In each district, 30 households were randomly selected to observe the family poultry management system and related issues. They also visited at least 5 poultry markets in each district. They discussed with family poultry farmers and took interview to develop insights about family poultry management system at rural Bangladesh. Poultry marketing and livestock-crop mixed cultivation system (rice-duck) were also checked in each district as this have been reported to play an important role in the introducing and spreading of H5N1 virus in some countries (Gilbert *et al.*, 2006; 2008; Sims, 2007; Cecchi *et al.*, 2008; Hop and Saatkamp, 2010; Chantong and Kaneene, 2011; Henning *et al.*, 2011). The collected information were transcribed and coded according to the standardized mutual performance of the surveyors.

RESULTS

Table 1 shows the base line information about flock size and purpose of family poultry. Approximately, two-third of farmers (67%) were rearing only chickens and remaining (33%) both chickens and ducks. Depending on the socio economic condition, there was wide variation with regard to the number of birds per family. The flock size ranged from 4 to 23 (average flock size 10±0.28) was observed at the survey areas (Table 1). Among the farmers, 7%, 12% and 81% were rearing birds for the source of protein, income and income plus protein, respectively.

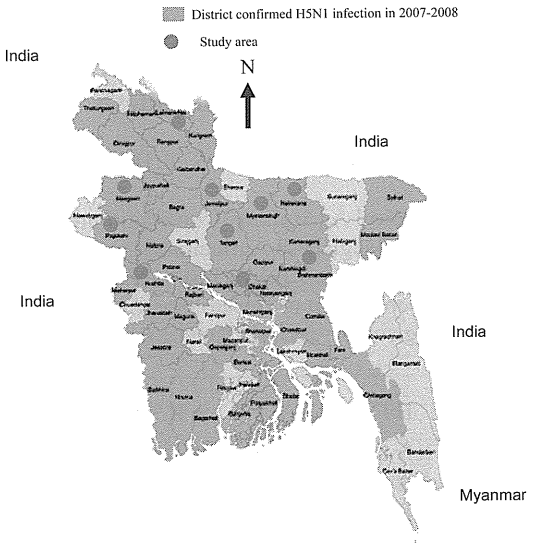

Fig. 1. Districts (administrative area) of Bangladesh selected for this study. The green circle indicated study area.

Table 1: Flock size and purpose of family poultry rearing

	No. of family (Total 300)	%
Types of birds		
Chicken	201	67.0
Chicken and duck	99	33.0
Number of birds		
≤ 5	21	7.0
6-10	182	60.7
11-15	75	25.0
≥ 16	22	7.3
Purpose of rearing		
Income	36	12
Source of protein	21	7
Income plus source of protein	243	81

The family members of rural farmers shared their bed room with the poultry. At night, the poultry were kept in a cage and put under their bed (24%) or an attachment (69%) where there was no biosecurity. This practice was observed in most of the farmer houses (93%) (Table 2). Some times, chickens and ducks were kept in the same cage. In others, there may be separate cages for chickens and ducks. Approximately three-quarters of farmers provided food to their birds on a daily basis that occurred inside the room or on verandas (Table 2).

During feeding of birds, no discrimination was made between healthy and sick birds. Most of the farmers (93.7%) gave food to them together. Usually, the children took part in poultry feeding. Also, the birds took food from the plates of the children at different times.

A large number of rural farmers (about 90%) were also using their living room for hatching of eggs. They sporadically put eggs under broody chickens or ducks and used them as hatcheries. Checking of hatching eggs and physical examination, feeding, cleaning droppings of broody birds occurred at unhygienic condition with no hand wash practices.

During day time, the chickens moved freely in the homestead areas of the farmers and nearby crop lands.

About 58.6% of farmers thought their ducks were mainly scavenging in the nearby ponds, rivers, lakes or other low lying areas of rural Bangladesh. Many migratory birds come to these areas in the winter season and about 15% farmers thought that there interactions among their ducks with migratory ones (Table 2 and 4). In addition, the rural farmers and their family members (92.3%) including female and children also used these watery areas for fishing, bathing, bathing of other domestic animals or other agricultural/aquacultural activities. Thus, rural life style favored an intimate contact among poultry, human and migratory birds. Another potential important place of interaction between chickens, domestic ducks and migratory birds was provided by mixed farming like chicken-duck-rice production system. About 18% farmers were practicing this farming system to allow the ducks to move freely in rice fields as pest controller (Table 2).

In the market, live birds were sold at minimal hygienic condition. The family poultry were put in close proximity of birds from commercial farms. The unsold or newly bought birds were brought back to framer's house or commercial farms at all instances (97.8%). It is a natural practice at rural Bangladesh to slaughter healthy or sick poultry and migratory/wild birds at their home with minimal hygienic condition within close proximity to the other birds and family members (Table 3). After the bird's throat is slit, it thrashes and its blood sprinkles all over the courtyard. The blood is not washed away, nor are the remains of the bird properly disposed off. Instead, almost all farmers (98%) were thrown away the uneatable portions of the birds to open place where wild birds, other poultry, dogs or cats may eat or children play with it.

DISCUSSION

Due to wide-spread outbreaks of H5N1 virus in early 21st century, some countries have banned family poultry farming to reduce infection (BBC News, 2007). However, considering the economical and social structures of comparatively improvised people of resource-restricted countries, it seems that this option would not contribute to prevent and control of avian influenza in Bangladesh. Rather, the effect may be counterproductive. On the contrary, attention should be given to find out ways and means to restrict or block infection of family poultry by H5N1 virus infection. In fact, public health approach to materialize this has not been well explored.

To draw a possible road map to block future infection of family poultry population by H5N1 virus or other similar infectious agent, it is needed to develop insights about ongoing practices of family poultry management at rural area. This study revealed that risks of H5N1 virus infection may increases at family poultry due to environmental, agro-ecological, physical, social and cultural factors. Avian influenza virus mostly spreads by direct contact with infected birds or contaminated feces, feeds, water, equipment, and clothing. Proper maintaining of bio-security measures at family poultry production level might be useful for stopping the spread of viruses. However, results from this study indicated that biosecurity, in its true sense, is neither possible nor feasible in almost all branches of family poultry production in Bangladesh.

