INTERNATIONAL COLLABORATIVE SURVEY ON EPIDEMIOLOGY OF HEPATITIS E VIRUS IN 11 COUNTRIES

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Abstract. We conducted seroepidemiological studies on antibody prevalence to hepatitis E virus (HEV) in 5,233 sera from 11 countries to ascertain the present state of HEV infection on a global basis. The prevalence of anti-HEV IgG increased with age in these tested countries, but the rate of antibody positivity was over 20% in the 16-30 year-old group in most of the participating countries, except for Japan, the USA, and Spain. Of patients with acute hepatitis of unknown etiology from Nepal, 56% (14/25) were positive for the IgM class of anti-HEV antibody. In addition, HEV RNAs in the serum from 3 Nepali patients who had the IgM antibody were detected by nested PCR and all of the HEV genes isolated belonged to genotype 1. Our results indicate that HEV is spreading worldwide, not only in developing countries, but also in more industrialized countries than previously thought.

INTRODUCTION

Hepatitis E virus (HEV), previously called enterically transmitted non-A, non-B hepatitis, is a major cause of acute hepatitis in many developing countries. This disease is spread frequently by fecally contaminated drinking water. The endemic areas of hepatitis E are located in developing nations where most people live under conditions of an inadequate water supply (Bradley, 1993; Krawczynski, 1993; Worm *et al*, 2002). Outbreaks of hepatitis E that involve several thousand cases have been observed in the developing counties of the Indian subcontinent, Asia, and Africa. Hepatitis E also accounts for a significant number of sporadic cases in endemic regions. The large number of cases involved, the frequency of epidemics, and the high mortality rates among infected pregnant women strongly suggest that hepatitis E is an important cause of morbidity and mortality in humans. Recent studies have reported that hepatitis E has also occurred among individuals in industrialized countries who have no history of travel to areas

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of endemicity (Takahashi *et al*, 2001, 2002a,b; Mizuo *et al*, 2002). The route of HEV infection in those patients is still unknown. Accordingly, HEV is an important public health concern, yet no means of preventing HEV infection, such as a vaccine or immunoglobulin, has been available.

Recently, a highly sensitive and specific enzyme-linked immunosorbent assay (ELISA) that uses recombinant virus-like particles (VLPs) of HEV produced by inserting a recombinant baculovirus vector into insect cells was developed by Li *et al* (1997, 2000). The recombinant VLPs possessed antigenicity similar to that of an authentic HEV particle and were used as an antigen probe to detect HEV-specific IgG and IgM responses.

Using this method, we conducted an international collaborative survey to ascertain the present state of HEV epidemiology in 11 countries that covered substantial territory worldwide, including Far East Asia (Japan and China), Southeast Asia (Vietnam, Myanmar and Thailand), South Central Asia (Nepal), Europe (Spain), Russia, the Middle East (Egypt), South America (Bolivia) and the USA.

MATERIALS AND METHODS

Study population

A total of 5,233 serum samples were tested from individuals in 11 different countries, including Japan [1,350 in Tokyo, aged from 0-69 years (mean = 49.5 ± 11.1 years), and 87 in Okinawa, aged from 1-86 years (mean 16.6 ± 16 year)]; Vietnam [280 in Hanoi, aged from 11-83 years (mean = 32.4 ± 13.8 years), and 220 in Ho Chi Minh City, aged from 18-73 years (mean = 41.5 ± 12.9 years)]; China [250 in Harbin, aged from 4-81 years (mean = 32.4 ± 12.8 years)]; Thailand [355 in Bangkok, aged from 18-77 years (mean = 44.3 ± 12.7 years)]; Myanmar [450 in Yangon, aged from 2-90 years (mean = 37.6 ± 14.2 years)]; Nepal (525 in Katmandu, aged from 13-85 years (mean = 28.3 ± 11.2 years)]; USA [150 in New York City, aged from 2-90 years (mean = 38.1 ± 13.2 years)]; Spain [103 in Barcelona, aged from 18-71 years (mean = 40.3 ± 14.5 years)]; Russia [371 in Moscow, age from 1-15 years (mean = 10.1 ± 3.6 years)]; Egypt [518 in Cairo, aged from 23-62 years (mean = 48 ± 9.4 years)]; and Bolivia [574 in Santa Cruz, aged from 17-56 years (mean = 29.8 ± 8.7 years)]. All sera investigated in this study were obtained from patients with chronic liver disease. In Nepal, the sera were obtained from 25 patients with non-A, non-B, non-C acute hepatitis. The sera were collected from 2000 to 2002 and stored at -40ºC or below until use. Informed consent for participation in this study was obtained from each individual.

