Genetic changes in hepatitis E virus of subtype 1a in patients with sporadic acute hepatitis E in Kathmandu, Nepal, from 1997 to 2002

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To investigate the genetic changes in hepatitis E virus (HEV) strains in the Kathmandu valley of Nepal, we compared the 412 nt sequence within open reading frame 2 of HEV among HEV isolates recovered from 16 patients in 1999, 14 patients in 2000 and 38 patients in 2002, and additional isolates recovered from 48 patients in 1997 whose nucleotide sequences have been previously published. All 116 HEV-viraemic samples were genotyped as 1 and subtyped further as 1a (n = 85, 73%), 1c (n = 29, 25%) and mixed infection of 1a and 1c (n = 2, 2%): subtype 1c was detected only in 1997. Among the 1a isolates, nucleotide sequence identity with the representative 1a isolate of Ne131-1997 was 96 ± 4% in 1997, 93 ± 1% in 1999, 92 ± 1% in 2000 and 91 ± 0% in 2002, indicating gradual diversification of HEV sequences. When phylogenetic analysis of the 87 subtype 1a isolates was performed, they further segregated into five clusters, with two predominant clusters of 1a-2 and 1a-3: the annual frequency of cluster 1a-2 isolates decreased from 63% in 1997, to 50% in 1999, to 7% in 2000 and no cases in 2002; cluster 1a-3 isolates were observed in all four years and its annual frequency increased from 5% in 1997 to 95% in 2002. Of the remaining three clusters, cluster 1a-1 was detectable only in 1997 and clusters 1a-4 and 1a-5 emerged in 2000 and 2002, respectively. These results indicate that genetic changes and takeover of HEV strains may contribute to the genetic variability of HEV in the community.

INTRODUCTION

Hepatitis E virus (HEV) frequently causes epidemics of acute hepatitis (hepatitis E) in many developing countries where sanitation is suboptimal. The virus is endemic in much of Asia and Africa, and one epidemic in Mexico has been documented (Purcell & Emerson, 2001). Epidemics of HEV infection have generally been caused by faecal contamination of drinking water and therefore they tend to occur during the rainy season in endemic countries such as Nepal. In the Kathmandu valley of Nepal, sporadic acute hepatitis E occurs year-round with a marked increase in incidence during the rainy season (June–August) (Kane et al., 1984; Clayson et al., 1997; Shrestha, 1987, 1991).

HEV is an unclassified non-enveloped virus. Its genome is a single-stranded, positive-sense RNA of approximately 7.2 kb. It consists of a short 5’ untranslated region (5’UTR) followed by three partially overlapping open reading frames (ORF1, ORF2 and ORF3) and then a short 3’UTR terminated by a poly(A) tract (Reyes et al., 1990; Tam et al., 1991). Although only one serotype has been recognized, extensive genomic diversity has been noted among HEV isolates, and HEV sequences have tentatively been classified into four genotypes (genotypes 1–4); the isolates in genotype 1 are further segregated into five subgroups (subtypes 1a–e) (Wang et al., 1999). Recent reports indicate that domestically infected hepatitis E also occurs among individuals in industrialized countries where hepatitis E had been believed to be non-endemic (Harrison, 1999; Purcell & Emerson, 2001; Smith, 2001). The majority of HEV infections in developing countries are caused by genotype 1 and only isolated cases of infection...
with HEV of genotype 3 or 4 have been described in industrialized nations (Schlauder & Mushahwar, 2001).

Although the prevalence of HEV infection has been reported in many developing and industrialized countries, the genetic changes that occur in HEV strains in a community over time are poorly understood. In the present study, the genomic variability among HEV strains isolated from 68 HEV-viraemic patients with acute hepatitis who were seen at a city hospital in the Kathmandu valley in 1999, 2000 and 2002 was investigated and compared with our previous data on HEV strains isolated from patients at the same hospital in 1997 (Shrestha et al., 2003) to understand better the molecular epidemiology of HEV in Nepal.

