2.6% (25/976). The antibody-positive rate was 1.2% in Hokkaido, 2.2% in Miyazaki, 3.1% in Iwate, 3.1% in Hyogo, and 3.6% in Miyagi. The difference among these prevalence was not statistically significant ($P > 0.05$).

Eighty-eight paired stool and serum samples from deer captured in Hokkaido (10), Iwate (23), Tochigi (7), Chiba (2), Nagano (5), Mie (4), Hyogo (8), Hiroshima (3), Oita (4), Fukuoka (1), Kumamoto (9), Miyazaki (6), and Kagoshima (6), 166 deer serum samples obtained in Aichi (18), Hyogo (28), Nagano (10), and Miyagi (110), and 159 deer liver tissue samples collected in Hyogo (50), Iwate (11) and Hokkaido (98) were tested by RT-PCR for HEV RNA. Total RNA was extracted with RNAzol-LS reagent (Invitrogen, Inc., Carlsbad, CA) using 200 µl of the deer serum, and 10% stool suspension Reverse transcription (RT) was performed at 42 °C for 50 min followed by 70 °C for 15 min in 20 µl reaction mixture containing 1 µl of Superscript™ II RNase H⁻ reverse transcriptase (Invitrogen, Inc., Carlsbad, CA), 1 µl of the oligo (dT) primer, 1 µl of RNaseOUT™, 2 µl of 0.1 M dithiothreitol, 4 µl of 5 × RT buffer, 1 µl of 10 mM deoxynucleoside triphosphates, 5 µl of RNA, and 5 µl of distilled water. Two microliters of the resulting cDNA was amplified in a 50 µl reaction mixture containing ExTaq DNA polymerase (Takara Shuzo Co., Ltd., Kyoto, Japan) with an external sense primer HEV-F1 (5′-GGBGTBGCNGAGGAGGAGGC-3′, nucleotide (nt) residues 5903–5922 of G1 Myanmar strain, D10330) and an external antisense primer HEV-R2 (5′-TGYTGGTTRTCRTARTCCTG-3′, nt residues 6486–6467 of G1 Myanmar strain, GenBank D10330), using the GeneAmp PCR System 9700 (PE Biosystems, Foster City, CA). Each cycle consisted of denaturation at 95 °C for 30 sec, primer annealing at 55 °C for 30 sec, and an extension reaction at 72 °C for 60 sec followed by a final extension at 72 °C for 7 min. The nested PCR was done by using 2 µl of the first PCR product with an internal sense primer HEV-F2 (5′-TAYCGHAA YCAAGGHTGGCG-3′; nt residues 5939–5958) and an internal antisense primer HEV-R1 (5′-CGACGAAATYAATTCTG TCG-3′, nt residues 6316–6297) under the same conditions [9, 10]. Total RNA in deer liver was extracted from 100 mg of the tissue using an RNeasy Mini Kit (Qiagen, Inc.) and dissolved in 50 µl nuclease-free distilled water. The nested RT-PCR was carried out as described by Takahashi et al. [20]. However, we were not able to amplify any HEV sequences in these samples.

An ELISA with recombinant VLPs was used to detect anti-HEV IgG in sika deer in Japan. This assay was previously shown to be capable of detecting anti-HEV antibodies in human and mongoose sera with high sensitivity and specificity [8, 10]. To establish a system for detecting anti-HEV IgG in deer, we first prepared a positive control serum by immunizing deer with the G1 VLPs. After two doses of immunization, an antibody whose titer was as high as 1:3,276,800 was obtained. The specificity of this antibody was confirmed by Western blot assay, indicating that G1 VLPs was an excellent antigen to induce a strong immune response in deer.

In the present study, we tested a total of 976 deer serum samples for the presence of anti-HEV IgG antibody and made the following observations. First, the antibody prevalence was low in sika deer in Japan. Only 25 of 976 (2.6%) samples were positive for anti-HEV IgG by ELISA, which is lower than the prevalence in pigs (58%) and wild boars (44%), both of which are thought to be reservoirs of HEV in Japan [10, 21]. Second, the OD value and titer of anti-HEV IgG were low in deer. The highest OD value was 0.486 and the highest titer was 1:400. This observation is also different from that in pigs and wild boars, where the highest OD values were greater than 3.000 and the titers were greater than 1:51200. Third, the distribution of OD values indicated that only one peak was less than 0.486. The bimodal distribution observed in pigs and wild boars was not seen in deer, indicating that the rate of infection by HEV under natural conditions is extremely low in deer, and suggesting that deer do not play an important role as a reservoir of HEV in Japan.

This study included 254 serum samples from deer captured in Hyogo, where the first deer positive for HEV RNA was found [24]. The prevalence of the antibody-positive rate was 3.1% (5/132) in female and 2.5% (3/122) in male deer, and the difference

between the sexes was not statistically significant ($P > 0.05$). These antibody-positive rates are also not significantly different from those in other areas, including Hokkaido, Iwate, and Miyazaki prefecture. The age of anti-HEV IgG-positive deer was 0–8 years, and no significant correlation between age and prevalence was observed. We also tested HEV RNA in 36 serum samples from deer captured in the same area in Hyogo prefecture where the deer that was positive for HEV RNA was reported. However, we were not able to amplify any HEV sequences in these samples.

Since wild boars are prevalent throughout Japan, with the exception of Hokkaido, and they seem to be eventually infected with HEV, the virus is spread throughout their habitat via their stools. Because wild deer and wild boars share this environment, wild deer might be exposed to HEV. Only low-titer anti-HEV IgG was detected in deer serum in this study, suggesting that either the antibody detected in this study was not induced by HEV infection or that deer have low sensitivity to HEV. If deer were to occasionally come into contact with a small amount of HEV, but were not susceptible to HEV, then a strong immune response to HEV might not be induced.

In summary, the prevalence of anti-HEV IgG in sika deer was lower than the prevalence in two possible reservoirs, pigs and wild boars, and no HEV RNA was detected in 254 sera, 88 stool and 159 liver tissue samples, indicating that wild deer may not be a reservoir of HEV in Japan.

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Sero-epidemiology of sporadic acute hepatitis in Bangladesh: high prevalences of infection with type-B, type-E and multiple types of hepatitis virus

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In a recent investigation of hepatitis in Bangladesh, the sera from 74 adult patients (aged 15–67 years) who had been clinically diagnosed as cases of sporadic acute hepatitis were collected at various hospitals in and around Dhaka. Five cases were positive for IgM antibody against the hepatitis A virus and 30 were positive both for the surface antigen of the hepatitis B virus (HBV) and for IgM antibody against the HBV core (HBc). The six cases found positive for antibodies against the hepatitis D virus were all also positive for the HBV surface antigen but negative for anti-HBc IgM. Thirteen patients harboured hepatitis C virus RNA and 29 were positive for IgM antibody against the hepatitis E virus (HEV). There were 14 non-A-to-E subjects, whose illness was of unknown aetiology. Of the 83 infections with hepatitis viruses detected in the other 60 patients, 6%, 36%, 16%, 7% and 35% were of types A, B, C, D and E, respectively. Each of 28 of the patients (47% of those confirmed to have viral hepatitis) had concomitant infection with more than one type of hepatitis virus. The predominance of HBV and HEV infections and the high prevalence of multiple infection seen among these Bangladeshi cases have not been observed among hepatitis cases in developed countries.