We assume that migratory birds might be responsible to initial introduction of H5N1 virus into Bangladesh because the H5N1 virus isolates from domestic chicken

Table 2: General management of family poultry

	No. of family (Total 300)	%
Place of night shelter		
Inside the owner's living room	72	24.0
Close proximate to owner living room with no or minimal attachment	207	69.0
Separate place from owner house	21	7.0
Times to provide foods		
One time per day	197	65.7
Two times per day	21	7.0
Not regularly	82	27.3
Feeding systems		
Provide feed separately to healthy and sick birds	19	6.3
Provide feed together to healthy and sick birds	281	93.7
Chicken scavenging system		
Scavenge around homestead area	132	44.0
Scavenge around homestead area and nearby crop fields	168	56.0
Duck scavenging system (out of 99 farmers)		
Scavenge around homestead area	0	0.0
Scavenge around homestead area and nearby crop fields	8	8.1
Scavenge nearby ponds, river and wet land area	58	58.6
Scavenge paddy field as weed and pest controller	18	18.2
Scavenge long distance and interact with migratory birds	15	15.1

Table 3: Poultry marketing and slaughtering systems

	No. of family (Total 300)	%
Live bird marketing system		
Marketing at home	21	7.0
Marketing at local market within vicinity	279	93.0
Unsold or newly bought poultry are back to home and keep in same box/sheds with other birds	293	97.8
Place to slaughter		
In side home	253	84.3
Outside home	47	15.7
Method to dispose of feathers and uneatable portion after slaughtering		
Buried in soil	5	1.7
Through to home garden	136	45.3
Through to open pit	103	34.3
Through to nearby pond	56	18.7

Table 4: Information about interaction with migratory birds

	No. of family (Total 300)	%
Have you seen the domestic ducks shared the same water land with migratory/wild birds		
Yes	47	15.7
No	253	84.3
You or any of your family members shared the same water land with migratory birds for bathing, washing cloth or fishing		
Yes	277	92.3
No	23	7.7
You or any of your family members catch the migratory birds for eating or entertainment		
Yes	13	4.3
No	287	95.7

are close to those from Mongolia and Russia (Biswas *et al.,* 2008). In fact, birds from Russia and Mongolia migrate to Bangladesh in winter season (Dolberg, 2008). Indeed, Bangladesh does not have any direct poultry trade with these countries; indicating that virus from migratory birds transmitted to poultry population of Bangladesh.

Above assumption was supported by the data from this study. More than 90% of rural poultry owners visited the same scavenging place of migratory/wild birds for their daily necessity (Table 4). About 18% farmers thought that their domestic ducks also have interaction with migratory birds either in watery area or paddy field (Table 2). These people and ducks share the living room or close proximate with no or minimal attachment with chickens indicating that either family members (especially when agricultural and fisheries tools or clothing are contaminated) or domestic ducks introduce viruses from migratory birds to farmers house. Subsequently, the virus may be transmitted to chicken, as they have very close and frequent interaction. Actually, special indication to free scavenging domestic ducks because they were infected with avian influenza virus and showed few clinical signs of the disease (Hulse *et al.*, 2005) but capable to shedding appreciable amounts of virus (Gilbert *et al.*, 2006; 2008; Henning *et al.*, 2011).

The prevailing marketing system of poultry might also be responsible for transmission of viruses. This system is characterized by comprehensive interactions among birds (chicken and ducks) for prolonged duration and unsold or newly bought birds were returned to farmer's house at all instances (97.8%). Moreover, the usual process of slaughtering healthy or sick birds was unhygienic that mostly practiced inside the residence (84.3%).

Findings from this study suggested that the viral circulation into poultry population might be facilitated by the interactions of the integrated agriculture which relies in the integration of farmer-livestock-fisheries-crop production in the presence of domestic ducks scavenging with migratory birds and by the connections with the live-bird marketing and home slaughtering at minimal hygienic condition. Thus, it is predicted that poultry and people might be easily infected by each others due to inadequate knowledge, awareness and information of rural farmers about zoonosis.

However, there are some limitations of this study. We could not show a direct evidence to support that domestic ducks or other animals as well as the members of famer's family transmitted the viruses from migratory birds to poultry population at Bangladesh by analyzing the viral genome. In fact, this observational study was planned to provide insights about importance of public health measures to control future H5N1 virus infection in poultry. If the rural farmers are provided with adequate knowledge about natural reservoir of viruses as well as danger of infection to their poultry, the present biosecurity condition may be improved. Another notable factor is to develop legal measure to block return of unsold poultry from markets. A stock pile of poultry reservoir may be developed at each market or designated market to preserve the unsold poultry. These unsold poultry may be sold in another day by a cooperative society. On the other word, the poultry can be bought by a marketing society on a daily basis to block return of unsold poultry.

In conclusion, this observation study, although far from drawing a conclusive conclusion, represents one of the first approaches to develop insights about rural farmer-based poultry development at Bangladesh. Some of these features may be shared by other developing countries. Analyses of this study may unveil a method to protect poultry from H5N1 virus infection.

REFERENCES

BBC NEWS, 2007. Backyard poultry ban in Jakarta. http://news.bbc.co.uk/2/hi/asia-pacific/6269941.stm. Accessed on August 14, 2011.

Biswas PK, JP Christensen, SS Ahmed, H Barua, A Das, MH Rahman, M Giasuddin, AS Hannan, MA Habib, A Ahad, AS Rahman, R Faruque and NC Debnath, 2008. Avian influenza outbreaks in chickens, Bangladesh. Emerg Infect Dis, 14: 1909-1912.

Brooks WA, AS Alamgir, R Sultana, MS Islam, M Rahman, AM Fry, B Shu, S Lindstrom, K Nahar, D Goswami, MS Haider, S Nahar, E Butler, K Hancock, RO Donis, CT Davis, RU Zaman, SP Luby, TM Uyeki and M Rahman, 2009. Avian influenza virus A (H5N1), detected through routine surveillance, in child, Bangladesh. Emerg Infect Dis, 15: 1311-1313.

Burgos S and SA Burgos, 2007. Avian influenza outbreaks in southeast asia affects prices, markets and trade: A short case study. Int J Poult Sci, 6: 1006-1009.

Cecchi G, A Ilemobade, Y Le Brun, L Hogerwerf and J Slingenbergh, 2008. Agro-ecological features of the introduction and spread of the highly pathogenic avian influenza (HPAI) H5N1 in northern Nigeria. Geospat Health, 3: 7-16.

Cecchinato M, A Comin, L Bonfanti, C Terregino, I Monne, M Lorenzetto and S Marangona, 2011. Epidemiology and control of low pathogenicity avian influenza infections in rural poultry in Italy. Avian Dis, 55: 13-20.

Chantong W and JB Kaneene, 2011. Poultry raising systems and highly pathogenic avian influenza outbreaks in Thailand: the situation, associations, and impacts. Southeast Asian J Trop Med Public Health, 42: 596-608.

Das SC, SD Chowdhury, MA Khatun, M Nishibori, N Isobe and Y Yoshimura, 2008. Poultry production profile and expected future projection in Bangladesh. Worlds Poult Sci J, 64: 99-118.

Dolberg F, 2008. Poultry sector country review: Bangladesh. FAO Animal Production and Health Division. ftp://ftp.fao.org/docrep/fao/011/ai319e/ai319e00.pdf. Accessed on August 14, 2011.

Ellis TM, LD Sims, HK Wong, CW Wong, KC Dyrting, KW Chow, C Leung and JS Peiris, 2006. Use of avian influenza vaccination in Hong Kong. Dev Biol (Basel), 124: 133-143.