METHODS

Sera from patients with acute hepatitis. Serum samples were obtained from a total of 280 consecutive patients (213 men, 67 women; mean age ± SD 29 ± 15 years; range 3–77 years) who were clinically diagnosed as having acute hepatitis during the periods from January to September 1999, from January to December 2000 and from April to October 2002. All patients received care at Bir Hospital, a city hospital in the Kathmandu valley of Nepal. This study included patients with an acute illness who presented with clinical signs or symptoms of hepatitis such as jaundice, dark urine, general fatigue, anorexia, nausea, vomiting and fever and who had a serum alanine aminotransferase level that was at least 2-5 times the upper limit of normal. Excluded from this study were patients with alcoholic liver disease, those with a history of blood transfusion within 6 months of the onset of the disease, those with a history of exposure to hepatotoxic drugs or chemicals and those with a past history of liver disease. Serum samples were obtained at the first visit and were stored at −20°C until they were tested for various serological markers of hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV) or HEV infection. This study was approved by the Committee of Ethics of the hospital and tested individuals gave informed consent. Sera from the patients were tested for IgM antibody against HAV (anti-HAV IgM) (IMX HAVAB-M; Abbott Japan) and for the IgM class of anti-HBc antibody (anti-HBc IgM) (IMx CORE-M; Abbott Japan) by ELISA. Antibodies to HCV (anti-HCV IgM) (IMx HAVAB-M; Abbott Japan) and for the IgM class of anti-HEV antibody (anti-HEV IgM and anti-HEV IgG) were assayed by the haemagglutination method (Abbott HCV PHA-II; Dainabot) and patients with anti-HCV were assayed for HCV RNA by RT-PCR using primers derived from well-conserved areas of the 5’UTR of the HCV genome (Okamoto et al., 1994).

Detection of IgM and IgG classes of antibodies to HEV. To detect anti-HEV IgG and anti-HEV IgM, ELISA was performed using purified recombinant ORF2 protein of HEV genotype 4 that had been expressed in the pupae of silkworm, as described previously (Mizuo et al., 2002). The absorbance of each sample was read at 450 nm. The cut-off value used for the anti-HEV IgG assay was 0-152 and that for the anti-HEV IgM assay was 0-353. Test samples with absorbance values for anti-HEV IgG and anti-HEV IgM equal to or greater than the respective cut-off values were considered positive for anti-HEV.

Detection of HEV RNA. RTC-PCR was performed for detection of HEV RNA. Total RNA was extracted from 100 µl serum, reverse transcribed and then subjected to nested PCR with the ORF2 primers as described previously (Mizuo et al., 2002). The size of the amplification product of the first-round PCR was 506 bp and that of the second-round PCR was 457 bp. The nested RT-PCR assay was performed in duplicate and reproducibility was confirmed. The specificity of the RT-PCR assay was verified by sequence analysis as described below. The sensitivity of the RT-PCR assay was comparable with that of the method reported by Clayson et al. (1995a).

Sequence analysis of PCR products. The amplification products were sequenced directly on both strands using the BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems). Sequence analysis was performed using Genetyx-Mac version 12.0.6 (Genetyx Corp.) and DNASTAR SeqMan II (DNASTAR, Madison, WI, USA) (Thompson et al., 1994). The phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) based on the partial nucleotide sequence of the ORF2 region (412 nt). Bootstrap values were determined on 1000 resamplings of the datasets (Felsenstein, 1985). The final tree was obtained using the TreeView program (version 1.6.6) (Page, 1996).