Current serological assays allow the viruses that can cause human hepatitis to be separated into at least five main types. The general prevalences of infection with each type of hepatitis virus have been clarified by recent world-wide research, although the relevant data are lacking for some developing countries, where endemic-type acute viral hepatitis is a major problem. The endemic types of hepatitis, A and E, have well characterised geographical distributions, with high prevalences in most

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developing countries such as those of South–east Asia, South Asia, China, the Middle East, Africa and parts of America (Gupta and Smetana, 1957; Belabbes *et al.*, 1985; Myint *et al.*, 1985; Velazquez *et al.*, 1990; Aye *et al.*, 1992; Shapiro and Margolis 1993; Krawczynski *et al.*, 2001). Aetiological analyses of acute hepatitis of the sporadic type have been conducted in most industrialized countries, and there have been quite a few similar studies in developing countries (Al-Knawy *et al.*, 1997; Gunaid *et al.*, 1997; Chadha *et al.*, 2003; Tsatsralt-Od *et al.*, 2006). It is not possible to determine the type of hepatitis virus causing human illness from the clinical features alone. Although, for cases of acute or aggressive hepatitis, it is important to identify the specific viral type so that the optimal therapy can be offered and an accurate prognosis can be made, it is sometimes difficult to perform the necessary tests in developing countries. In Bangladesh, for example, the limited resources, and the relatively high costs of the appropriate serology, make aetiological analyses difficult in many hospitals.

Although diseases of the gastro–intestinal tract and hepato–biliary system are very common in Bangladesh — accounting for approximately 25% of patients at medical-college hospitals (Hasan and Khan, 1997) — there has been surprisingly little research on hepatitis in the country. The main aim of the present study was to use a mixture of serology and molecular biology to determine the types of hepatis virus causing sporadic acute hepatitis in and around the capital city of Dhaka.

MATERIALS AND METHODS

Subjects

Between November 1995 and October 1996, sera from 74 adult patients with sporadic, non-endemic, acute hepatitis were collected at various hospitals in and around Dhaka and stored, frozen at −20°C, at the Hospital of Bangabandhu Sheikh Mujib Medical University (BSMMU), in Dhaka. In November 1996, these sera were transported on dry ice to Nihon University in Tokyo and there stored at −80°C until they could be tested further.

The study subjects had to show a typical prodrome of viral hepatitis, with a history of persistent or progressive jaundice of <6 months' duration, and biochemical evidence of hepatocellular necrosis or injury. Patients who had suffered with jaundice or shown any viral markers for >6 months, those who had developed ascites and/or encephalopathy, and those showing clinical, endoscopic or ultrasonographic evidence of established cirrhosis of the liver or bile-duct obstruction were excluded. Not all of the sera investigated were obtained during the peak stages of the patients' illnesses.

The ages of the sample donors ranged from 15–67 years (mean=29.5 years), with 11 (five males), 27 (21 males), 24 (21 males), nine (six males), zero and three (two males) in their 2nd, 3rd, 4th, 5th, 6th and 7th decades, respectively. There were 55 male donors (74%) and 19 female (26%).

Serum samples were collected after obtaining informed consent, and the study protocol was approved by the Ethics Committee of Nihon University's School of Medicine.

Serology and Molecular Biology

Commercial kits were used, according to their manufacturers' instructions, to detect evidence of infection with hepatitis viruses of types A (HAV), B (HBV), C (HCV) and D (HDV). Whole-immunoglobulin and IgM-specific anti-HAV, HBV surface antigen (HBsAg), IgM antibody against the HBV core antigen (anti-HBc) and anti-HBc in 200-fold-diluted sera were each investigated in radio-immuno-assays from Dainabot (Tokyo), who also made the enzyme-immuno-assay used to detect anti-HDV antibody. The concentration of HCV RNA in each serum was explored using the

relevant Amplicore kit (Roche Diagnostics, Basel, Switzerland), while titres of IgG and IgM antibody against hepatitis E virus were determined using the ELISA described by Li *et al.* (2000), which uses a recombinant antigen based on empty virus-like particles of HEV.

Only the sera found negative for anti-HBc IgM and positive for HBsAg were tested for anti-HBc after being diluted 200-fold, and only the sera found positive for HBsAg or anti-HBc IgM were assayed for anti-HDV.

Statistical Analysis

Unpaired Student's *t*-tests were used to explore the significance of the relationships between donor age, the results for anti-HAV IgM, HBsAg, HCV RNA, anti-HDV and anti-HEV IgM and the seroprevalence of 'multiple' infection (i.e. infection with more than one type of hepatitis virus). The significance of the relationships between gender and each of these factors was similarly explored in χ^2 tests. A *P*-value of <0.05 was considered indicative of a statistically significant difference.

RESULTS

In terms of the evidence of infection with hepatitis viruses A–E, 15 different patterns were recognised among the 74 serum donors (Table 1). Five cases were positive for anti-HAV IgM and were classified as having acute hepatitis A. All five were, however, also positive for other hepatitis viral markers, indicating their co-infection with two or more types of hepatitis virus. The remaining 69 cases (93%) were positive for anti-HAV immunoglobulin.

Thirty of the serum donors were each positive for both HBsAg and anti-HBc IgM and they were classified as cases of acute hepatitis B. Among these 30, 13 were also positive for other markers of viral hepatitis.

Another 14 cases were positive for HBsAg but negative for anti-HBc IgM. When diluted 200-fold, the sera from all of these cases showed the high inhibition titres (>95%) that indicated that they had high levels of anti-HBc (Wada *et al.*, 1980), but all 14 sera also harboured markers of hepatitis viruses other than type B (i.e. anti-HAV IgM, HCV RNA, anti-HDV, and/or anti-HEV IgM). The cause of the acute hepatitis in these 14 cases was recorded as infection with hepatitis viruses other than type B, acutely superimposed on HBV carrier status. None of the patients investigated was found negative for HBsAg but positive for anti-HBc IgM.

Thirteen cases had HCV RNA in their sera but nine of these 13 cases were also positive for one or more markers of non-C viral hepatitis. The aetiology of the acute hepatitis seen in these 13 cases was difficult to determine. As all 74 patients investigated had been clinically diagnosed as cases of sporadic acute hepatitis, the four found positive only for HCV RNA were considered to be suffering from acute hepatitis C. The nine cases found positive for both HCV RNA and one or more markers of non-C hepatitis were considered to be possible (but unconfirmed) cases of acute infection with HCV.

Sera from six cases were positive for both anti-HDV and HBsAg and showed high inhibition titres (>95%) when diluted 200-fold and tested for anti-HBc, although they were negative for anti-HBc IgM. Two (of the six) cases were also both positive for the markers of non-B and non-D viral hepatitis. Since all of the subjects had been clinically diagnosed as having sporadic acute hepatitis, acute infection with HDV was considered to be confirmed in four of these six cases and possible but unconfirmed in the other two cases. Evidence of HDV infection was found in six (43%) of the 14 cases with HBV carrier status.

