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Seroepidemiology of water-borne hepatitis in India and evidence for a third enterically-transmitted hepatitis agent

VIDYA A. ARANKALLE*†, MANDEEP S. CHADHA*, SERGEI A. TSAREV‡, SUZANNE U. EMERSON‡, ARUN R. RISBUD*, KALYAN BANERJEE*, AND ROBERT H. PURCELL‡

*National Institute of Virology, Indian Council of Medical Research, Pune 411001, India; and ‡Hepatitis Viruses Section, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

Contributed by Robert H. Purcell, December 27, 1993

ABSTRACT **Many epidemics of water-borne hepatitis have occurred throughout India. These were thought to be epidemics of hepatitis A until 1980, when evidence for an enterically transmitted non-A, non-B hepatitis was first reported. Subsequently, hepatitis E virus was discovered and most recent epidemics of enterically transmitted non-A, non-B hepatitis have been attributed to hepatitis E virus infection. However, only a limited number of cases have been confirmed by immuno electron microscopy, polymerase chain reaction, or seroconversion. In the present study we have performed a retrospective seroepidemiologic study of 17 epidemics of water-borne hepatitis in India. We have confirmed that 16 of the 17 epidemics were caused at least in part by serologically closely related hepatitis E viruses. However, one epidemic, in the Andaman Islands, and possibly a significant minority of cases in other epidemics, appears to have been caused by a previously unrecognized hepatitis agent.**

Enterically transmitted water-borne hepatitis is recognized as a major public health problem in many developing countries (1). The discovery of enterically transmitted non-A, non-B (ET-NANB) hepatitis, now designated hepatitis E, resulted from the use of highly sensitive and specific assays for the diagnosis of hepatitis A and hepatitis B to exclude these latter two types of hepatitis as the cause of several epidemics of water-borne hepatitis in India (2, 3). Although the disease was discovered in 1980 (2, 3) it was not until 1983 that the putative etiologic agent, hepatitis E virus (HEV), was visualized by immuno electron microscopy (IEM) and IEM was used to establish the serologic uniqueness of this agent and to confirm its transmission to humans and nonhuman primates (4). However, a major impediment to progress in the study of HEV was the paucity of virus that could be obtained from feces of patients and experimentally infected primates for use as an antigen in serologic tests. In 1990, Reyes *et al.* (5) cloned and sequenced part of the HEV genome. Subsequently, an enzyme-linked immunosorbent assay (ELISA) was developed that used recombinant proteins expressed in *Escherichia coli* from open reading frames (ORFs) 2 and 3 of HEV as the source of antigen (6). Recently, Tsarev *et al.* (7) developed an ELISA for the detection of IgM and IgG antibodies to HEV (anti-HEV) that uses a recombinant protein expressed in insect cells from ORF-2 of HEV as the source of antigen. This assay is highly sensitive and specific for the diagnosis of HEV infections and therefore useful for seroepidemiologic studies (8).

In the past we have investigated 25 epidemics of water-borne viral hepatitis in India and have demonstrated that none of these was caused by hepatitis A virus (HAV). They were therefore classified as ET-NANB hepatitis. In the present study, we have revisited the question of the etiology of ET-NANB hepatitis by evaluating patients from 17 of these epidemics for serologic evidence of recent HEV infection. We have confirmed that HEV was the cause of the 1955 Delhi epidemic that was integral to the discovery of hepatitis E and have discovered evidence for another enterically transmitted hepatitis agent that may be the cause of significant morbidity in India.

MATERIALS AND METHODS

Epidemics. Seventeen epidemics of hepatitis were included in the present study (Table 1). All except the Delhi epidemic were investigated by an experienced team from the National Institute of Virology, Pune (refs. 2 and 9–12; unpublished data). All 17 of the epidemics were explosive, water-borne outbreaks, and fecal contamination of the drinking water was documented in each epidemic. Cases occurred mainly in young adults, except for the Andaman Islands epidemic, in which 46% of the cases were in children.