Gilbert M, X Xiao, DU Pfeiffer, M Epprecht, S Boles, C Czarnecki, P Chaitaweesub, W Kalpravidh, PQ Minh, MJ Otte, V Martin and J Slingenbergh, 2008. Mapping H5N1 highly pathogenic avian influenza risk in Southeast Asia. Proc Natl Acad Sci USA, 105: 4769-4774.

Gilbert M, T Chaitaweesub, T Parakamawongsa, S Premashthira, T Tiensin, W Kalpravidh, H Wagner and J Slingenbergh, 2006. Free-grazing ducks and highly pathogenic avian influenza Thailand. Emerg Infect Dis, 12: 227-234.

Gueye EF, 2007. Evaluation of the impact of HPAI on family poultry production in Africa. Worlds Poult Sci J, 63: 391-400.

Henning J, KA Henning, JM Morton, NT Long, NT Ha, T Vu le, PP Vu, DM Hoa and J Meers, 2011. Highly pathogenic avian influenza (H5N1) in ducks and in-contact chickens in backyard and smallholder commercial duck farms in Viet Nam. Prev Vet Med, 101: 229-240.

Hop GE and HW Saatkamp, 2010. A PathWayDiagram for introduction and prevention of avian influenza: Application to the Dutch poultry sector. Prev Vet Med, 97: 270-273.

Hulse-Post DJ, KM Sturm-Ramirez, J Humberd, P Seiler, EA Govorkova, S Krauss, C Scholtissek, P Puthavathana, C Buranathai, TD Nguyen, HT Long, TS Naipospos, H Chen, TM Ellis, Y Guan, JS Peiris and RG Webster, 2005. Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. Proc Natl Acad Sci USA, 102: 10682-10687.

Khan SI, SM Akbar, ST Hossain and MA Mahtab, 2009. Swine influenza (H1N1) pandemic: developing countries' perspective. Rural Remote Health, 9: 1262.

Martin R, A Conseil, A Longstaff, J Kodo, J Siegert, AM Duguet, P Lobato de Faria, G Haringhuizen, J Espin and R Coker, 2010. Pandemic influenza control in Europe and the constraints resulting from incoherent public health laws. BMC Public Health, 3: 532-542.

OIE-Office International des Epizootics, World Organisation for Animal Health, 2011. Follow-up report No: 33. Report reference: OIE Ref: 10572, Country: Bangladesh. http://web.oie.int/wahis/reports/en_fup_0000010572_20110512_154018.pdf. Accessed on August 14, 2011.

Sims LD, J Domenech, C Benigno, S Kahn, A Kamata, J Lubroth, V Martin and P Roeder, 2005. Origin and evolution of highly pathogenic H5N1 avian influenza in Asia. Vet Rec, 157: 159-164.

Sims LD, TM Ellis, K Liu, K Dyrting, H Wong, M Peiris, Y Guan and KF Shortridge, 2003. Avian influenza in Hong Kong 1997-2002. Avian Dis, 47: 832-838.

Sims LD, 2007. Experience in control of avian influenza in Asia. Dev Biol (Basel), 130: 39-43.

Sonaiya EB, 2007. Family poultry, food security and the impact of HPAI. Worlds Poult Sci J, 63: 132-138.

The World Bank, 2008. Avian and human pandemic influenzas update. http://siteresources.worldbank.org/INTTOPAVIFLU/Resources/OPCS_AvianInfluenzaIssueBrief_Dec14_2007.pdf. Accessed on August 14, 2011.

WHO-The World Health Organization, 2011. Cumulative number of confirmed human cases of avian influenza A/ (H5N1) reported to WHO. http://www.who.int/csr/disease/avian_influenza/country/cases _table_2011_08_09/en/index.html. Accessed on August 14, 2011.

Hepatology Research

Hepatology Research 2012; 42: 870–878 doi: 10.1111/j.1872-034X.2012.01006.x

Original Article

Three cases of acute or fulminant hepatitis E caused by ingestion of pork meat and entrails in Hokkaido, Japan: Zoonotic food-borne transmission of hepatitis E virus and public health concerns

Kencho Miyashita,[1,3] Jong-Hon Kang,[2] Akiyoshi Saga,[3] Kazuaki Takahashi,[5] Tsuyoshi Shimamura,[4] Atsushi Yasumoto,[1] Hiraku Fukushima,[1] Susumu Sogabe,[1] Kouhei Konishi,[1] Takumi Uchida,[1] Akira Fujinaga,[1] Takeshi Matsui,[2] Yasuo Sakurai,[2] Kunihiko Tsuji,[2] Hiroyuki Maguchi,[2] Masahiko Taniguchi,[4] Natsumi Abe,[5] Sheikh Mohammad Fazle Akbar,[5] Masahiro Arai[5] and Shunji Mishiro[5]

[1]*Department of Gastroenterology, Abashiri Kosei General Hospital, Abashiri,* [2]*Center for Gastroenterology, Teine Keijinkai Hospital,* [3]*Department of Gastroenterology, Aiiku Hospital,* [4]*Department of General Surgery, Graduate School of Medicine, Hokkaido University, Sapporo, and* [5]*Department of Medical Sciences, Toshiba General Hospital, Tokyo, Japan*

Aim: In developed countries including Japan, the transmission route of indigenous hepatitis E virus (HEV) infection is obscure. Accordingly, public health implications of indigenous HEV infection have not been well addressed. The aim of this study was to clarify the route of transmission of a small outbreak of acute hepatitis E and assess the public health implications of indigenous zoonotic HEV transmission.

Methods: Three patients with non-A, B and C acute hepatitis, two of whom presented in a critical condition, were assessed for HEV infection using polymerase chain reaction and their route of infection; the genome sequences of the infecting HEV were also analyzed. A phylogenetic tree based on the full, or near full, HEV RNA sequences were constructed by neighbor-joining method.

Results: All three patients ingested grilled pork meat and entrails at the same barbecue restaurant in Abashiri, Hokkaido, Japan. When comparing partial to entire, or nearly entire, nucleotide sequences of HEV detected in these patients, they were 99.9–100% identical to each other. These genotype 4 isolates had great resemblance to the genome sequences of the isolates from the mini-outbreak in 2004 in Kitami, a city adjacent to Abashiri. These Kitami/Abashiri strains were segregated into a single cluster on the phylogenetic tree of HEV genotype 4 indigenous to Japan.

Conclusion: Indigenous HEV transmission via a zoonotic food-borne route has been demonstrated in Kitami and Abashiri via pork meat and entrails contaminated with virulent HEV strains. Because a similar outbreak can recur in the future, infection sources and distribution routes should be clarified rapidly for public health.