Overall, 29 of the 74 cases were found ELISA-positive for anti-HEV IgM, and all 29 (but none of the other patients investigated) were found positive for anti-HEV IgG. These 29 were considered to have

TABLE 1. *The serological results for the 74, clinically diagnosed, Bangladeshi cases of sporadic acute hepatitis*

Result of the assay for:								
HBsAg	Anti-HBc IgM	Anti-HBc in 200-fold diluted sera	Anti-HAV IgM	HCV RNA	Anti-HDV	Anti-HEV IgM	No. of cases	Possible diagnoses
+	+	ND	−	−	−	−	17	Acute hepatitis B
+	+	ND	+	−	−	−	2	Acute hepatitis A and B
+	+	ND	−	−	−	+	7	Acute hepatitis B and E
+	+	ND	−	+	−	−	2	Acute hepatitis B and C, or acute hepatitis B superimposed on HCV carrier status
+	+	ND	−	+	−	+	2	Acute hepatitis B, C and E, or acute hepatitis B and E superimposed on HCV carrier status
+	−	+*	+	+	−	+	2	Acute hepatitis A, C and E superimposed on HBV carrier status, or acute hepatitis A and E superimposed on carrier status for both HBV and HCV
+	−	+*	−	+	−	−	2	Acute hepatitis C superimposed on HBV carrier status
+	−	+*	−	−	+	−	4	Acute hepatitis D superimposed on HBV carrier status
+	−	+*	−	−	−	+	4	Acute hepatitis E superimposed on HBV carrier status
+	−	+*	−	−	+	+	1	Acute hepatitis D and E superimposed on HBV carrier status, or acute hepatitis E superimposed on carrier status for both HBV and HDV
+	−	+*	−	+	+	+	1	Acute hepatitis C, D and E superimposed on HBV carrier status, acute hepatitis D and E superimposed on carrier status for both HBV and HCV, acute hepatitis C and E superimposed on carrier status for both HBV and HDV, or acute hepatitis E superimposed on carrier status for HBV, HCV and HDV
−	−	ND	+	−	ND	+	1	Acute hepatitis A and E
−	−	ND	−	+	ND	−	4	Acute hepatitis C
−	−	ND	−	−	ND	+	11	Acute hepatitis E
−	−	ND	−	−	ND	−	14	Acute hepatitis with cause other than hepatitis viruses A–E

HBsAg, Surface antigen of the hepatitis B virus; HBc, core of the hepatitis B virus; HAV, hepatitis A virus; HCV, hepatitis C virus; HDV, hepatitis D virus; HEV, hepatitis E virus; ND, not determined; HBV, hepatitis B virus.
*High inhibition titre (>95%).

acute hepatitis E, although 18 of them were also positive for at least one marker of non-E viral hepatitis.

Fourteen of the 74 subjects investigated in the present study were negative for all of the markers of viral hepatitis investigated except anti-HAV immunoglobulins (all 14) and anti-HEV IgG (eight cases) and were therefore classified as cases of acute non-A-to-E hepatitis.

The prevalences of apparent infection with hepatitis viruses of types A to E among the other 60 subjects are shown in Table 2. Of these 60 subjects, 28 cases (47%) appeared to be co-infected with two, three or four types of hepatitis virus.

None of the links between the carriage of anti-HAV IgM, HBsAg, HCV RNA, anti-HDV or anti-HEV IgM and multiple infection, donor age or donor gender appeared to be statistically significant ($P>0.05$ for each).

DISCUSSION

Two characteristic findings were revealed by this study. First, HBV and HEV infections were the most common and equally significant aetiologies of clinically diagnosed cases of acute sporadic viral hepatitis in Bangladesh, each of these viruses accounting for about half of the cases in which the probable cause was identified (Table 2). Acharya and Panda (2006) reported that HEV was the major aetiological agent for acute hepatitis and acute liver failure in endemic regions, and Purcell and Emerson (2008) found hepatitis E to be either the most important or the second most important cause of acute clinical hepatitis in adults throughout Asia, the Middle East and Africa. Although all the subjects of the present study came from Dhaka or its environs, the seroprevalence of HEV infection recorded among them is very similar to the values previously reported among Bangladeshi adults with fulminant hepatitis (Sheik *et al.*, 2002; Mahtab *et al.*, 2008), and similar to the corresponding values recorded in India, Nepal and other HEV-endemic areas (Coursaget *et al.*, 1998; Shrestha *et al.*, 2003; Acharya and Panda, 2006). That HEV infection is a major cause of the acute and fulminant hepatitis seen in Bangladesh probably reflects the often-inadequate levels of public sanitation and hygiene in the country, as well as the frequency of natural disasters such as typhoons and flooding, which is much higher in Bangladesh than in most industrialized countries.

As evidence of acute HEV infection, anti-HEV antibodies were investigated in the present study, instead of HEV RNA, since infected humans remain seropositive for anti-HEV IgM for about 100 days (Li *et al.*, 2000) but harbour detectable HEV RNA in their sera for no more than 40 days (Takahashi *et al.*, 2005). The HEV-infected sera detected in the present study were subsequently used for the isolation of HEV clones. When the nucleotide and amino-acid sequences of these clones were

TABLE 2. *Final diagnoses for the 60 patients showing evidence of infection with hepatitis viruses A, B, C, D and/or E (including the 28 who were each found positive for two to four types of hepatitis virus)*

	Diagnosis				
	Acute infection with HAV	Acute infection with HBV	Possible acute infection with HCV	Possible acute infection with HDV	Acute infection with HEV
No. and (%) of the 60 cases	5 (8)	30 (50)	13 (22)	6 (10)	29 (48)
% of the 83 infections detected	6	36	16	7	35

HAV, Hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HDV, hepatitis D virus; HEV, hepatitis E virus.

analysed, genotype I was found to be the prevalent form of HEV circulating in Bangladesh (Sugitani *et al.*, 2009).

In contrast to the relative commonness of HBV and HEV observed in the present study, acute infection with HAV (as indicated by the presence of anti-HAV IgM in the serum) — another virus transmitted via the faecal–oral route — appeared rare. It seems that HAV has little significance in the aetiology of sporadic acute hepatitis among Bangladeshi adults. Acute HAV infection is, however, considered to be prevalent in childhood in Bangladesh (Sheik *et al.*, 2002), and such early infection probably produces lifelong immunity to re-infection. This would explain why almost all (93%) of the adult subjects of the present study carried antibodies against HAV.

Given the present results, in which HBV accounted for about half of the cases in which the probable cause was identified (Table 2), it is surprising that Mahtab *et al.* (2007) concluded that HBV accounted for only 10%–35% of the acute hepatitis cases seen in Bangladesh. Differences in the sensitivities of the methods used to detect HBV-related antigens and antibodies may account for this apparent discrepancy. Another possible explanation is that the present results were affected by a gender bias, with most (74%) of the samples analysed in the present study coming from men. Further investigations are clearly necessary to elucidate the true state of HBV infection in Bangladesh.

The superimposition of HBV carrier status on infection with hepatitis viruses of types A, C, D or E was frequently detected in the present study, being recorded in 14 (23%) of the cases in which the probable cause was identified. Infection with HBV is considered to be moderately endemic in Bangladesh (Mahtab *et al.*, 2007). In Taiwan, a country considered to be hyperendemic for HBV, Chu *et al.* (1999) found the prevalence of HBsAg carrier status among 334 cases of sporadic acute hepatitis to be even higher (53.0%).

In the present study, the measurement of the serum concentrations of HBV DNA would probably have been advantageous, especially for the HBV-positive cases who were co-infected with other types of hepatitis virus, including those suspected to have HBV carrier status. Unfortunately, however, this was not possible because the serum volumes available were inadequate.