Serology. Serum samples collected from hepatitis patients involved in the various epidemics were initially screened for hepatitis B surface antigen (HBsAg) with a noncommercial ELISA and for IgM antibodies to HAV (IgM anti-HAV) and IgM antibodies to hepatitis B core antigen (IgM anti-HBc) with the HAVAB-M RIA and CORZYME-M tests (Abbott), respectively. Patients negative for HBsAg, IgM anti-HAV, and IgM anti-HBc were diagnosed as having NANB hepatitis [since patients with acute or chronic hepatitis B virus (HBV) infection were excluded, we did not test for markers of hepatitis D virus infection]. Serum from patients in the Andaman Islands epidemic was also tested for IgM antibodies to leptospira by ELISA (Leptase; Bradsure Biologicals, Leicestershire, U.K.). After initial screening all the serum samples were stored at −20°C. Randomly selected serum samples from each epidemic were tested for the presence of IgM and IgG antibodies to HEV (IgM anti-HEV and IgG anti-HEV) with an ELISA (8) based on the use of recombinant ORF-2 antigen (13). They were also tested for antibodies to hepatitis C virus (HCV) with a second-generation ELISA (Abbott) and confirmed with a supplemental second-generation recombinant immunoblot assay (RIBA II; Chiron). Samples positive for anti-HCV were excluded from the study. IgM anti-HEV- and IgG anti-HEV-positive samples were titrated at 1:100, 1:1000, and 1:10,000 dilutions. All samples were assayed in duplicate. The negative control antigen, from insect cells infected with recombinant baculovirus lacking the HEV sequence, was purified by following the protocol for purification of ORF-2 protein. The samples

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Abbreviations: ET-NANB, enterically transmitted non-A, non-B; IEM, immuno electron microscopy; ORF, open reading frame; GMT, geometric mean titer; HAV, hepatitis A virus; HEV, hepatitis E virus.

†To whom reprint requests should be addressed at: National Institute of Virology, Indian Council of Medical Research, 20-A, Dr. Ambedkar Road, Pune 411001, India.

Table 1. Water-borne epidemics of hepatitis reexamined in this study

Epidemic			This study				
Location	Year	No. of patients with hepatitis	No. of patients studied	No. (%) with recent HEV infection*	Interval,† days	IgM anti-HEV reciprocal GMT	IgG anti-HEV reciprocal GMT
Delhi	1955	29,000	28	28 (100)	NA‡	343	1668
Ahmedabad	1976	2,572	9§	8 (88.9)	41.9 ± 33.8	2063	4500
Kolhapur	1981	1,169	12§	10 (83.3)	24.5 ± 18	1122	3870
Ahmedabad	1982	1,072	17	10 (58.8)	23.2 ± 17.4	200	1000
Ahmedabad	1984	118	22	20 (90.9)	12.1 ± 5.1	1122	1000
Baroda	1984	3,005	16	15 (93.8)	10.0 ± 5.1	1540	1585
Surat	1985	1,395	17	13 (76.5)	18.7 ± 10.1	1000	3162
Ahmedabad	1986	1,015	18	14 (77.8)	15.3 ± 8.2	811	866
Ahmedabad	1987	2,215	33	23 (69.7)	16.9 ± 12.4	1105	848
Andaman Islands	1987	307	75	0 (0)	27.1 ± 11.4	1	1
Khadakwasla	1989	276	19	13 (68.4)	11.5 ± 4.6	2154	1000
Akluj	1990	139	20	18 (90)	12.3 ± 7.1	618	2254
Beed	1990	>3,000	28	26 (92.9)	17.9 ± 10.7	1905	1390
Rewa	1990	517	27	23 (85.2)	33.0 ± 21.6	257	2254
Bijapur	1990	132	17	17 (100)	18.1 ± 7.2	582	1000
Kolhapur	1991	1,442	21	19 (90.5)	12.9 ± 4.7	1274	891
Karad	1993	2,427	27	27 (100)	10.5 ± 5.3	1978	1817

GMT, geometric mean titer.
*Positive for IgM anti-HEV or high titer (≥1:1000) of IgG anti-HEV.
†Interval between onset of clinical symptoms and collection of serum sample (mean ± SD).
‡Not available.
§Paired sera were tested.

positive for IgM or IgG anti-HEV were tested with this control antigen in ELISA; none was reactive, demonstrating that the ELISA was specific for anti-HEV.

Stool Samples. Stool samples were collected during the early acute phase of the disease from NANB patients involved in the epidemics at Ahmedabad (1976, 1984, and 1987), Kolhapur (1981 and 1991), and Akluj (1990) and were stored at −70°C.

PCR. Stool samples were tested in nested reverse transcription–PCRs as described (14, 15). Sequencing and computer analyses were as described (15).