RESULTS

Aetiology of acute hepatitis

In the present study, the prevalence of hepatitis virus infections among 280 patients with acute hepatitis who were seen at Bir Hospital in Nepal during the indicated periods in 1999, 2000 and 2002 was investigated by testing serum samples for the IgM class of antibodies against hepatitis viruses (HAV, HBV and HEV) and for the RNAs of HCV and HEV. The results are summarized in Table 1. Table 1 also shows the prevalence of hepatitis virus infection among 154 patients with acute hepatitis who were seen at the same hospital in 1997 (Shrestha et al., 2003). Hepatitis A (anti-HAV IgM positive) was diagnosed in only 8, 3 and 3% of the patients with acute hepatitis in 1999, 2000 and 2002, respectively, which were similar to the prevalence of 3% among patients with acute hepatitis in 1997 (Shrestha et al., 2003). Type B acute hepatitis (anti-HBc IgM positive) was diagnosed in 10, 12 and 3 patients in 1999, 2000 and 2002, respectively, with a slightly higher prevalence in 1999 (15%) and 2000 (15%) compared with that in 1997 (3%) and 2002 (2%). Both anti-HCV and HCV RNA were detectable in one, four and one patients in 1999, 2000 and 2002, respectively, and in four patients in 1997, suggesting that they contracted type C acute hepatitis, although we could not rule out the possibility that they were persistent HCV carriers. Anti-HEV IgM was detected in 47% (n = 58) of the patients in 2002, which was similar to its prevalence in 1997 (30% or 77/254), but it was detected in only 21% (n = 16) and 19% (n = 15) of the patients in 1999 and 2000, respectively. Similarly, HEV RNA was detected in 31% (n = 38) of the patients in 2002, which was the same frequency as that in 1997 (31% or 48/154), but it was detected in only 21% (n = 16) and 18% (n = 14) of the patients in 1999 and 2000, respectively. Among the 116 patients with HEV RNA, 23 (20%) were negative for anti-HEV IgM (nine in 1997, seven in 1999, two in 2000 and five in 2002); four of these 23 patients had neither anti-HEV IgG nor anti-HEV IgM (two in 1997 and...
two in 2002). One hundred and sixty-six patients were positive for anti-HEV IgM, but 73 (44 %) of them had no detectable HEV RNA. As a result, hepatitis E was diagnosed in 189 patients (44 %). One hundred and ninety-three patients (44 %) (34 % in 1997, 52 % in 1999, 58 % in 2000 and 43 % in 2002) were serologically negative for all known markers of hepatitis viruses and they were tentatively categorized as ‘non-A to E’ cases in the present study.

**Distribution of HEV subtypes**

In an attempt to analyse the genomic heterogeneity of HEV and to compare the distribution of HEV genotypes/subtypes in 1997, 1999, 2000 and 2002 in Nepal, a portion of ORF2 of HEV RNA corresponding to nt 5944–6355 of the Nepali isolate of TK15/92 (AF051830) was directly sequenced in the amplification products from the 68 HEV-viraemic patients in 1999, 2000 and 2002. The previously reported HEV strains isolated in 1997 (Shrestha et al., 2003) were renamed with the original name followed by a hyphen and then the year of isolation (1997); for example, Ne014 was changed to Ne014-1997.

All 116 HEV RNA-positive samples were homologous to known genotype 1 isolates including a Nepali isolate (TK15/92) and they were subtyped further as 1a (n=85, 73 %), 1c (n=29, 25 %) and mixed infection of 1a and 1c (n=2, 2 %): subtype 1c was detected only in 1997. Fig. 1 depicts the phylogenetic tree that was constructed based on the 412 nt ORF2 sequence of the 68 HEV isolates obtained from patients in 1999, 2000 and 2002 in the present study and the reported 50 isolates obtained from patients at the same hospital in 1997, including two clones each from two patients with mixed subtype (1a and 1c) (Shrestha et al., 2003), as well as 16 reported isolates of genotype 1 whose common 412 nt sequence is known, using a Mexican isolate of genotype 2 as an outgroup. Among the 50 isolates obtained from patients at Bir Hospital in 1997, other than the isolates from the two patients who were co-infected with HEV 1a and HEV 1c, 17 isolates were classified into subtype 1a and 29 isolates were classified into subtype 1c. The 68 isolates obtained from patients in 1999, 2000 and 2002 were classified into subtype 1a, suggesting that only a single subtype of HEV was circulating in the Kathmandu valley of Nepal in 1999 and thereafter.