After HBV and HEV, the third most significant infectious agent in sporadic acute viral hepatitis among Bangladeshi adults appears to be HCV. Although HDV infection appeared to have little role in the poor health of the present subjects, HDV can replicate only in HBV-infected individuals and the prevalence of HDV infection among the 14 cases considered to have HBV carrier status was quite high, at 43%. This value is markedly higher than the seroprevalences of HDV infection detected, by Zaki *et al.* (2003), among other Bangladeshi carriers of HBsAg, whether those carriers were symptomatic or not (21.8%–25.6%). As all the samples used in the present study were collected from clinically established cases of sporadic acute hepatitis, however, a simple comparison of the present data with those collected by Zaki *et al.* (2003) might be inappropriate. The number of subjects with HBV carrier status in the present study was also small. Further investigations of HDV prevalence and estimation of the total number of HBV-infected subjects in Bangladesh remain necessary.

In the present study, the hepatitis seen in 14 subjects could not be attributed to hepatitis viruses of types A–E. These subjects may have been suffering from other viral or bacterial infections or the effects of drugs, auto-immunity, toxicity, ischaemia, shock etc. (Alcohol abuse was an unlikely cause of their illness because the ingestion of alcohol goes against the religious views of most Bangladeshis.)

A notable result of the present study was the high frequency of co-infection with two to four types of hepatitis virus. Although rare in the industrialized world,

such multiple infection is commonly seen among cases of acute and/or fulminant hepatitis in other areas where HBV and/or HEV are endemic (Wu *et al.*, 1994; Arora *et al.*, 1996; Coursaget *et al.*, 1998), where it might play an important role in the aetiology of sporadic acute viral hepatitis.

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HEPATOLOGY

Detection of hepatitis E virus RNA and genotype in Bangladesh

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Key words
Bangladesh, genotype, hepatitis E virus (HEV), HEV RNA, IgM specific anti-HEV.

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Abstract

Background/Aims: Hepatitis E virus (HEV) in Bangladesh has not been adequately documented. We report HEV RNA and genotype detection in Bangladesh.

Methods: In total, 82 samples were used; 36 sporadic acute hepatitis (AH), 12 fulminant hepatitis (FH), 14 chronic liver disease (CLD) and 20 from an apparently healthy population (HP) positive for both immunoglobulin (Ig) M and IgG specific anti-HEV antibodies (anti-HEV). The male/female ratio was 61/21, ages 12–67 (mean 30.4) years. RNA was extracted, transcribed to cDNA and amplified in nt 6345–6490 (ORF2) of HEV. Nucleic and amino acid sequences were determined. Homology comparison between Bangladesh clones and other representative HEV clones and phylogenetic tree analyses were done. Relations between HEV RNA-positivity and clinical factors were analyzed.

Results: HEV RNA was positive in 9/36 (25.0%) of AH cases, 4/12 (33.3%) FH, 3/14 (21.4%) CLD and 0/20 (0%) HP samples; total 16/82 (19.5%). Four factors correlated significantly with HEV RNA-positivity (Mann-Whitney U test); alanine aminotransferase (ALT) ($P = 0.0229$), aspartate aminotransferase (AST) ($P = 0.0448$), and titers of IgG ($P = 0.0208$) and IgM ($P = 0.0095$) specific anti-HEV. The 16 HEV clones were divided mainly into two groups, A and B, including six different cDNA sub-groups.

Conclusion: HEV RNA was found in sporadic AH and FH and sub-clinical CLD cases, but not in HP. HEV RNA-positivity was significantly related to values of ALT and AST and titers of IgG and IgM specific anti-HEV, with IgM specific anti-HEV showing the most significant relationship. All clones were genotype I, which is prevalent in South Asia.

Introduction

Hepatitis E virus (HEV) is a single-stranded, approximately 32 to 34 nm in diameter, positive sense, 7.2 kb RNA virus. Hepatitis E virus infection causes both endemic and sporadic types of acute hepatitis (AH) and fulminant hepatitis (FH) transmitted via the oral-fecal route. Outbreaks of acute hepatitis E are common, mainly in developing countries with inadequate environmental sanitation, with contaminated drinking water considered to be the main source of infection. The first documented outbreak of acute hepatitis E was in New Delhi[1,2] in 1955. Subsequently, similar outbreaks were reported worldwide, for example in Southeastern[3] and Central[4] Asia, China,[5] Africa[6] and Mexico.[7] Furthermore, hepatitis E was recognized as being the major etiological agent for acute hepatitis and acute liver failure in endemic regions.[8]

Though numerous endemic or sporadic types of acute hepatitis E have been reported in India,[9] Pakistan,[10] Nepal[11] and Burma,[12] the state of hepatitis E in Bangladesh has not been adequately documented. As there are many rivers in Bangladesh, floods are common during the rainy season. Therefore, waterborne hepatitis has long been suspected to exist in Bangladesh. Our previous report[13] on the etiologies of Bangladesh FH and a report[14] describing a peacekeeping soldier who suffered from hepatitis E while traveling in Bangladesh, support the assumption that waterborne hepatitis E does exist in this area. Additionally, infection with hepatitis A virus (HAV), which is also transmitted via the oral-fecal route, occurs in 98% of the population by the age of 15 in Bangladesh.[13] Given the life long immunity to HAV, hepatitis A was not considered to be significant in Bangladeshi adults.

Table 1 Summary of parameters assessed in cases positive for both IgG and IgM specific anti-HEV

	AH	FH	CLD	HP	Total
Number of Cases	36	12	14	20	82
Male/Female	24/12	8/4	11/3	18/2	61/21
Age (mean, years)	17–67 (29.8)	22–60 (32.8)	12–60 (31.7)	16–62 (29.2)	12–67 (30.4)
ALT (mean, U/L)	23–1648 (176)	25–679 (169)	14–806 (130)	17–53 (31)	14–1648 (132.5)
AST (mean, U/L)	8–513 (63)	14–748 (129)	4–330 (45)	9–27 (16)	4–748 (58.1)
Total Bilirubin (mean, mg/dL)	0.3–31.2 (6.4)	0.8–19.5 (8.5)	0.3–6.0 (1.9)	0.2–1.0 (0.4)	0.2–31.2 (4.5)
Titer of IgG specific anti-HEV (mean)	0.506–3.613 (2.942)	0.769–3.691 (3.048)	1.242–3.742 (2.563)	0.512–3.535 (2.234)	0.506–3.742 (2.720)
Titer of IgM specific anti-HEV (mean)	0.184–2.911 (1.712)	0.209–3.338 (2.116)	0.136–1.375 (0.596)	0.124–2.356 (0.468)	0.124–3.338 (1.277)

AH, acute hepatitis; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CLD, chronic liver disease; FH, fulminant hepatitis; HEV, hepatitis E virus; HP, healthy population; IgG, immunoglobulin G; IgM, immunoglobulin M.

We obtained sera positive for both immunoglobulin (Ig) G and IgM specific anti-HEV antibodies (anti-HEV), as demonstrated by ELISA,[15] from patients with sporadic AH, FH, chronic liver diseases (CLD) and an apparently healthy population (HP) in Bangladesh. The reason why we examined anti-HEV in sera from the HP group was to assess subclinical HEV infection in Bangladesh. We measured anti-HEV in sera also from subjects with CLD. First, we wanted to determine whether or not HEV co-infection exists in sera from chronically HBV- and/or HCV-infected Bangladeshi subjects. Second, we wanted to obtain HEV-infected sera for the purpose of HEV cloning. Our next step was to investigate HEV RNA in the above sera and statistically analyze the relationships between HEV RNA-positivity and clinical parameters. We then determined nucleic and amino acid sequences and the HEV genotype prevalent in Bangladesh.