Statistics. The χ^2 test for significance was employed. GMTs were calculated by using an arbitrary titer of 1:1 for the negative sera.

RESULTS

Fig. 1 depicts the geographic locations of the 17 epidemics and the year each occurred. The epidemics occurred in urban, rural, and insular (Andaman) settings. The city of Ahmedabad experienced water-borne epidemics in 1976, 1982, 1984, 1986, and 1987. The city of Kolhapur experienced epidemics in 1981 and 1991. Different parts of the two cities were affected in the different epidemics and the patients in each epidemic reported they had not had clinical hepatitis during the previous epidemic(s). Serological studies conducted during the investigation of these 17 epidemics indicated a NANB etiology (refs. 2 and 9–12; unpublished data).

We used PCR specific for HEV to analyze a limited number of fecal samples collected from patients during six of the epidemics and were able to detect HEV genomes in seven samples and to amplify the ORF-3 region from three of the seven. By employing primers covering the entire ORF-3 region, we showed that there were no deletions in ORF-3 compared with the Burmese strain (16) in the three strains we studied, in contrast to a previous report of a 246-bp deletion in ORF-3 of Indian strains (17). Sequences were obtained for isolates of HEV from three of the above epidemics (Ahmedabad 1976 and 1984; Akluj 1990) as well as from four other Indian epidemic or sporadic cases. Most of the seven Indian strains partially sequenced were more closely related to a Burmese strain (16) (98.5–99.3% nucleotide identity) than to a Pakistani strain (15) (94.8–95.5% nucleotide identity) and were very similar to one another (98.9–99.9% nucleotide identity). One strain (Ahmedabad 1976) was equidistant between these two reference strains in sequence similarity. Thus we were able retrospectively to identify HEV as an etiologic agent in epidemics at Ahmedabad (1976, 1984, and 1987), Kolhapur (1981 and 1991), and Akluj (1990).

Development of a specific and sensitive ELISA for the detection of IgM and IgG anti-HEV (7) made it possible to study a much larger number of patients and to compare the prevalences of HEV infection in the 17 epidemics. The

FIG. 1. Geographic distribution and year of water-borne hepatitis epidemics studied.

frequency with which IgM anti-HEV was detected varied from epidemic to epidemic and ranged from 0% to 100% of tested sera (Table 1). Although the detection of IgM anti-HEV proved that HEV was an etiologic agent in most of the hepatitis epidemics, these discrepant data suggested that more than one etiologic agent was involved in some of the epidemics.

The epidemic of water-borne hepatitis in the Andaman Islands provided the most striking evidence for another hepatitis agent. Even though the mean interval between onset of illness and collection of blood from patients in this epidemic was similar to that for the other epidemics studied (Table 1), none of the 75 patients tested was positive for anti-HEV.

A detailed account of the Andaman epidemic was published previously by Chadha *et al.* (11). A total of 307 hepatitis cases, including 183 males and 124 females, were reported (Fig. 2). The diagnosis of viral hepatitis was made by the investigating team from the National Institute of Virology in Pune on the basis of clinical signs and symptoms and presence of bile salts and bile pigments in the urine. The symptoms were not consistent with those caused by the common Gram-negative enteric bacilli, non-typhoid *Salmonella*, or the typhoid bacillus. Only low-grade fever of short duration during the prodrome was reported. The icteric patients showed no evidence of bradycardia or splenomegaly and all recovered without the aid of antibiotics. Eighty-five percent of the patients were icteric, 84% had dark urine, 74% were anorexic, 52% had nausea and vomiting, and 48% had abdominal pain.

Blood samples were collected from 81 patients. Serological analysis by ELISA demonstrated that 6 patients were suffering from hepatitis A (these were excluded from the present study), but none of the 81 suffered from acute hepatitis B or leptospiral infection. Unlike other epidemics of water-borne NANB hepatitis previously reported in India, 46% of the patients were children under the age of 15 years.