Among the 87 subtype 1a isolates, the nucleotide sequence identity with the representative 1a isolate of Ne131-1997 was 96.4±2.4 % (mean±SD) in 1997, 93.9±1.7 % in 1999, 92.2±1.0 % in 2000 and 91.7±0.5 % in 2002 (Table 2), indicating gradual diversification of the HEV sequence during the 5 year period.

**Prevalence of five HEV 1a clusters**

As shown in Fig. 1, the 87 Nepali HEV strains of subtype 1a isolated in 1997, 1999, 2000 and 2002 were further grouped into five clusters, which were tentatively designated in the present study as 1a-1, 1a-2, 1a-3, 1a-4 and 1a-5, with the two predominant clusters being 1a-2 and 1a-3. Cluster 1a-1 comprised six strains isolated in 1997; cluster 1a-1 comprised six strains isolated in 1997; cluster 1a-3 comprised 29 strains isolated in 1999; cluster 1a-4 comprised 17 strains isolated in 2000; and cluster 1a-5 comprised 33 strains isolated in 2002.

### Table 1. Demographic and viral features of patients with acute hepatitis who were seen at Bir Hospital in Nepal between 1997 and 2002

<table>
<thead>
<tr>
<th>Feature</th>
<th>1997 (n=154)†</th>
<th>1999 (n=77)</th>
<th>2000 (n=79)</th>
<th>2002 (n=124)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean years±SD)</td>
<td>29±13</td>
<td>29±17</td>
<td>30±17</td>
<td>29±12</td>
</tr>
<tr>
<td>Male</td>
<td>103 (67%)</td>
<td>60 (78%)</td>
<td>61 (77%)</td>
<td>92 (74%)</td>
</tr>
<tr>
<td>Aetiology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HAV</td>
<td>4 (3%)</td>
<td>6 (8%)</td>
<td>2 (3%)</td>
<td>4 (3%)</td>
</tr>
<tr>
<td>HBV</td>
<td>4 (3%)</td>
<td>10 (13%)</td>
<td>12 (15%)</td>
<td>3 (2%)</td>
</tr>
<tr>
<td>HCV</td>
<td>4 (3%)</td>
<td>1 (1%)</td>
<td>4 (5%)</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>HEV</td>
<td>86 (56%)</td>
<td>23 (30%)</td>
<td>17 (22%)</td>
<td>63 (51%)</td>
</tr>
<tr>
<td>Anti-HEV IgM (+)</td>
<td>77 (50%)</td>
<td>16 (21%)</td>
<td>15 (19%)</td>
<td>58 (47%)</td>
</tr>
<tr>
<td>Anti-HEV IgG (+)</td>
<td>124 (81%)</td>
<td>62 (81%)</td>
<td>50 (63%)</td>
<td>83 (67%)</td>
</tr>
<tr>
<td>HEV RNA (+)</td>
<td>48 (31%)</td>
<td>16 (21%)</td>
<td>14 (18%)</td>
<td>38 (31%)</td>
</tr>
<tr>
<td>Non-A to E</td>
<td>53 (34%)</td>
<td>40 (52%)</td>
<td>46 (58%)</td>
<td>53 (43%)</td>
</tr>
</tbody>
</table>

*Serum samples were obtained from patients who were clinically diagnosed as having acute hepatitis during the periods from January to September 1999, from January to December 2000 and from April to October 2002.
†Adopted from our previous data (Shrestha et al., 2003). Anti-HAV IgM and anti-HBc IgM were retested by the same commercial kits as those used for serum samples obtained in 1999, 2000 and 2002 and the same results were obtained as in the previous study.
Table 2. Distribution of HEV subtypes among 116 HEV-viraemic patients who were seen between 1997 and 2002

<table>
<thead>
<tr>
<th>HEV subtype</th>
<th>1997 (n=48)</th>
<th>1999 (n=16)</th>
<th>2000 (n=14)</th>
<th>2002 (n=38)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>17 (35%)</td>
<td>16 (100%)</td>
<td>14 (100%)</td>
<td>38 (100%)</td>
</tr>
<tr>
<td>1c</td>
<td>29 (60%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1a + 1c</td>
<td>2 (4%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Mean ± SD of nucleotide sequence identity with the Ne131-1997 isolate.
†Mean ± SD of nucleotide sequence identity with the Ne137-1997 isolate.