Detection of anti-HEV antibodies by ELISA

A recombinant open reading frame (ORF) 2 protein of HEV, which was expressed by a recombinant baculovirus, was used as the antigen in ELISA as previously described (Li *et al*, 1997, 2000). Briefly, serum samples were diluted at 1:200 and added to assay plates that were coated with the recombinant HEV ORF2 protein. Horseradish peroxidase (HRP)-conjugated goat anti-human IgM with 1:1000 dilution and anti-human IgG with 1:5000 dilution (Cappel, Durham, NC, USA) were used to detect antigen-bound human IgM and IgG, respectively. Human serum that is known to be positive for both anti-HEV IgG and IgM was included in every assay plate as a positive control. The cutoff value was set at 0.2 of OD492 because the mean + 3 SD values of human sera known to be negative for both anti-HEV IgG and IgM never exceeded 0.2 in the above-mentioned assays.

HEV RNA detection

HEV RNA was detected by the nested RT-PCR. We targeted the ORF3 of HEV gene for PCR and designed the primer sequences to be covered for all genotypes of HEV for screening. Total RNAs were extracted from 100μl of the serum using a SepaGene RV-R Kit (Sanko-Junyaku, Tokyo, Japan). The resulting pellet was resuspended in 50 μl RNase-free water, following the manufacturer's instructions. Extracted nucleic acids were stored at -20ºC until use. Five μl of nucleic acid were used for amplification of HEV RNA by the nested RT-PCR. PCR was carried out using a set of primers with 5′-GTW CAT AAC CTK ATT GGB ATG C-3′ [E3; sense, nucleotide (nt) 4996-5017] and 5′-RAA GGG GTT GGT TGG ATG-3′ (E3R; antisense, nt 5315-5332) for

the outer primer pairs (337 bases), and 5′-CGG GHD GAA TGA ATA ACA TG-3′ (E4; sense, nt 5098-5117) and 5′-AAG GGC TGA GAA TCA RCC C-3′ (E4R; antisense, nt 5280-5298) for the inner primer pairs (201 bases). The nucleotide positions of each primer corresponded to those of the HEV isolate of genotype 1 (DDBJ/GenBank/EMBL accession # AF051830).

Nucleotide sequence and phylogenetic analysis

We cloned to obtain partial sequences of HEV. The PCR products were separated by 1% agarose gel electrophoresis and purified by using the a QIAquick gel extraction kit (Qiagen, Chatsworth, CA, USA). Purified PCR products were subjected to direct sequencing from both directions using the ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Norwalk, CN, USA). Sequences of amplified DNA were determined using a sequencer (ABI model 3130; Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis

Nucleotide sequences were multiple aligned using CLUSTAL W (version 1.4). The distance matrix of the nucleotide substitutions between each clone was estimated by the eight-parameter method (Rzhetsky, 1995), and phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei, 1987) from the matrix. These procedures were computed using Phylo_win (version 1.2) (Galtier *et al*, 1996) on a DEC alpha 2000 server, and the trees were drawn by TreeView (version 1.5) (Page, 1996). The reliability and topology of each tree branch was tested by bootstrap analysis (Billis and Bull, 1993) of the data of 100 bootstrap resamplings of the columns in the ORF3 gene alignment.

RESULTS

Anti-HEV IgG prevalence

The rate of anti-HEV IgG detection is described in Table 1. The rate of HEV infection increased with age in most of the countries investigated (Table 2). In the developing countries, including Vietnam, China, Thailand, Myanmar, Nepal, Egypt, and Bolivia, the rates of antibody positivity were already over 20% in the 16-30 year-old groups. In Russia, although only infants and children were surveyed, the antibody was already at the 18.8% level in subjects less than 15 years of age.

Table 1
Prevalence of anti-HEV IgG in 11 countries.