Materials and methods

Background of the study

Sera from patients with clinically diagnosed sporadic AH or FH and with CLD were collected at the Department of Gastroenterology, Internal Medicine, Bangabandhu Sheikh Mujib (BSM) Medical University (formerly the Institute of Post Graduate Medicine and Research) Hospital, Dhaka, Bangladesh, while the HP subjects were selected randomly from various walks of life of Dhaka, Bangladesh, in 1995 and 1996. Study subjects with AH, FH, CLD and HP were enrolled based on the following criteria. Inclusion criteria for sporadic AH were a typical prodrome of viral hepatitis from history, persistence or progressive jaundice of less than 6 months duration, and biochemical evidence of hepatocellular necrosis or injury. The FH criteria were presence of a typical prodrome of viral hepatitis from history and clinical observation of jaundice, biochemical evidence of severe hepatocellular injury and the presence of hepatic encephalopathy within 8 weeks of the development of jaundice. Criteria for CLD including chronic hepatitis (CH), liver cirrhosis (LC) and hepatocellular carcinoma (HCC) were persistence of viral markers of HBV, HCV or HDV for more than 6 months. Other CLD criteria were as follows: CH, persistent or intermittent jaundice for more than 6 months and history of acute hepatitis 6 or more months previously with persistence of prodromal symptoms of acute hepatitis; LC, clinical features suggestive of liver cirrhosis based on symptoms, signs, physical examination and laboratory findings; HCC, a space occupying lesion in the liver on ultrasonographic examination, elevated alpha-fetoprotein level and no other primary malignancy anywhere in the body. The study did not require separating CH, LC and HCC, therefore, the combined group of CLD was used. Criteria for the HP were to be unrelated to the patients included in this study though living in a similar hygienic environment, and to be apparently healthy and have no past history of jaundice, liver dysfunction, blood transfusion, or exposure to alcohol or other hepatotoxic drugs.

Sera were immediately frozen at BSM Medical University in Dhaka, packed in dry ice, and then transported to Japan for examination. Out of the total of 549 samples from AH, FH, CLD and HP subjects, 97 (40 AH, 14 FH, 23 CLD and 20 HP) were positive for both IgG and IgM specific anti-HEV. Among the 97 subjects, 15 samples were not suitable for the present examination due to inadequate sample volumes.

Samples

Samples from 82 subjects in total, 36 AH, 12 FH, 14 CLD and 20 HP, were used in this study. The male/female ratio was 61/21, the age range was 12–67 (mean 30.4) years, and ranges for IgG and IgM specific anti-HEV titers were 0.506–3.742 (mean 2.720) and 0.124–3.338 (mean 1.277), respectively. Positive cut-off titers for IgG and IgM specific anti-HEV were over 0.150 and 0.110, respectively.[15] Sera from AH and FH subjects examined in this study had not always been collected at the peak of disease severity. All 14 CLD subjects were positive for HBV surface antigen, and four were also positive for HCV RNA in sera. Sex, age, values of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total bilirubin, as well as titers of IgG and IgM specific anti-HEV, in the AH, FH, CLD and HP groups, are summarized in Table 1. All serum samples were collected after obtaining informed consent and appropriate approval.

Assays for IgG and IgM specific anti-HEV

IgG- and IgM-specific anti-HEV antibodies (anti-HEV) were determined by in-house enzyme-linked immunosorbent assay

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(ELISA).[15] In brief, a recombinant antigen of empty virus like particles (VLPs) of HEV was used for measurement of anti-HEV. These VLPs possess antigenicity similar to that of authentic HEV particles and are highly sensitive for detecting HEV-specific IgM and IgG antibodies. Serum samples were added to microplate wells and the reaction procedures were performed in the ordinary way. Finally, the absorbance at 492 nm was measured. Sera from hepatitis patients, previously confirmed by serological tests as well as reverse transcriptase polymerase chain reaction (RT-PCR) to be positive for hepatitis E viral infection, served as positive controls. Sera from healthy Japanese individuals, negative for both anti-HEV IgM and IgG, served as negative controls.

Detection of HEV RNA and sequencing

RNA was extracted from the sera of AH, FH, CH and HP subjects in Bangladesh using TRIzol LS (Invitrogen Corporation, Carlsbad, CA, USA) by a modification of the acid guanidinium thiocyanate-phenol-chloroform method,[16] and transcribed to complementary DNA (cDNA) using Super Script II reverse transcriptase (Invitrogen) by incubation at 42°C for 60 min. The cDNA was amplified by AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) using primers designed to amplify nucleotides 6369–6466 (length of amplified cDNA: 98 bp excluding primers) from the 5′ end of the open reading frame of HEV. Nucleic acid sequences of sense and anti-sense primers for the first PCR were GCC GAC AGA ATT GAT TTC GTC GGC (nucleotides 6295–6318) and GTC CTG CTC ATG TTG GTT GTC ATA (6496–6473), those for the second PCR CCG TCG TCT CAG CCA ATG GCG AGC (6345–6368) and CTC ATG TTG GTT GTC ATA ATC CTG (6490–6467), respectively. Nested PCR was performed as follows. Samples were incubated for denaturation at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 30 s. The first denaturation was performed for 9 min, the last extension for 7 min. The first and second PCR were carried out with 35 cycles each. One tenth of the volume of the first PCR products was used for the second PCR. The second PCR products were analyzed by electrophoresis on a 3% agarose gel, stained with ethidium bromide and examined under UV light.

DNA was extracted and purified and nucleic acid sequences were determined using an automated DNA sequencer (Applied Biosystems) in both directions in all HEV cDNA-positive samples. The amino acid sequence was then analyzed on the basis of the nucleic acid sequence. Comparisons of cDNA homology between Bangladesh clones and other representative HEV isolates were carried out. The isolate names and GenBank accession numbers of the reported sequences were as follows: M73218 from Burma, AF185822 and M80581 from Pakistan, X99441, X98292 and AF076239 from India, M94177, D11093 and AJ272108 from China, AF051830 from Nepal, AY230202 from Morocco, M74506 from Mexico and AF060668 from the United States. Phylogenetic trees were constructed by the neighbor-joining method,[17] based on the above partial nucleotide sequence in the ORF2 region of HEV. The final tree was drawn using DNASIS Pro software (Hitachi Software Engineering Co., Ltd., Tokyo, Japan).

Statistical analysis

Relationships between HEV RNA-positivity and clinical parameters were statistically analyzed. For the gender and clinical diagnosis analyses, the chi-square test was used, and for those of age, values of ALT, AST and total bilirubin and IgG and IgM specific anti-HEV titers, the Mann-Whitney U test was used. Concomitant infection was frequently found in AH and FH. These infections were assessed in 27 subjects (75.0% of 36) in the AH and 5 (41.7% of 12) in the FH group. Therefore, relationships between HEV RNA-positivity and concomitant infection were analyzed, using the chi-square test, only in the AH and FH groups. A P-value below 0.05 was considered significant.