Key words: fulminant hepatitis, genotype 4, hepatitis E, Kitami/Abashiri strains, zoonosis

INTRODUCTION

EVIDENCE NOW SHOWS that the hepatitis E virus (HEV) infection is no longer confined to developing countries. HEV transmission routes specific to industrialized societies have been eagerly investigated.[1–5] These studies have shown a zoonotic transmission of HEV from ingestion of the meat of deer, wild boars and pigs in industrialized countries.[3,6–9] It is unclear, however, whether these instances of zoonotic HEV transmission have occurred randomly. Mini-outbreaks of HEV infection may inevitably repeat themselves even in developed countries, especially if the society concerned has a history of zoonotic transmission specific to the regional livestock cultivation and distribution industries.

Correspondence: Dr Jong-Hon Kang, Center for Gastroenterology, Teine Keijinkai Hospital Maeda, 1-12 Teine-ku, Sapporo 006-8555, Japan. Email: kanjh@keijinkai.or.jp

Conflict of interest: none.

Received 18 October 2011; revision 6 March 2012; accepted 13 March 2012.

A mini-outbreak of acute HEV infection in Abashiri, a city in the northern Japanese prefecture of Hokkaido, has occurred. In the course of the investigation for the transmission route of indigenous HEV, attention has been given to a similar mini-outbreak of hepatitis E occurring 2 years previously at Kitami, another city situated 50 km from Abashiri.[8,9] This finding has provided an opportunity to address the public health implications of sporadic community-acquired cases of HEV infection in Japan by comparing viral sequences of Abashiri's acute HEV patients with those of Kitami's patients.

In the present study, the route of transmission in a mini-outbreak of acute hepatitis E is clarified, and the public health implications of indigenous zoonotic HEV transmission in Japan is also investigated.

METHODS

Patients

THREE PATIENTS EXHIBITING the features of non-A, B and C acute hepatitis presented in Hokkaido, Japan. Although different physicians treated the patients at different institutions, all of them ate pork meat and entrails together at a barbecue party in a restaurant at Abashiri on 1 February 2006. The ingested pork meat and entrails were grilled by gas cookers during concurrent alcohol consumption. It was unclear whether they were thoroughly cooked or not.

The clinical profiles of the patients are shown in Table 1. Serum anti-hepatitis B core antigen immunoglobulin (Ig)M, hepatitis C virus RNA, and anti-hepatitis A IgM were not detected in any patient although patient

Table 1 Characteristics and clinical features in patients with acute HEV infection

Patient characteristics, laboratory data and outcome	Patient number		
	1	2	3
Age (years)/sex	53/male	58/male	56/male
Symptoms	Jaundice, malaise	Appetite loss	Fever
Estimated time of infection	1 February	1 February	1 February
Time of onset	Fourth week of March	First week of March	First week of March
Underlying liver disease†	None	None	Inactive HBV carrier
Alcohol intake	30 g/day, 33 years	30 g/day, 20 years	30 g/day, 30 years
Peak AST (IU/L)	297	9045	3266
Peak ALT (IU/L)	929	5297	4468
Peak total bilirubin (mg/dL)	12.6	2.6	10.3
Lowest prothrombin time (%), INR	74.0, 1.25	38.0, 2.04	16.6, 4.82
HEV RNA/genotype	+/4	+/4	+/4
Anti-HEV IgG/OD‡ value	+/2.170	+/2.878	+/1.761
Anti-HEV IgM/OD value	+/3.118	+/2.878	+/1.761
Anti-HAV IgM	<0.8	<0.8	<0.8
HBsAg (IU/mL)	0.01	0.05	10.74
Anti-HBc IgM	1.7	<0.09	<0.09
HBV DNA (log copies/mL)	<2.6	n.t.§	<2.6
Anti-HCV (U)	<1.0	<1.0	<1.0
HCV RNA (IU/mL)	<50	<50	<50
Disease progression	Self-limited hepatitis	Acute severe hepatitis¶	Fulminant hepatitis††
Outcome	Survived	Survived	Survived

Normal range: AST, aspartate aminotransferase (10–40 U/L); ALT, alanine aminotransferase (5–45 U/L); total bilirubin (0.2–1.0 mg/dL); prothrombin time (80–100%, 0.84–1.14); anti-HEV IgG, hepatitis E virus immunoglobulin G (<0.191); anti-HEV IgM (<0.447); anti-HAV, hepatitis A virus IgM (<0.8); HBsAg, hepatitis B surface antigen (<0.06); anti-HBc, hepatitis B core IgM (<0.9); HBV, hepatitis B virus DNA (<2.6 log copies/mL); anti-HCV, hepatitis C virus (<1.0); HCV RNA (<50 IU/mL).
†Underlying liver disease defined as disease which had been diagnosed before onset of hepatitis E.
‡"OD", optical density.
§"n.t.", not tested.
¶"acute severe hepatitis" defined as hepatitis with ≤40% in lowest prothrombin time without hepatic coma.
††"fulminant hepatitis" defined as hepatitis with hepatic coma within 8 weeks after onset.

3 had hepatitis B surface antigen. The clinical courses of these patients differed considerably with patient 1's showing a self-resolving course, patient 2's presenting with serious coagulopathy due to severe hepatitis and patient 3's developing fulminant hepatitis with recovery within 3 months after hospital admission.

Among the nearly 40 customers having eaten the grilled pork meat and entrails at the same restaurant in Abashiri on the same day, 11 of them (nine men and two women) voluntarily agreed to be checked for HEV. The biochemistry, anti-HEV IgM and IgG, and HEV RNA were checked using blood sample collections at 25–29 weeks after the dinner on 1 February 2006.

Informed consent was obtained from all patients and volunteers after explaining the nature and purpose of the study; approval for this study was obtained from the hospital's institutional review board. The study protocol conformed to guidelines provided in the Declaration of Helsinki for clinical trials.

Detection of HEV-related antibodies

Serum anti-HEV IgG and IgM were determined by using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Viragent HEV-Ab; Cosmic, Tokyo, Japan).

Detection of the full-length sequences of HEV isolates

Detection and nucleotide sequencing of the serum HEV RNA were performed by methods described previously.[10,11] Briefly, the nucleic acids were extracted from the serum with commercial kits (Smitest EX-R&D; Genome Science, Fukushima, Japan). The nucleotide sequences of HEV were reverse transcribed to cDNA and amplified by polymerase chain reaction (PCR) in 17 overlapping regions with 20-mer primers deduced from the nucleotide sequences of HEV deposited in the international DNA Data Bank of Japan (DDBJ)/GenBank/European Molecular Biology Laboratory (EMBL) database. Reverse transcription was performed, and the first and second round of PCR was carried out in the presence of Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA). The final products were sequenced in a 377 DNA sequencer. The sequences rich in G-C were amplified, and those not amplifiable by the above PCR methods were subjected to PCR with primers deduced from adjacent 5′- and 3′-sequences. The 5′- and 3′-terminal sequences were amplified with 5′-Full RACE Core Set (TaKaRa Bio, Shiga, Japan) and Oligo (dt) 20 primer (Invitrogen), respectively.