Table 3. Distribution of HEV 1a clusters among 87 patients infected with HEV 1a who were seen between 1997 and 2002

<table>
<thead>
<tr>
<th>HEV 1a cluster</th>
<th>1997 (n=19)</th>
<th>1999 (n=16)</th>
<th>2000 (n=14)</th>
<th>2002 (n=38)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a-1</td>
<td>6 (32%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1a-2</td>
<td>12 (63%)</td>
<td>8 (50%)</td>
<td>1 (7%)</td>
<td>0</td>
</tr>
<tr>
<td>1a-3</td>
<td>1 (5%)</td>
<td>8 (50%)</td>
<td>11 (79%)</td>
<td>36 (95%)</td>
</tr>
<tr>
<td>1a-4</td>
<td>0</td>
<td>2 (14%)</td>
<td>1 (3%)</td>
<td>1 (3%)</td>
</tr>
<tr>
<td>1a-5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Mean ± SD of nucleotide sequence identity with the Ne131-1997 isolate.
†Mean ± SD of nucleotide sequence identity with the Ne026-1997 isolate.
‡Mean ± SD of nucleotide sequence identity with the Ne146-1997 isolate.
§Mean ± SD of nucleotide sequence identity with the Ne059-2000 isolate.
DISCUSSION

In our previous study conducted among 154 patients who developed sporadic acute hepatitis in the Kathmandu valley of Nepal in 1997, 86 (56%) of the patients studied were diagnosed as having hepatitis E. In contrast, in the present study, only 30% (23/77) and 22% (17/79) of the patients were diagnosed with hepatitis E in 1999 and 2000, respectively, although hepatitis E accounted for 51% (63/124) of the patients in 2002. One of the reasons for this fluctuating prevalence of hepatitis E in Kathmandu, Nepal, could be the small sample size for the 1999 and 2000 cases. However, the changing epidemiological pattern of HEV infection in Nepal may result in fewer clinical cases and a lower potential for outbreaks. Whether this changing pattern is related to high genetic variability of HEV, changes in hygiene conditions, the size of the susceptible population or a combination of these and other factors remains to be established. Nevertheless, it is presently beyond doubt that hepatitis E is the predominant type of acute hepatitis in Nepal, highlighting the importance of hepatitis E as a public health problem in this geographic region.

In contrast with the results of the study of Clayson et al. (1995b) who reported that eight (11%) of the 76 patients with acute hepatitis in Nepal in 1993 were without any evidence of HAV, HBV, HCV, or HEV infection (collectively, non-A to E), 43-58% of the patients from 1999 to 2002 in the present study had no serological or molecular markers of HAV, HBV, HCV or HEV, comparable with that in our previous study on patients with acute hepatitis in 1997 (34%) (Shrestha et al., 2003). The reason for this difference is unclear. However, the data in our studies were based on single serum samples and those of Clayson et al. (1995b) were based on studies of replicate serum and faecal samples. Considering the transient occurrence of the acute markers of hepatitis viruses, the possibility that the higher percentage of non-A to E cases observed in our studies might be attributed in part to higher false negatives cannot be ruled out. As for other possibilities, the involvement of a third, previously unrecognized, enterically transmitted hepatitis agent, whose existence was suggested by Arankalle et al. (1994), and non-viral agents such as Gram-negative bacilli that are known to cause hepatitis (El-Newihi et al., 1996), need to be taken into consideration in some patients with acute hepatitis of unknown aetiology, since the clinical picture, age distribution and seasonal distribution of patients with non-A to E hepatitis in Nepal were similar to those of patients with hepatitis E (data not shown). The observed difference in the prevalence of hepatitis E between 1993 and 2002 may also be attributable to the changing epidemiological pattern(s) of acute hepatitis in the Kathmandu valley of Nepal.