Furthermore, two cluster groups of HEV clones were identified and the relationships of these clusters to clinical diagnoses were also statistically analyzed using the chi-square test.

Results

Ratios of the number of subjects positive for HEV RNA to those examined were 9/36 (25.0%) in the AH, 4/12 (33.3%) in the FH, 3/14 (21.4%) in the CLD and 0/20 (0%) in the HP group; 16/82 (19.5%) in total. The male/female ratios for HEV RNA-positive subjects were 7/2, 3/1 and 2/1 in the AH, FH and CLD groups, respectively; 12/4 in total. The age ranges of HEV RNA-positive subjects were 17–67 years (mean 35.8), 22–50 years (mean 31.2) and 18–60 years (mean 36.7) in the AH, FH and CLD groups, respectively, and that for all subjects was 17–67 years (mean 34.9). On one hand, there were no significant relationships between HEV RNA-positivity and either gender or clinical diagnosis (chi-square test), nor between HEV RNA-positivity and age (Mann-Whitney U test). On the other hand, significant correlations were identified between HEV RNA-positivity and ALT and AST values ($P = 0.0229$ and $P = 0.0448$, respectively, Mann-Whitney U test), while total bilirubin was not related to HEV RNA positivity.

Titers of IgG and IgM specific anti-HEV in HEV RNA-positive subjects were 0.506–3.742 (mean 2.998) and 0.192–3.338 (mean 1.994), respectively. In HEV RNA-positive subjects, mean titers of IgG specific anti-HEV in the AH, FH and CLD groups were 2.852, 3.477 and 2.798, respectively, whereas those of IgM specific anti-HEV were 1.996, 2.810 and 0.636, respectively. Significant correlations between these IgG and IgM titers and HEV RNA-positivity were demonstrated ($P = 0.0208$ and $P = 0.0095$, respectively, Mann-Whitney U test).

Concomitant infections with HEV and other hepatitis viruses were detected in 6 of 9 samples from HEV RNA-positive subjects with AH but in none of the four from HEV RNA-positive subjects with FH. Among the six AH cases, HEV was super-imposed on HBV carrier status in five, and one case with HCV infection was also a HBV carrier. On the other hand, concomitant infections were found in 21 of 27 samples from HEV RNA-negative subjects with AH, and in 5 of 8 samples from HEV RNA-negative subjects with FH. The ratio of concomitant infection with hepatitis viruses was high in both the HEV RNA-positive and the HEV RNA-negative group. There were no significant correlations between HEV RNA-positivity and concomitant infection with either AH or FH (chi-square test).

Nucleic and amino acid sequences of 16 samples are shown in Tables 2 and 3, respectively. Changes in nucleic acids showed a scattered distribution. Six subgroups (A1, A2, A3, A4, B1 and B2) of HEV clones from Bangladesh were found by nucleic acid analysis, and were classifiable into two main (A and B) groups. However, only two groups with minor changes were identified by

Table 2 Nucleic acid sequence and number of Bangladesh HEV clones found in this study

Clone	Nucleic acid sequence	Number of clones
A1	5′ CAACTGTTAAGCTGTATACATCTGTAGAGAATGCTCAGCAGGATAAGGG TATTGCAATCCCGCATGACATTGATCTTGGAGAATCTCGTGTGGTTATT 3′	6
A2	5′ ----------T---------- 3′	5
A3	5′ ----------T----------G-- 3′	1
A4	5′ ----------G---------- 3′	1
B1	5′ -G-----G-----A----------T---------- ---C----------T-----C--C----------A------ 3′	2
B2	5′ -G---------- ---C----------T-----C--C----------A------ 3′	1

Table 3 Reduced amino acid sequence and number of Bangladesh HEV clones found in this study

Clone	Reduced amino acid sequence	Number of clones
A1	TVKLYTSVENAQQDKGIAIPHDIDLGESRVVI	6
A2	----------V	5
A3	----------V	1
A4	----------	1
B1	----------	2
B2	----------	1

amino acid analysis (Table 3) and were not related to the A and B groups demonstrated by nucleic acid analysis. Homology comparisons between Bangladesh clones and other representative HEV clones with the same DNA sequence areas are summarized in Table 4. Bangladesh clones showed high homology with South Asian, Southeast Asian and Chinese clones of genotype I in comparison to those of genotype II, III and IV clones. Therefore, Bangladesh clones were recognized as belonging to genotype I, in terms of the nucleic acid sequence, rather than the other genotypes. The phylogenetic tree (Fig. 1) supports these results.

There were no significant associations between these two clusters of HEV clones (Groups A and B) and clinical diagnoses (chi-square test).

Discussion

In Bangladesh, HEV infection, serologically indicated by positivity for both IgG and IgM specific anti-HEV, was detected in adolescents and adults of both genders. In this study, HEV RNA was detected in 9 (25.0%) of 36 samples positive for both IgG and IgM specific anti-HEV in sporadic AH, in 4 of 12 (33.3%) FH, and in 3 of 14 (21.4%) CLD, but in none of the 20 sera positive for both antibodies from HP subjects with subclinical HEV infection. HEV RNA detection ratios were in the order of symptom severity for these three diseases. HEV RNA was reportedly detected in sera from AH and FH cases in an endemic HEV infection area, while co-existence of HEV infection in CLD was shown, at the nucleic acid level, to be rare. None of the HP samples in this study were positive for HEV RNA, though all samples came from an endemic HEV infection area. Mitsui *et al.*[18] reported subclinical HEV infection in healthy individuals with evidence of transient viremia using periodically collected sera. Examination using sera collected only once, rather than periodically, might not be adequate to detect HEV RNA in HP. Mean titers of IgM specific anti-HEV in the AH, FH, CLD and HP groups were 1.712, 2.116, 0.596 and 0.468, respectively. That of the HP group was lower than those of the AH and FH groups, both of which had marked clinically symptomatic HEV infection. However, these titers did not differ significantly between the CLD and HP groups. The reason for no HEV RNA-positive cases being detected in the HP group was unclear, though a few samples examined in this study did have high titers of IgM specific anti-HEV. These findings might be attributable to HEV RNA disappearing much earlier than IgM specific anti-HEV in HP sera. HEV is considered to persist longer in stool than in sera. Therefore, if examinations were performed using stool collected at the same time as sera, from subjects with HP, HEV RNA might be detectable in HP. Other possibilities include a cross-reactive or a somewhat more remote infection affecting the anti-HEV results. Finally, HEV RNA-positivity was significantly related to four parameters, values of ALT and AST and titers of IgG and IgM specific anti-HEV. Of these four parameters, the *P*-value for IgM specific anti-HEV was by far the smallest, below 0.01, indicating the IgM specific anti-HEV titer to be the parameter with the most significant relationship to HEV RNA-positivity. The duration of anti-HEV IgM-positivity in sera is about 100 days according to Li *et al.*,[15] while that of HEV RNA is 40 days at the longest according to Takahashi *et al.*[19] Serum positive for IgM specific anti-HEV is thus not always positive for HEV RNA. Therefore, the time elapsed between HEV infection and blood collection was considered to be a significant factor in HEV RNA detection. Concomitant infection is characteristic of areas of endemic HEV infection such as South Asia, in contrast to industrialized countries. Therefore, relationships between HEV RNA-positivity and concomitant infection in AH and FH cases are of interest in areas of endemic HEV infection, though no significant correlations with either AH or FH were detected using the chi-square test in this study. Further studies are required to investigate HEV and concomitant infections.