Phylogenetic analyses of HEV isolates

A phylogenetic tree based on the full, or nearly full, HEV RNA sequence was constructed by the neighbor-joining method. Analyses were performed with the use of computer software (GENETYX-MAC ver. 13.0; Genetyx, Tokyo, Japan).

RESULTS

Diagnosis of acute HEV infection in 4 patients

SERUM HEV RNA was detected in all three patients in the early phases of hepatitis. Given the clinical presentations, absence of markers of acute hepatitis A, B and C, and the presence of HEV RNA and anti-HEV, these patients were diagnosed with acute hepatitis E (Table 1) infection. In addition, among the 11 volunteers who attended the same barbecue party, a 51-year-old man who was a colleague of patient 3 showed an elevation of both serum anti-HEV IgG and IgM; he, however, had normal alanine aminotransferase levels and no HEV RNA by PCR for which he was, therefore, diagnosed with an asymptomatic HEV infection. None of the four patients above dined together again after 1 February 2006.

Similarity of nucleotide sequences of the HEV in three patients with acute hepatitis E in Abashiri

Full-length sequences in 7255 nucleotides were determined for genotype 4 HEV from patient 2 (JMM-Aba06C) and patient 3 (JKO-Aba-FH06C). Also, partial nucleotide sequences containing 432 nucleotides in the replicase region and 1136 nucleotides in the ORF2 region of HEV were analyzed for patient 1 (JKU-Aba06). Comparison of the full-length sequence between JMM-Aba06C and JKO-Aba-FH06C revealed only six nucleotide differences out of 7255 nucleotides with a sequence homology of 99.92% (Table 2). In addition, when these three isolates were compared among each other for 1568 nucleotide sequences, JKU-Aba06 (patient 1) showed 100% identicalness with JKO-Aba-FH06C (patient 3) and 99.94% with JMM-Aba06C (patient 2). The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences of HEV isolates were AB291967–70.

Table 2 Nucleotide sequence analyses for the hepatitis E virus strains from the mini-outbreaks in Kitami and Abashiri

Isolate names/regions	Overlap match (%) where mini-outbreak occurred				
	JKU-Aba06	**JMM-ba06C**	**JKO-Aba-FH06**	HRC-HE14C	JTC-Kit-FH04L
JKU-Aba06/Abashiri					
JMM-Aba06C/Abashiri	99.94				
JKO-Aba-FH06C/Abashiri	100	99.92			
HRC-HE14C/Kitami	99.11	99.38	99.43		
JTC-Kit-FH04L/Kitami	98.92	99.43	99.49	99.86	
JST-Kit04C/Kitami	99.11	99.38	99.43	99.99	99.99

Full, or nearly full, length nucleotide sequences were determined and compared to each other except for JKU-Aba06.
Among JKU-Aba06 and 5 other strains, 1568 nucleotide sequences containing 432 nucleotides in the replicase region and 1136 nucleotides in ORF2 were compared.
Bold letters represents the strains separated in Kitami and Abashiri regions in Hokkaido Prefecture, Japan.

Close relationship of HEV genome between mini-outbreaks in Abashiri in 2006 and Kitami in 2004

To assess any relationship between HEV isolates of the present mini-outbreak in Abashiri in 2006 with those in Kitami in 2004, partial or entire genome sequences of Abashiri isolates such as JKU-Aba06, JMM-Aba06C and JKO-Aba-FH06C were compared with Kitami isolates HRC-HE14C, JTC-Kit-FH04 and JST-Kit04C.[8,9] JMM-Aba06C and JKO-Aba-FH06C showed 99.38–99.49% identicalness with HRC-HE14C, JTC-Kit-FH04L and JST-Kit04C (Table 2). These Abashiri isolates, JMM-Aba06C and JKO-Aba-FH06C, revealed merely 37 or 41 nucleotide differences from JTC-Kit-FH04L and 41 or 45 nucleotide differences with JST-Kit04C. The chronological intervals of blood sampling were 513–538 days in these two mini-outbreaks.

Persistence of similar HEV genome at Kitami and Abashiri

In addition to the mini-outbreak of acute hepatitis E at Kitami in 2004, sporadic cases of the same disease were also reported around Kitami and Abashiri. A comparison of the entire, or nearly entire, nucleotide sequences of the HEV genomes of the seven isolates separated in Kitami and Abashiri and one isolate identified in Monbetsu (70 km from Kitami and 90 km from Abashiri) was made (Table 3). Two HEV strains, HE-JA36 and HE-JA41, were isolated from the patients in Kitami, and HE-JA28 was separated from the patient in Monbetsu (H. Okamoto, Jichi Medical School, Tochigi, Japan, pers. comm.). The data demonstrated 99.3–99.9% homology among these eight isolates. The constructed phylogenetic tree based on the full-length genome analysis confirmed that strains obtained in Kitami and Abashiri belonged to genotype 4 indigenous to Japan. A single cluster segregated from adjacent strains separated in other parts of Hokkaido, Japan (Fig. 1b) was gathered. The Kitami/Abashiri strains showed only 84–88.3% sequence homology with HEV genotype 4 isolates detected from other regions in Japan (Fig. 1b, Table 3). The genome sequence of case 1 was not included in this phylogenetic tree because only partial sequences of the HEV genome were done.

Absence of nucleotide substitution at nucleotide 1816 and 3148 in Kitami/Abashiri strains responsible for fulminant hepatitis

Because nucleotide substitution at nucleotide 1816 and 3148 in genotype 4 HEV was reported to be significantly associated with fulminant hepatitis,[12] nucleotide sequences were compared in JKO-Aba-FH06C and JTC-Kit-FH04L. By analyzing full-length genome sequences, both T at nt 1816 and C at nt 3148 were observed in JKO-Aba-FH06C and JTC-Kit-FH04L.

DISCUSSION

TO DATE, SPORADIC indigenous hepatitis E infections have been reported throughout the industrialized world.[20–24] The transmission routes of HEV in these countries remain obscure although a zoonotic foodborne route has been shown in several instances ascertained molecularly.[3,7,9] Autochthonous HEV infection, therefore, seems to be a growing public health concern even in developed countries.