Five serum samples from the Andaman epidemic previously had been tested for IgM anti-HEV by Western blot and two had been found to be weakly positive at a serum dilution of 1:20 (18). Neither serum was positive by the present ELISA when tested at a dilution of 1:100, the dilution routinely employed for this assay (7). To assure that we were not missing weak anti-HEV responses in these patients, we reanalyzed the data with a lower cutoff value for OD (0.2 vs. 0.35). Only two patients were weakly reactive (titer of 1:100) for IgM anti-HEV only, and two patients were similarly weakly reactive for IgG anti-HEV only. In addition, we retested all 75 sera at a 1:20 dilution for IgM and IgG anti-HEV by ELISA, using the standard cutoff value. Four additional test sera showed higher OD values, but a comparable number of negative control sera also showed similar OD values. The new samples with the high OD values did not include the two sera previously found to be positive by Western blot. Although the Western blot assay correlated well with the ELISA when samples from other epidemics were tested, we presume that the previous weak positive results with the Andaman samples were nonspecific. Since the vast majority of the Andaman sera were nonreactive in the ELISA, we conclude that the Andaman epidemic was not caused by an agent serologically related to HEV.

To ascertain whether the differences in prevalence of anti-HEV among the epidemics were due to variations in the interval between the onset of clinical symptoms and collection of blood samples, the data were analyzed by week after onset of illness (Fig. 3). During the first week of the clinical disease, 86% of NANB patients were positive for IgM anti-HEV (GMT, 1:1129). Similar results were noted through the third week. Subsequently the percent of sera positive for IgM anti-HEV declined sharply, as did the GMT. By the end of week 12 only 21.8% of the patients were IgM anti-HEV positive and the GMT had decreased to 1:3.7. Meanwhile, the percent of sera positive for IgG anti-HEV and the GMT, respectively, for this group of patients rose from 81.4% and 1:405 (week 1) to 86.7% and 1:1817 (week 4). When these data were compared with the interval between onset of illness and collection of serum from anti-HEV-negative patients, it was apparent that the seronegative patients were bled, on aver-

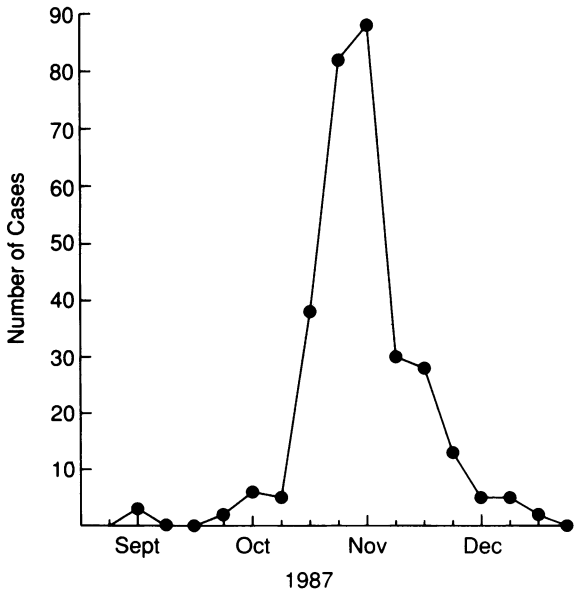

FIG. 2. Epidemic curve of hepatitis cases (no. per week), North Andaman Island, 1987.

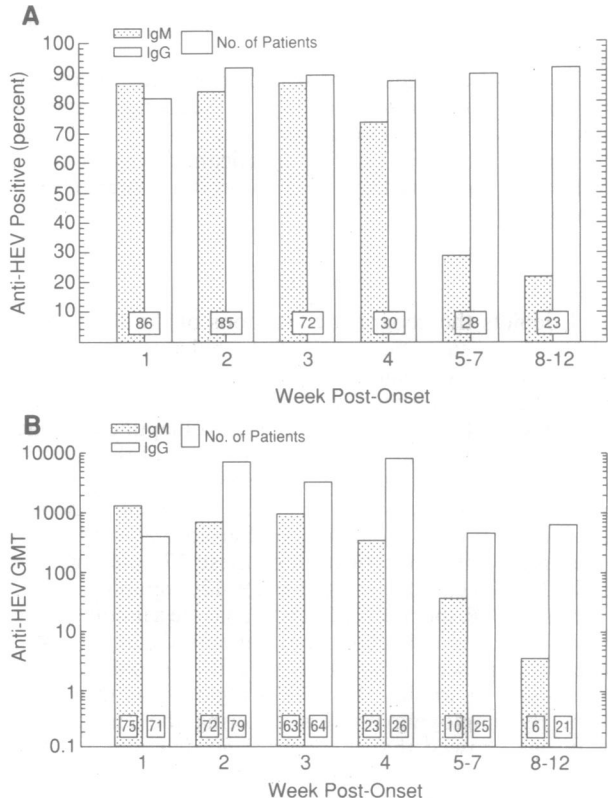

FIG. 3. (*A*) Percent of patients positive for IgM or IgG anti-HEV, by week after onset of symptoms. All patients are included. (*B*) GMT of IgM and IgG anti-HEV by week after onset of symptoms. All anti-HEV-positive patients are included.

age, at a time when they would be expected to be strongly positive for IgM and/or IgG anti-HEV, had their hepatitis been hepatitis E.