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Table 4 Homology percentages within Bangladesh clones, and between Bangladesh clones and previously reported HEV clones. Figures in parentheses are GenBank accession numbers

		Bangladesh clones					
		A1	A2	A3	A4	B1	B2
Bangladesh clones	A1	100					
	A2	98.97	100				
	A3	97.95	98.97	100			
	A4	98.97	97.95	96.93	100		
	B1	90.81	89.79	88.77	90.81	100	
	B2	93.98	92.85	91.83	92.85	96.93	100
Genotype I	Burma B1 (M73218)	95.91	94.89	93.87	94.89	92.85	95.91
	Pakistan Abb-2B (AF185822)	95.91	94.89	93.87	94.89	92.85	95.91
	India 2 Madras (X99441)	95.91	94.89	93.87	94.89	90.81	93.87
	Nepal TK1592 (AF051830)	94.89	93.87	92.85	93.87	95.91	98.97
	China HeBei (M94177)	93.87	92.85	91.83	92.85	92.85	95.91
	India hev037 (X98292)	93.87	92.85	91.83	92.85	90.81	93.87
	India Hyderabad (AF076239)	93.87	92.85	91.83	92.85	92.85	95.91
	China Uigh179 (D11093)	92.85	91.83	90.81	91.83	90.81	93.87
	Pakistan SAR-55 (M80581)	91.83	90.81	89.79	92.85	91.83	93.87
	Morocco (AY230202)	89.79	88.77	87.75	88.77	87.75	89.79
Genotypes II, III & IV	Mexico (M74506) Genotype II	84.69	83.67	82.65	85.71	81.63	80.61
	US1 (AF060668) Genotype III	76.53	75.51	74.48	76.53	75.51	78.57
	China (AJ272108) Genotype IV	77.55	78.57	77.55	77.55	79.59	73.46

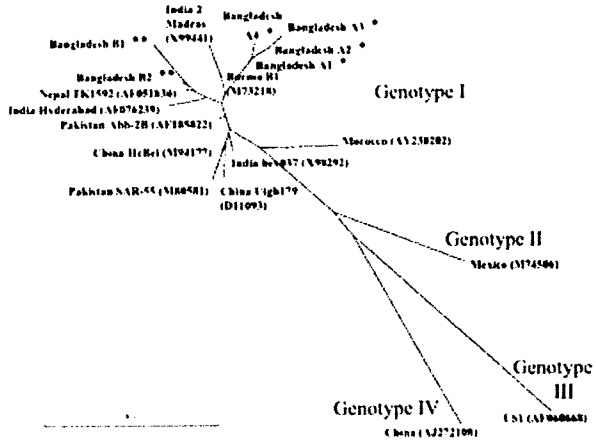

Figure 1 Phylogenetic tree analyses: * and ** indicate Bangladesh HEV clones, groups A and B, respectively. Isolates classified as genotype I are shown in the upper left area, those of genotypes II, III and IV in the lower right area. GenBank accession numbers are in parentheses. The bar in the lower left area indicates genetic distance.

Six different subgroups, that is clones with minor changes, were detected in 16 HEV Bangladesh clones by nucleic acid analysis. However, only two groups were identified by amino acid analysis, indicating most nucleic acid changes to be silent in these clones. Several HEV genotype nomenclatures[20–22] have been proposed based on the phylogenetic relationships of various HEV isolates. In this study, the widely recognized genotype I-IV system[22] was used. All six distinct clones found in this study were classified as genotype I, the most prevalent HEV genotype in South Asia. According to homology comparisons and phylogenetic tree analysis of the six clones, Bangladesh Group A (A1, A2, A3 and A4) clones were similar to the clone groups of Burma (M73218) and India (X99441), while the Group B (B1 and B2) clones resembled those of Nepal (AF051830) and India (AF076239) in genotype I. Thus, Bangladesh HEV isolates in this study were divided into two groups (A and B). The possibility was raised that two distinct HEV origins within genotype I are prevalent in Bangladesh. However, the nucleic acid sequences analyzed in this study were not particularly long. Therefore, this finding is not conclusive. However, different genotypes or subgenotypes were reportedly found in the same area, for example, genotypes III and IV in Japan,[23] genotypes I and II in Namibia[24,25] and genotypes I and IV in China.[21,26] Therefore, further studies of the genetic diversity of Bangladesh HEV are anticipated. Most Bangladesh Group A clones were derived from AH and FH patient sera, whereas Group B clones were mainly derived from CLD sera. In view of the clinical diagnosis, the severities of disease symptoms in the two groups apparently differed. However, there were no statistically significant relations between isolate clusters and clinical diagnosis.

We have presented herein six partial sequences of Bangladesh HEV clones, for comparison with the many HEV isolates identified in other countries. To our knowledge, this is the first study focusing on the analysis of previously unknown Bangladesh HEV isolates.

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NOTE *Virology*

Mice are Not Susceptible to Hepatitis E Virus Infection

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ABSTRACT. To determine whether or not mice are susceptible to hepatitis E virus (HEV) infection, C57BL/6 mice were experimentally infected with genotypes 1, 3 and 4 HEV by intravenous injection. Serum and stool samples were collected and used to detect HEV RNA and anti-HEV antibodies by RT-PCR and ELISA. The virus infection was monitored up to two months after inoculation; however, none of the serum or stool samples was positive for virus replication, demonstrating that C57BL/6 mice were not susceptible to HEV.

KEY WORDS: C57BL/6, hepatitis E, hepatitis E virus, HEV, mouse.

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Hepatitis E is a serious public health concern in many developing countries, and recognized as sporadic and endemic acute hepatitis in many industrialized countries. Pregnant women have a high risk associated with hepatitis E, with a high mortality rate (up to 20%) [5, 25]. The causative agent of hepatitis E is hepatitis E virus (HEV), and this virus transmits primarily via the fecal-oral route through contaminated drinking water [1, 6]. HEV is the sole member of the genus *Hepevirus* in the family *Hepeviridae*. HEV is a small round non-enveloped virus, 27–34 nm in diameter, containing an RNA genome approximately 7.2 kb in length [2, 3]. The RNA consists of a single-strand RNA molecule containing three discontinuous and partially overlapping open reading frames (ORFs). The 3' terminus of the RNA is polyadenylated. HEV isolates were grouped into at least four major genotypes, genotypes 1, 2, 3 and 4 (G1, G2, G3 and G4) on the basis of nucleotide and deduced amino acid sequences [3, 6, 24]. Because G3 and G4 HEV were isolated from pigs and wild boars in addition to humans, and much direct and indirect evidence has indicated that HEV transmits from pigs or wild boars to humans, hepatitis E is recognized as a zoonotic disease [8, 18, 23]. Many studies have reported the detection of HEV RNA and the HEV-specific antigen (HEV-Ag) in pig and wild boar stool and serum specimens, and suggested the active circulation of this virus among these animals [18, 20, 26]. HEV-specific antibodies have been detected in many animals including sheep, cows, dogs, cats, wild rats, wild deer and mongoose, in addition to pigs and wild boars [9, 12, 14, 15, 19]. However, it is obscure whether or not HEV substantially replicates in these animals. In this study we infected C57BL/6 mice with G1, G3 and G4 HEV, and monitored the virus growth to determine the susceptibility of mice to HEV infection.