Hepatitis E virus isolates identified from the three symptomatic acute hepatitis E patients who had eaten

Table 3 Comparison of entire-length nucleotide sequences in Kitami/Abashiri strains with those in other HEV isolates

Genotype	Isolate name	Accession no.	Host	Diagnosis	Collection date (year/month/day)	Habitat (city, prefecture, country)	Nucleotide length	Nucleotide JKO-Aba-FH06C	Identity (%) JMM-Aba06C	Reference
4	**JKO-Aba-FH06C**	**Current study**	**Human**	**FH**	**2006/3/10**	**Abashiri, Hokkaido, Japan**	**7255**		**99.9**	**Current study**
4	**JMM-Aba06C**	**Current study**	**Human**	**AH**	**2006/3/9**	**Abashiri, Hokkaido, Japan**	**7255**	**99.9**		**Current study**
4	**HE-JA36**	**AB220977**	**Human**	**AH**	**2004/1/6**	**Kitami, Hokkaido, Japan**	**7266**	**99.5**	**99.4**	**Inoue *et al.*[12]**
4	**HRC-HE14C**	**AB291965**	**Human**	**Blood Donor**	**2004/9/20**	**Kitami, Hokkaido, Japan**	**7255**	**99.4**	**99.4**	**Matsubayashi *et al.*[9]**
4	**JST-KitAas04C.**	**AB291966**	**Human**	**AH**	**2004/10/12**	**Kitami, Hokkaido, Japan**	**7255**	**99.4**	**99.3**	**Matsubayashi *et al.*[9]**
4	**JTC-Kit-FH04L**	**AB291959**	**Human**	**FH**	**2004/9/24**	**Kitami, Hokkaido, Japan**	**7209**	**99.4**	**99.3**	**Matsubayashi *et al.*[9]**
4	**HE-JA28**	**AB220976**	**Human**	**AH**	**2002/12/13**	**Monbetsu, Hokkaido, Japan**	**7266**	**99.4**	**99.4**	**Inoue *et al.*[12]**
4	**HE-JA41**	**AB220979**	**Human**	**AH**	**2004/8/17**	**Kitami, Hokkaido, Japan**	**7265**	**99.4**	**99.3**	**Inoue *et al.*[12]**
4	JSM-Sap95	AB161717	Human	AH	1995/3/28	Sapporo, Hokkaido, Japan	7202	98.3	98.2	Takahashi *et al.*[13]
4	HE-JF4	AB220972	Human	FH	2002/10/2	Sapporo, Hokkaido, Japan	7271	97.6	97.5	Inoue *et al.*[12]
4	JKK-Sap	AB074917	Human	AHS	2000/11/10	Sapporo, Hokkaido, Japan	7235	97.6	97.5	Takahashi *et al.*[11]
4	JTS-Sap02	AB161718	Human	AH	2002/9/14	Sapporo, Hokkaido, Japan	7202	97.5	97.5	Takahashi *et al.*[11]
4	JYW-Sap02	AB161719	Human	AH	2002/8/30	Sapporo, Hokkaido, Japan	7202	97.5	97.5	Takahashi *et al.*[11]
4	HE-JF5	AB220973	Human	FH	2002/12/2	Sapporo, Hokkaido, Japan	7270	97.5	97.5	Inoue *et al.*[12]
4	HE-JA19	AB220975	Human	AH	2002/12/24	Sapporo, Hokkaido, Japan	7262	97.3	97.3	Inoue *et al.*[12]
4	HE-JA37	AB220978	Human	AH	2004/1/30	Sapporo, Hokkaido, Japan	7281	97.1	97	Inoue *et al.*[12]
4	HE-JA1	AB097812	Human	AH	1997/12/6	Sapporo, Hokkaido, Japan	7258	88.4	88.4	Nishizawa *et al.*[14]
4	HE-JF3	AB220971	Human	FH	1998/6/19	Mito, Ibaraki, Japan	7262	88.3	88.2	Inoue *et al.*[12]
4	swJ13-1	AB097811	Swine			Hokkaido, Japan	7258	88.3	88.3	Nishizawa *et al.*[14]
4	JSN-Sap-FH02C	AB20239	Human	FH	2002/3/21	Sapporo, Hokkaido, Japan	7251	88.1	88.1	Takahashi *et al.*[15]
4	JYN-Sap01C	AB193177	Human	AH	2001/12/28	Sapporo, Hokkaido, Japan	7256	88.1	88.1	Takahashi *et al.*[15]
4	HE-JK4	AB099347	Human	AH	2002/4/25	Tochigi, Japan	7250	88.1	88	Kuno *et al.*[16]
4	JYN-Nii02L	AB193178	Human	AH	2002/4/30	Niigata, Japan	7154	88.1	88	Takahashi *et al.*[11]
4	JAK-Sai	AB074915	Human	AH		Saitama, Japan	7236	88.1	88	Takahashi *et al.*[13]

4	JSF-Tot03C	AB193176	Human	FH	2003/3/12	Tottori, Japan	7251	88	88	Takahashi *et al.*[15]
4	swJB-H7	AB481227	Swine			Japan	7253	87.8	87.8	
4	HE-JI4	AB080575	Human	AH	1905/6/22	Tochigi, Japan	7186	87.4	87.3	Takahashi *et al.*[17]
4	HE-JA2	AB220974	Human	AH	1998/9/4	Sapporo, Hokkaido, Japan	7268	86.7	86.6	Inoue *et al.*[12]
4	CCC220	AB108537	Human	AH	2000/6	Changchun, Jilin, China	7193	85.4	85.4	Liu *et al.*[18]
4	E087-SAP04C	AB369688	Human	AH		Sapporo, Hokkaido, Japan	7227	85.2	85.2	
4	IND-SW-00–01	AY723745	Swine			India	7262	84.6	84.6	
4	E067-SIJ05C	AB369690	Human	AH		Shinjuku, Tokyo, Japan	7236	84.5	84.4	
4	swCH31	DQ450072	Swine			China	7248	84.3	84.3	
4	SH-SW-zs1	EF570133	Swine			China	7293	84.3	84.2	
4	swCH25	AY594199	Swine			Xinjian, China	7270	84.2	84.2	
4	T1	AJ272108	Human			Beijing, China	7232	84.2	84.2	
4	KNIH-hHEV4	FJ763142	Human	AH		South Korea	7260	84.1	84.1	
4	swCH189	FJ610232	Swine			Gansu, China	7284	84.1	84.1	
4	swGX40	EU676178	Swine			China	7267	84	84	
4	JYI-ChiSai01C	AB197674	Human	AH	2001/4/12	Shanghai, China	7260	84	84.4	Koike *et al.*[19]
4	JKO-ChiSai98C	AB197673	Human	AH	1998/10/27	Xian, Shaanxi, China	7257	84	83.9	Koike *et al.*[19]
4	HEVN2	AB253420	Human			Okinawa, Japan	7253	84	84	
4	Ch-S-1	EF077630	Human			China	7261	83.9	83.8	
4	swGX32	EU366959	Swine			China	7281	83.8	83.8	
4	DQ1	DQ279091	Swine			China	7234	83.8	83.7	
1	B1	M73218	Human			Burma	7207	75.4	75.3	
3	US1	AF060668	Human			USA	7202	75.3	75.3	Schlauder *et al.*[20]
2	M1	M74506	Human			Mexico	7180	73.9	73.9	

Bold letters represents the strains separated in Kitami and Abashiri regions in Hokkaido Prefecture, Japan.
AH, acute hepatitis; ASH, acute severe hepatitis; FH, fulminant hepatitis.