To determine whether the differences in percent of patients with serologic evidence of HEV infection in different epidemics were the result of serologic heterogeneity of HEV strains, leading to different sensitivities of the ELISA for different epidemics, we compared the titers of IgM anti-HEV for patients from different epidemics. As seen in Fig. 4, the titers of IgM anti-HEV for patients from epidemics with low prevalences of anti-HEV positivity were indistinguishable from those of patients from epidemics with high prevalences of anti-HEV, indicating that the differences were probably not due to serologic variation among the epidemic strains of HEV.

As a further test of whether IgM anti-HEV-negative patients might actually have represented missed diagnoses of hepatitis E, we reinvestigated the serologic status of three patients with acute-phase sera that were negative for IgM anti-HEV and who had been rebled 41–77 days after onset of illness. These convalescent sera, from one patient in the Ahmedabad 1976 outbreak and two patients in the Kolhapur 1981 epidemic, were also negative for both IgM and IgG anti-HEV, thus confirming that these cases of hepatitis were indeed not etiologically related to known strains of HEV. Paired sera were not available from other anti-HEV-negative patients. However, from the absence of both IgM anti-HEV and high-titered IgG anti-HEV (≥1:1000), we conclude that 44 patients from 13 of the remaining 16 epidemics may have had hepatitis not related to HEV (Table 2). Ten of the 44 patients had low-titered IgG anti-HEV, but we have included them in the list of possible cases of non-E hepatitis because patients with low levels of IgG anti-HEV were found in a previous study to be protected against hepatitis E, probably by virtue of an infection with HEV at some time in the past (8). Therefore, these 44 cases of hepatitis, occurring in conjunction with water-borne epidemics and not serologically related to any of the five recognized hepatitis viruses, may have been caused by a previously unrecognized hepatitis agent.

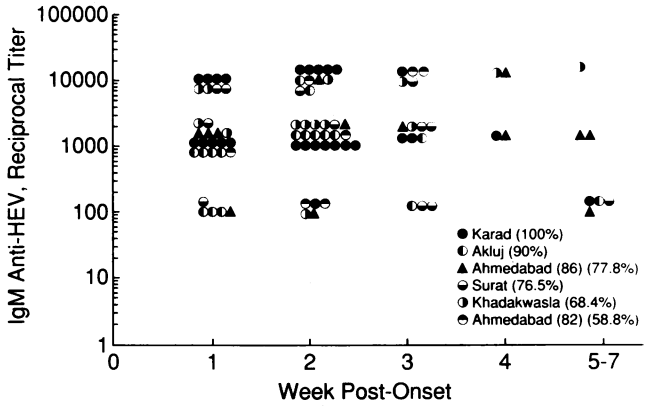

FIG. 4. Individual IgM anti-HEV titers of patients from epidemics with a high proportion, intermediate proportion, or low proportion of hepatitis E cases, by week after onset of symptoms. There is no difference among the epidemics.

DISCUSSION

In 1980, Purcell and colleagues (2) reported evidence for a previously unrecognized hepatitis virus. This conclusion was based, in part, on a retrospective analysis of sera saved from the massive epidemic of water-borne hepatitis that occurred in Delhi in 1955–1956 (1). Similarly, Khuroo (3) provided evidence that a previously unrecognized hepatitis agent was the cause of epidemic hepatitis in northern India. Both studies were based on exclusion of HAV and were made possible by the development of sensitive and specific serologic tests for HAV, the only water-borne hepatitis virus recognized at that time.

Subsequent to the identification of hepatitis E virus by IEM (4), attempts were made to characterize ET-NANB epidemics by this technique. Epidemics from India, Nepal, the former Union of Soviet Socialist Republics, Burma, Pakistan, Algeria, and Mexico were all shown to be associated with serologically related viruses. Only Doroshenko *et al.* (19) suggested that there was more than one agent causing epidemic ET-NANB hepatitis, based on the results of IEM.