Country (City)	No.	Anti-HEV IgG (%)
Japan (Tokyo)	1,350	120 (8.9)
(Okinawa)	87	30 (34.5)
Vietnam		
(Hanoi)	280	174 (62.1)
(Ho Chi Minh)	220	92 (41.8)
China (Harbin)	250	73 (29.2)
Thailand (Bangkok)	355	96 (27)
Myanmar (Yangon)	450	158 (35.1)
Nepal (Katmandu)	525	347 (66)
USA (New York City)	150	32 (21.3)
Spain (Barcelona)	103	35 (34)
Russia (Moscow)	371	75 (20.2)
Egypt (Cairo)	518	415 (80)
Bolivia (Santa Cruz)	574	73 (12.7)
	5,233	

Anti-HEV IgM prevalence among patients with non-A, non-B, non-C acute hepatitis

Among patients with non-A, non-B, non-C acute hepatitis from Nepal, the IgM class of anti-HEV was detected in 14 out of 25 (56%) cases examined. All patients who were positive for anti-HEV IgM were also positive for anti-HEV IgG, with a high titer (OD492 ≥ 3.0).

HEV RNA detection and phylogenetic analysis

Among the anti-HEV IgM-positive patients with acute hepatitis in Nepal, HEV RNA was detectable by nested PCR in the serum of 3 patients. Using these amplicons, we sequenced and confirmed the specificity to the HEV ORF-3 gene. Based on these sequences, all isolates in Nepal belonged to genotype 1 of HEV by phylogenetic analysis (Fig 1).

DISCUSSION

Hepatitis E is endemic in many subtropical and tropical areas, and over 50 outbreaks have been reported in Southeast and central Asia, the Middle East, northern and western parts of Af-

Table 2
Age-specific prevalence of anti-HEV IgG in 11 countries.

Country	No.	Age group					
		0-15 yrs	16-30 yrs	31-40 yrs	41-50 yrs	51-60 yrs	≥61 yrs
Japan	846	1/240 (0.4)	5/84 (5.9)	10/130 (7.7)	15/127 (11.8)	25/145 (17.2)	17/120 (14.1)
USA	148	NA	4/32 (12.5)	3/23 (13)	8/34 (23.6)	5/30 (16.7)	7/29 (24.1)
Spain	87	NA	1/14 (7.1)	5/20 (25)	8/26 (30.8)	10/27 (37)	NA
Vietnam							
Hanoi	280	1/4 (25)	24/70 (34.3)	30/53 (56.7)	67/81 (82.7)	33/41 (80.5)	19/31 (61.3)
Ho Chi Minh	93	NA	6/13 (46.2)	15/25 (60)	27/34 (79.4)	7/10 (70)	8/11 (72.7)
China	222	2/11 (18.2)	13/58 (22.4)	16/53 (30.2)	15/43 (34.9)	8/31 (25.8)	7/26 (26.9)
Thailand	299	NA	16/54 (29.6)	19/69 (27.5)	25/93 (26.9)	16/65 (24.6)	6/18 (33.3)
Myanmar	370	1/16 (6.3)	18/77 (23.3)	32/79 (40.5)	43/93 (46.2)	22/54 (40.7)	24/51 (47.1)
Nepal	505	3/4 (75)	192/292 (65.8)	77/100 (77)	37/63 (58.7)	23/27 (85.2)	11/19 (58)
Russia	341	64/341 (18.8)	NA	NA	NA	NA	NA
Egypt	150	NA	23/27 (85.2)	40/42 (95.2)	28/40 (70)	29/32 (90.7)	8/9 (88.9)
Bolivia	326	NA	40/192 (20.8)	22/89 (24.7)	6/40 (15)	1/5(20)	NA

Number in parentheses indicate percentage. NA=not available

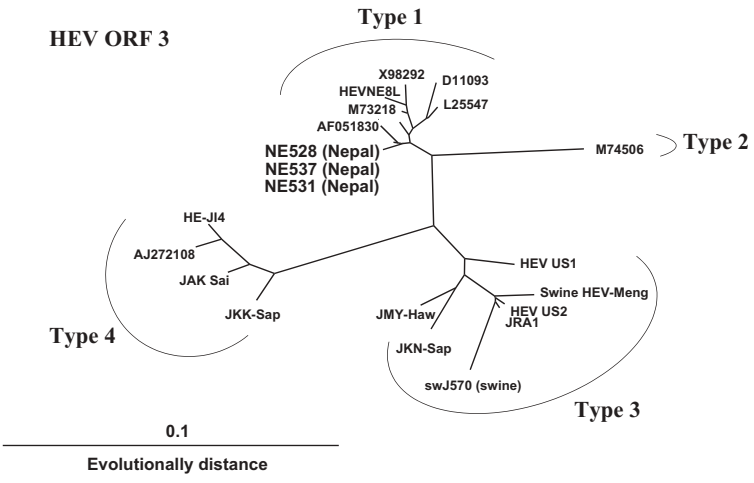

Fig 1–Phylogram generated by neighbor-joining analysis of genetic distances in the ORF3 sequence of HEV isolates.