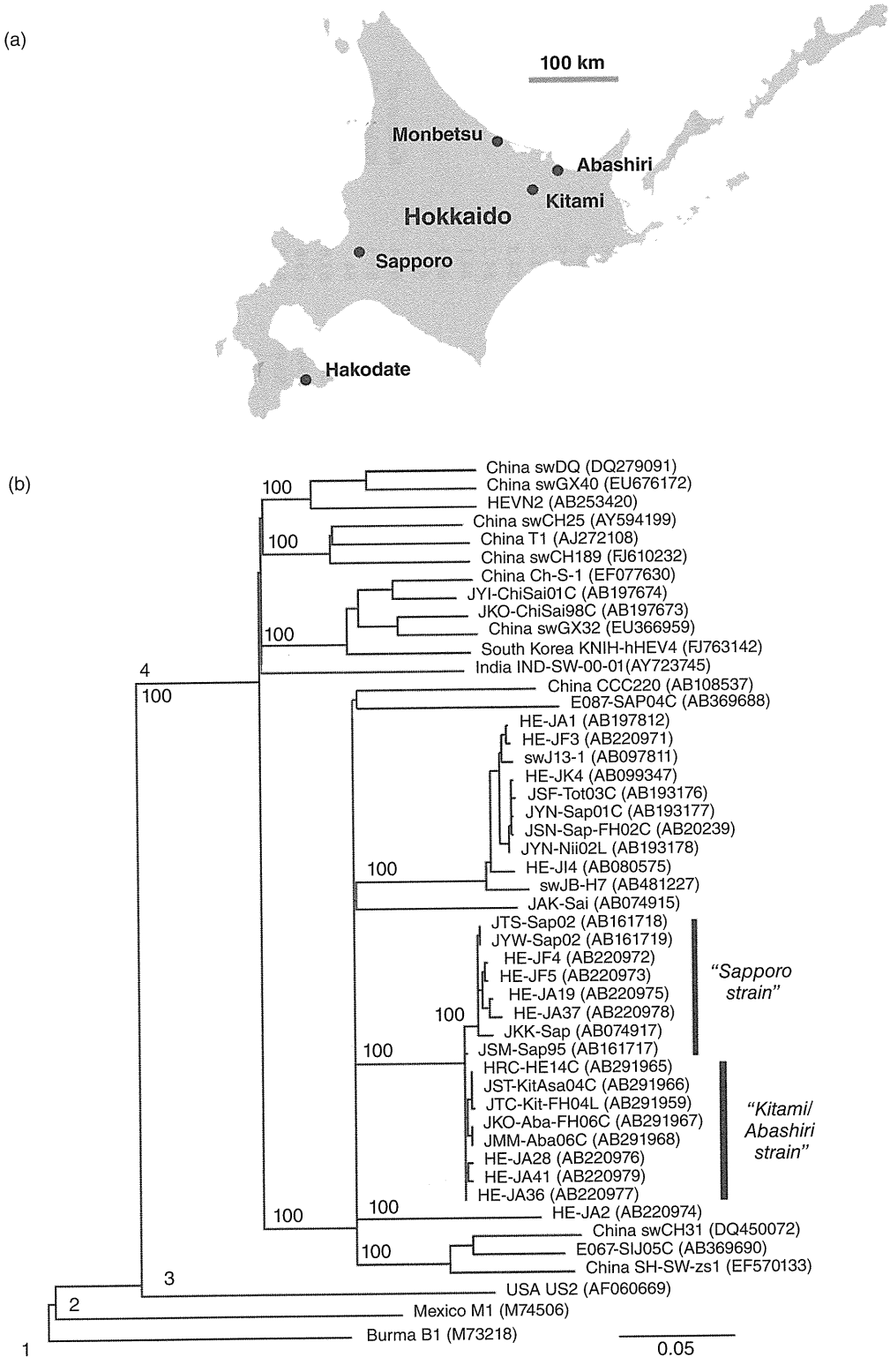

Figure 1 (a) Three symptomatic and one asymptomatic patient with acute infection of hepatitis E virus (HEV) attended the same barbecue restaurant in Abashiri, Hokkaido, Japan. Among them, two were admitted in Sapporo and one in Abashiri. (b) Phylogenetic tree based on the full, or nearly full, length nucleotide sequence of HEV genotype 4 by neighbor-joining method. Two of the three isolates, JKO-Aba-FH06 and JMM-Aba06C, analyzed in this study were segregated into a unique cluster ("Kitami/Abashiri strains") distinct from others in genotype 4 indigenous to Japan.

grilled pork meat and entrails at the same party had extremely high sequence homology among the HEV isolates in genomic analyses (Table 2), suggesting a zoonotic transmission of indigenous Japanese HEV strains from pigs to humans in Japan.

In addition, this study demonstrates the persistent presence of virulent strains of HEV in the Kitami/Abashiri region of Hokkaido, Japan. The HEV strain in the Kitami cases of 2004 shows high sequence homology with the Abashiri cases of 2006, as described above. Among the strains, JKO-Aba-FH06C, isolated from patient 3 in Abashiri, and JTC-Kit-FH04L, isolated from Kitami in 2004, demonstrate the strongest resemblance in full-length sequence of nucleotides (Table 2).

The isolates identified from the Kitami and Abashiri patients have also been segregated into a single cluster on the phylogenetic tree of HEV genotype 4 (Fig. 1). They show subtle, but distinct, differences in nucleotide identity from other strains in Hokkaido Prefecture, such as JSM-Sap95 and JKK-Sap[11] isolated at Sapporo (Fig. 1, Table 3). Given that the causative strains in the above Abashiri cases have a close resemblance to those in Kitami in 2004 and that the two areas are contiguous to each other, these HEV instances may share the same channels of distribution and production of pork meat.

The public health impact of the above findings demonstrates significance because the HEV strains of Kitami/Abashiri belong to genotype 4, which is associated with development of fulminant acute hepatitis E.[24] In fact, one of the three HEV patients during the mini-outbreak at Kitami in 2004 has died of fulminant hepatitis.[8,9] Among the six total hepatitis E patients in Kitami and Abashiri, two patients have developed fulminant hepatitis while one has presented with severe hepatitis. Given these facts, suspicion arises that the Kitami/Abashiri strains may be associated with disease progression. In the meantime, another case of fulminant hepatitis due to infection with HEV genotype 4 has been reported in Hakodate, Hokkaido, approximately 400 km from Abashiri and Kitami;[25] the nucleotide sequence of the HEV isolate has shown extreme homology with the Kitami/Abashiri strains. These facts indicate that the Kitami/Abashiri strains of HEV may be spreading to different parts of Hokkaido Prefecture, and further expansion of this strain to other islands of the Japanese archipelago may be possible.