G1 HEV strain was derived from stool specimens from a cynomolgus monkey (*Macaca fascicularis*), born and grown in the Tsukuba Primate Center for Medical Science, National Institute of Infectious Diseases (NIID), which had been experimentally infected with an Indian strain [10]. The G3 HEV strain (DQ079632) was derived from stool specimens collected on a pig farm in Japan. The G4 HEV strain (DQ079628) was from a stool specimen collected from a wild boar caught in Aichi prefecture, Japan. The stool specimens were used to prepare 10% (w/v) suspensions as described [10]. These suspensions were positive for HEV RNA by reverse-transcription polymerase chain reaction (RT-PCR). The concentrations of the G1, G3, and G4 HEV were 5×10^4, 2×10^4 and 1×10^5 copies per one m*l* of suspension, respectively, by real time RT-PCR (unpublished).

To confirm the infectivity of these stool specimens, 3 cynomolgus monkeys (4 year-old males) were inoculated intravenously with 2 m*l* of one of the suspensions, and the stools were collected daily, and used to detect HEV RNA and HEV-Ag. Sera were collected weekly before and after the inoculation to detect HEV RNA, HEV-Ag, and HEV-specific IgG antibodies. The sera were also used to determine ALT values. All monkey experiments were reviewed by the Institute's ethical committee and carried out according to "Guides for animal experiments performed at NIID" under codes 990058, 000019 and 504006. The primates were individually housed in BSL-2 facilities. Detection of HEV RNA, HEV-Ag, and IgG has been described previously [8, 10, 11]. The ALT value was measured as described [10]. As shown in the figure, HEV RNA and HEV-Ag were detected within one week in the sera (A) and stools (B) of all three monkeys after inoculation, and ALT values increased more than three-fold compared with that of pre-inoculation in infected monkeys, though the increase was slow and the values were low in G1 HEV- and G3 HEV-infected animals (C), indicating that all three HEV strains, G1, G3 and G4, were infectious. Furthermore, drastic increases of IgG antibody titers, probably due to extensive replication of the virus, were demonstrated in these animals (D). These results confirmed that the HEVs used in these experiments were indeed infectious.

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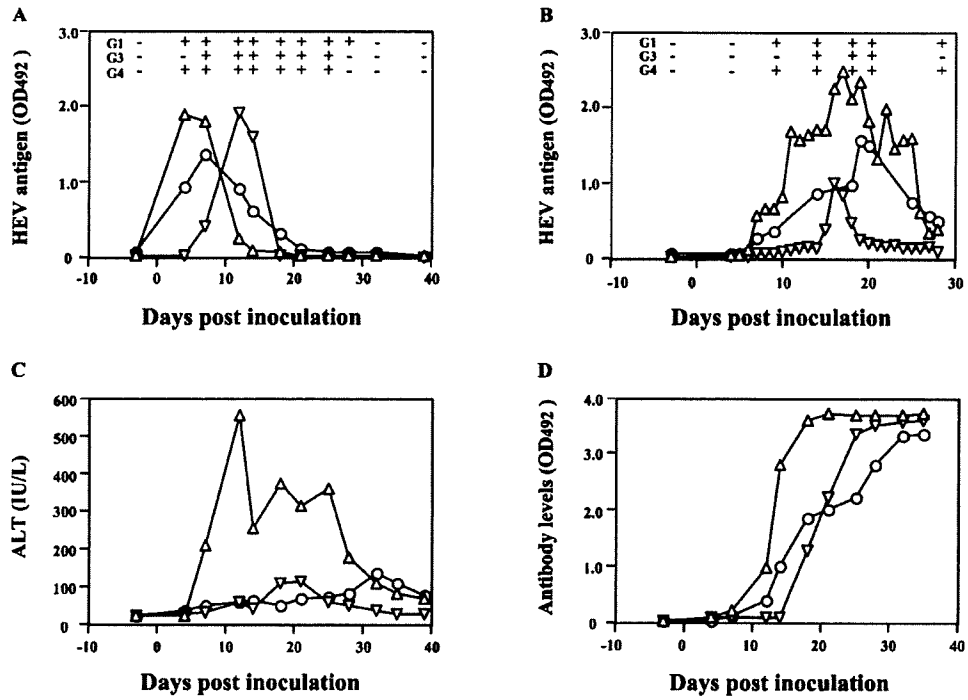

Fig. 1. Kinetics of biochemical, serological, and virological markers in monkeys after inoculation. HEV-Ag and HEV RNA in sera (A) and stools (B), was measured by an antigen ELISA and RT-PCR. ALT was indicated (C), and IgG antibody was measured by an antibody-ELISA (D). ○, monkey inoculated with G1 HEV; ▽, monkey inoculated with G3 HEV; △, monkey inoculated with G4. HEV RNA was monitored by RT-PCR . +, Positive; −, Negative.

Six 4-week-old and five 9-month-old C57BL/6 mice, negative for anti-HEV antibody and HEV RNA, were inoculated intravenously with 100 μl of 10% fecal suspensions. Serum and fecal samples were obtained at 1 week before, and at 1, 2, 3, 4, 6, 8, and 10 weeks after inoculation, and HEV RNA and anti-HEV IgG antibodies were measured. However, neither serum nor fecal specimens were positive for HEV infection, clearly indicating that HEV did not replicate in C57BL/6 mice (data not shown). In other words, the C57BL/6 mice were not susceptible to hepatitis E virus.

Anti-HEV IgM and/or IgG antibody and HEV RNA are frequently detected in pigs and wild boars in various countries, and these 2 animals are recognized as the main reservoirs of HEV. Although the infection is asymptomatic when G3 and G4 HEVs are used to inoculate pigs, it is obvious that pigs are susceptible to HEV infection [17]. Interestingly, pigs were resistant to experimental infection with G1 and G2 HEVs [16]. Although experimental data is not available for wild boars, these animals are genetically close to pigs, and wild boars are likely to be susceptible to HEV. Direct and indirect evidence of HEV transmission from wild boars and pigs to humans has been reported in Japan, suggesting that these animals are the main zoonotic reservoirs in this country [8, 27]. Chimpanzees, rhesus monkeys, cynomolgus monkeys, and marmosets have been used for experimental infection and to evaluate the efficacy of HEV vaccines, and HEV has been used as a challenge virus, indicating that these monkeys are susceptible to HEV infection [13, 22, 29, 30]. In addition to these animals, anti-HEV IgG antibody has been detected in dogs, cats, cows, goats, sheep, and rodents including rats [4, 7, 14, 19], and anti-HEV IgG antibody and HEV RNA were detected from mongoose and wild deer [9, 21, 28]. However, the susceptibility of these animals to HEV infection has not been fully evaluated, and whether or not HEV replicates *in vivo* in these animals is unknown.

We evaluated the susceptibility of B57C/6 mice by directly inoculating infectious HEV through intravenous injection. Although two different age groups, at 4 weeks and 9 months, were used, none of the mice was successful in producing *in vivo* HEV replication. Our study clearly demonstrated that C57BL/6 mice are resistant to HEV infection. By contrast, our preliminary results indicated that HEV is capable of replicating in chimeric mice harboring replaced human hepatocyte cells when exactly the same amount of the G1, G3 and G4 HEV suspension is used (manuscript in preparation). These results indicate that the human hepatocyte is a major target cell for HEV infection, and HEV is not capable of replicating in mice.

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