In order to gain insight into the genetic variability and mode of evolution of HEV in Nepal, we performed an analysis of the sequence data of the ORF2 region of HEV strains isolated in 1999, 2000 and 2002 in Nepal. All HEV strains seen in the Kathmandu valley, including those obtained from patients in 1997, were of genotype 1 and were subgrouped further into subtypes 1a and 1c: HEV 1c was identified in 1997, but not in 1999 and thereafter. The presence of HEV 1a among individuals infected during the hepatitis outbreaks in Nepal between 1987 and 1995 has previously been reported by Gouvea et al. (1997, 1998) and Shrestha et al. (2003), but HEV 1c had not been identified in Nepal. To date, HEV 1c has been isolated in India and mainland China (Wang et al., 1999), suggesting that the 1c strain(s) was imported from India or China to Nepal in 1997 or before, but was taken over by the co-circulating 1a strains in 1999. As HEV 1a strains were isolated from patients in all four years studied, comparative sequence analysis was restricted to HEV 1a isolates. When the 412 nt ORF2 sequences of 87 HEV 1a isolates obtained in our previous and present studies were compared with that of the representative 1a isolate (Ne131-1997), the nucleotide sequences of the 1a isolates recovered from patients in each of the four years studied were found to have gradually diverged from 96.4% in 1997 to 91.7% in 2002. An amino acid substitution emerged only among the 2002 1a strains and, among the 38 1a strains isolated in 2002, 24 (63%) had a single non-silent mutation (Thr to Ser) and 33-36 silent mutations, and two (5%) had a non-silent mutation (Thr to Ala) and 36 silent mutations on comparison with the representative 1a isolate of Ne131-1997. The remaining 61 isolates of subtype 1a, including the Ne131-1997 isolate, had an identical amino acid sequence, indicating that the majority of mutations observed in the 1a isolates were silent mutations resulting in no change in the amino acid sequence. This suggests that the pattern of divergence observed in a part of the ORF2 gene encoding a capsid protein is due to selective forces that do not allow amino acid substitutions, as was reported for the HAV VP1 protein (Costa-Mattioli et al., 2002). Only a single serotype is recognized for HAV, although HAV strains are classified into multiple genotypes, similar to HEV (Hollinger & Emerson, 2001). As a consequence, the mode of evolution of HEV may partly explain the presence of only one serological group of HEV.

Eighty-seven Nepali HEV 1a isolates obtained in the previous and present studies were further grouped into five clusters (1a-1 to -5). The observed changing prevalence of the five 1a clusters according to the year of disease onset suggests that the genetic variability of HEV in a community is due to continuously occurring genetic changes and that takeover of existing strain(s) possibly occurs by the selected variant having an advantage in transmission in the community or variant(s) that was imported from other communities. An alternative hypothesis for the variability observed in field isolates is ongoing evolution in alternative host species, since increasing evidence has indicated that hepatitis E is a zoonosis (Harrison, 1999; Erker et al., 1999; Meng, 2000; Meng et al., 1997, 1998; Halbur et al., 2001; Okamoto et al., 2001; Smith, 2001; Takahashi et al., 2003; Tei et al., 2003), whether or not hepatitis E is common in the resident human population.
In conclusion, genetic variability was observed among HEV strains and even among HEV strains of the same subtype (1a) isolated from patients with acute hepatitis E in Nepal in all observed years of 1997, 1999, 2000 and 2002. HEV continuously evolves in infected individuals in Nepal, leading to genomic variability of HEV in the community. The fact that no significant amino acid substitutions were recognized in the HEV strains isolated during a 5 year period indicates that genomic mutations of HEV may occur naturally in infected individuals without immunological pressure from the host and that selective forces that do not allow amino acid substitutions may be involved in the observed pattern of divergence. Taking into account that partial sequencing of a selected genomic region was employed, a definitive picture of the biological significance of these and other possible changes in the entire genome needs to be obtained from more in-depth studies.

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