The association between the genomic distinction and fulminant hepatitis in genotype 4 of HEV remains unclear. Because the T at nucleotide 1816 and C at nucleotide 3148 have remained in the JTC-Kit-FH04L and JKO-Aba-FH06C responsible for fulminant hepatitis, no apparent correlation exists between the nucleotide substitutions C1816 and U3148[12] and disease progression in patients infected with the Kitami/Abashiri strains.

In order to prevent HEV infection caused by ingestion of pork meat and entrails, the regional community may need further instruction on proper food handling and cooking techniques. Individual indigenous sources and routes of infection should be clarified. Moreover, administrative precautionary measures must be taken for protecting the livestock and distribution industries as well as regional consumers' health.

ACKNOWLEDGMENTS

THIS WORK HAS been partially supported by a grant from the Ministry of Health, Labor, and Welfare of Japan. The authors thank Hiroaki Okamoto (Jichi Medical Scholl, Tochigi, Japan) for the information on the dwelling places and dates of blood sampling in patients in whom HE-JA1, HE-JA2, HE-JA19, HE-JA28, HE-JA36, HE-JA37, HE-JA41, HE-JF3, HE-JF4 and HE-JF5 have been identified. We are also grateful to Dr Christine Kwan for her help in preparing the manuscript.

REFERENCES

1 Kwo PY, Schlauder GG, Carpenter HA *et al.* Acute hepatitis E by a new isolate acquired in the United States. *Mayo Clin Proc* 1997; **72**: 1133–6.
2 Meng XJ. Zoonotic and xenozoonotic risks of the hepatitis E virus. *Infect Dis Rev* 2000; **2**: 35–41.
3 Tei S, Kitajima N, Takahashi K *et al.* Zoonotic transmission of hepatitis E virus from deer to human beings. *Lancet* 2003; **362**: 371–3.

4 Amon JJ, Drobeniuc J, Bower WA *et al.* Locally acquired hepatitis E virus infection, El Paso, Texas. *J Med Virol* 2006; **78**: 741–6.
5 Li TC, Miyamura T, Takeda N. Detection of hepatitis E virus RNA from the bivalve Yamato-Shijimi (Corbicula japonica) in Japan. *Am J Trop Med Hyg* 2007; **76**: 170–2.
6 Matsuda H, Okada K, Takahashi K *et al.* Severe hepatitis E virus infection after ingestion of uncooked liver from a wild boar. *J Infect Dis* 2003; **188**: 944.
7 Tamada Y, Yano K, Yatsuhashi H *et al.* Consumption of wild boar linked to cases of hepatitis E. *J Hepatol* 2004; **40**: 869–70.
8 Kato M, Taneichi K, Matsubayashi K. A mini-outbreak of HEV infection in those who enjoyed *Yakiniku* party: one died of fulminant hepatitis. *Kanzo* 2004; **45**: 688.
9 Matsubayashi K, Kang J-H, Sakata H *et al.* A case of transfusion-transmitted hepatitis E caused by blood from a donor infected with hepatitis E virus via zoonotic food-borne route. *Transfusion* 2008; **48**: 1368–75.
10 Takahashi K, Iwata K, Watanabe N *et al.* Full-genome nucleotide sequence of a hepatitis E virus strain that may be indigenous to Japan. *Virology* 2001; **287**: 9–12.
11 Takahashi K, Kang J-H, Ohnishi S *et al.* Full-length sequences of six hepatitis E virus isolates of genotype III and IV from patients with sporadic acute or fulminant hepatitis in Japan. *Intervirology* 2003; **46**: 308–18.
12 Inoue J, Nishizawa T, Takahashi M *et al.* Analysis of the full-length genome of genotype 4 hepatitis E virus isolates from patients with fulminant or acute self-limited hepatitis E. *J Med Virol* 2006; **78**: 476–84.
13 Takahashi K, Kang J-H, Ohnishi S, Hino K, Mishiro S. Genetic heterogeneity of hepatitis E virus recovered from Japanese patients with acute sporadic hepatitis. *J Infect Dis* 2002; **185**: 1342–5.
14 Nishizawa T, Takahashi M, Mizuo H, Miyajima H, Gotanda Y, Okamoto H. Characterization of Japanese swine and human hepatitis E virus isolates of genotype IV with 99% identity over the entire genome. *J Gen Virol* 2003; **84**: 1245–51.
15 Takahashi K, Okada K, Kang JH *et al.* A lineage of hepatitis E virus within Genotype IV, associated with severe forms of hepatitis. *Kanzo* 2005; **46**: 389–90.
16 Kuno A, Ido K, Isoda N *et al.* Sporadic acute hepatitis E of a 47-year-old man whose pet cat was positive for antibody to hepatitis E virus. *Hepatol Res* 2003; **26**: 237–42.
17 Takahashi M, Nishizawa Y, Yoshikawa A *et al.* Identification of two distinct genotypes of hepatitis E virus in a Japanese patient with acute hepatitis who had not travelled abroad. *J Gen Virol* 2002; **83**: 1931–40.
18 Liu Z, Chi B, Takahashi K, Mishiro S. A genotype IV hepatitis E virus strain that may be indigenous to Changchun, China. *Intervirology* 2003; **46**: 252–6.
19 Koike M, Takahashi K, Mishiro S *et al.* Full-length sequences of two hepatitis E virus isolates representing an Eastern China-indigenous subgroup of genotype 4. *Intervirology* 2007; **50**: 181–9.
20 Schlauder GG, Dawson GJ, Erker JC *et al.* The sequence and phylogenetic analysis of a novel hepatitis E virus isolated from a patient with acute hepatitis reported in the United States. *J Gen Virol* 1998; **79**: 447–56.
21 Mansuy JM, Peron JM, Abravanel F *et al.* Hepatitis E in the south west of France in individuals who have never visited an endemic area. *J Med Virol* 2004; **74**: 419–24.
22 Ijas S, Arnold E, Banks M *et al.* Non travel-associated hepatitis E in England and Wales: demographic, clinical and molecular epidemiological characteristics. *J Infect Dis* 2005; **192**: 1166–72.
23 Widdowson MA, Jaspers WJ, van der Poel WH *et al.* Cluster of cases of acute hepatitis associated with hepatitis E virus infection acquired in the Netherlands. *Clin Infect Dis* 2003; **36**: 29–33.
24 Abe T, Aikawa T, Akahane Y *et al.* Demographic, epidemiological, and virological characteristics of hepatitis E virus infection in Japan based 254 human cases collected nationwide. *Kanzo* 2006; **47**: 384–91.
25 Sugawara N, Yawata A, Takahashi K, Abe N, Arai M. The third case of fulminant hepatitis associated with "Kitami/Abashiri strain" of hepatitis E virus genotype 4. *Kanzo* 2009; **50**: 473–4.