rica, and Mexico thus far (Bradley, 1993). In these areas, hepatitis E has occurred both in epidemic and sporadic forms. Although antibodies to HEV have been found in sera from individuals in developing countries, data have been scarce from the countries that participated in the present study. At present, only two ELISA systems are broadly available commercially: the Genelabs®-EIA that uses four short recombinant proteins derived from the 3′ termini of ORF2 (42aa) and ORF3 (33aa) from Burmese and Mexican prototype sequences, and the Abbott®-EIA that uses two recombinant proteins derived from complete ORF3 (123aa) and from a sequence of ORF2 (327aa) from the Burmese prototype strain. The specificity and sensitivity of these tests for detecting convalescence-phase IgG have not been precisely established, which limits the reliability of results from seroepidemiological studies. This is one of the main reasons why no seroepidemiological survey of HEV at a worldwide level has been attempted thus far. Recently, Li and others (Li *et al*, 1997, 2000) developed an ELISA for anti-HEV detection that uses highly purified empty VLPs. HEV VLPs have been generated by expressing the truncated ORF2 at its N-terminus of the genome through the use of recombinant baculovirus vector in insect cells. The sensitivity and specificity of this assay system have been confirmed (Li *et al*, 2000).

In the present study, we applied this method for the investigation of HEV epidemiology in various countries and found that HEV was distributed widely in developing countries as well as in developed counties. Surprisingly, our data showed a very high prevalence of anti-HEV IgG (>60%) in Vietnam (Hanoi), Nepal, and Egypt. These results indicated that HEV is still an important etiological agent in these counties, and the development of an effective vaccine for HEV protection is urgently required. Until recently, HEV was believed to have had a limited geographic distribution. However, recent serological investigations suggest that HEV may be endemic in the industrialized countries although it infrequently causes overt disease in these regions (Takahashi *et al*, 2001,2002a,b; Mizuo *et al*, 2002). It has been said that Japan is not an endemic area of HEV infection because acute hepatitis patients infected with HEV are rare, and most patients with acute hepatitis E have recently traveled to countries where HEV is highly endemic. In this study, however, none of the individuals found to have the anti-HEV antibody in Japan had had a recent history of visiting countries where HEV was endemic. Conversely, compared with the Tokyo area, there was a higher prevalence of anti-HEV IgG in Okinawa prefecture, which is an island at the southern end of Japan. Several recent studies have suggested that there are indigenous HEV strains in Japan because several HEV strains were recovered from Japanese patients with acute hepatic failure of unknown etiology who had not traveled abroad (Takahashi *et al*, 2001, 2002a,b; Mizuo *et al*, 2002).

The route of HEV infection in those patients is still unknown. It has been reported that swine (Meng *et al*, 1997; Okamoto *et al*, 2001) and rodents (Kabrane-Lazizi *et al*, 1999; Favorov *et al*, 2000; Arankalle *et al*, 2001) may be reservoirs of HEV, but the exact role of animals in the transmission of HEV to humans remains obscure. Recently, we found evidence for widespread infection of HEV among wild rats and Japanese monkeys living in Japan (Hirano *et al*, 2003a,b). The role of transmission of HEV to humans from these animals should be considered in order to resolve these important problems. We are now conducting a study to clarify these issues.

In this study, we tested the detection rate of anti-HEV IgM among patients with acute hepatitis of unknown etiology (clinically diagnosed as non-A, non-B, non-C hepatitis) from Nepal and found that 56% were positive for the IgM antibody. Furthermore, we also detected the HEV RNA in serum from three patients. HEV has been classified into genotypes 1 through 4 and have been shown to have a geographic distribution. It is known that genotype 1 is found mainly in Southeast Asia. The Nepali HEV isolates recovered in this study were also confirmed by phylogenetic analysis to belong to genotype 1.

In conclusion, our results suggested that HEV was distributed over a wide area, particularly in developing counties, but also in developed countries that until now have not been regarded as endemic for HEV. Clarification of the infection route and the establishment of prevention measures such as vaccine development are needed. Use of the VLPs of HEV would be a useful tool for achieving these purposes.

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