In the present work, we have used a sensitive and specific test for anti-HEV to study 17 water-borne epidemics occurring in India during the past 38 years. We confirmed that the Delhi epidemic was, indeed, caused by HEV and showed that 15 of the other 16 epidemics studied were caused predominantly or exclusively by HEV, confirming that HEV is an important human pathogen in India. Although the current study encompassed epidemics of hepatitis caused by many different strains of HEV, the magnitudes and temporal relationships of the IgM and IgG anti-HEV responses were remarkably similar to those described by Bryan *et al.* (8) in the epidemic that occurred in Sargodha, Pakistan, in 1987. In that epidemic, from which the antigen used for ELISA in the present study was derived, 92% of 131 cases were identified as cases of hepatitis E. This suggests that most, if not all, epidemics of hepatitis E in this region are caused by serologically closely related strains of HEV. Similarly, in a study of three epidemics of NANB hepatitis in villages in Somalia, Mushahwar *et al.* (20) noted that 77.8–94% of patients were suffering from recent HEV infection. Although the expression systems were different in the two studies, the results obtained were remarkably similar (20).

However, while underlining the importance of HEV infections in India, our data also suggested that some of these Indian epidemics may have been caused at least in part by a previously unrecognized agent that is spread by fecal–oral means. Evidence for such an agent came from several observations. First, the epidemics studied varied markedly in the proportion of hepatitis cases that could be ascribed to HEV. Most compelling was the absence of anti-HEV in

Table 2. Characteristics of patients with water-borne hepatitis in India

Type of hepatitis	No. of patients	Age range (mean, median), yr	Sex M/F ratio	Interval,* days	No. of epidemics
Andaman	75	2–51 (18.5, 17.5)	44/31 (1.4)	27.1 ± 11.4	1
Other non-E	44†	3–60 (25.4, 22)	30/14 (2.1)	16.0 ± 10.5‡	13
E	259§	2–84 (25.4, 23)	197/62 (3.2)	18.5 ± 12.7	15

*Interval between onset of clinical symptoms and collection of serum sample (mean ± SD).
†Thirty-four patients negative for IgM and IgG anti-HEV and 10 patients positive only for low-titer IgG anti-HEV (1:100).
‡Only 8 of the 44 patients were bled during the first week after onset of symptoms.
§Excluding the Delhi epidemic, for which data were not available.

patients from the Andaman epidemic in which there was a 31% attack rate and 307 cases of hepatitis (11). These differences could not be explained by the interval between onset of illness and collection of the serum sample, the age or storage conditions of the sera, or the age of the patients. Nor was there evidence that the differences in prevalence of anti-HEV among the epidemics studied were caused by serologic differences among the HEV strains associated with the epidemics. Sequence analyses of PCR products demonstrated that viruses recovered from epidemics representing high and low proportions of hepatitis E cases were all genetically closely related. A major deletion of 246 bp in the genome of two Indian strains of HEV has been described (17). This might affect an ELISA based on ORF-3 but should not affect the ELISA used here, which was based on ORF-2. In addition, we were able to show that the reported deletion was not present in three Indian strains we studied. Although most Indian strains were more closely related to a Burmese strain than to the Pakistani strain of HEV used as the source of antigen for ELISA in this study, the anti-HEV response to even the most distantly related (Mexican) strain of HEV, as well as to the more closely related Burmese strain, can be detected by this ELISA with a sensitivity equal to that of the Pakistani strain (ref. 7 and unpublished data). Thus, we do not believe that the results we describe here can be explained as an artifact of the assay.

In conclusion, we have provided evidence for the existence of a sixth human hepatitis agent and the third agent to be spread by fecal contamination. The Andaman epidemic appears to have been almost totally caused by an agent other than HAV or HEV. In India overall, one or more such agents appear to cause epidemic disease somewhat less frequently than HEV (70% of the 406 ET-NANB cases studied were diagnosed as hepatitis E). However, the infection rate could be higher than the estimate of 30% if the new agent causes infection at an earlier age than that believed to be typical for HEV, since many viruses more commonly cause inapparent infections in the very young. For example, infection with HAV remains by far the most common hepatitis virus infection in India but most infections, occurring in the very young, are clinically inapparent and documented only by serological means.

As with hepatitis A and hepatitis E previously, the answers to many questions must await the development of sensitive and specific assays for the putative agent and antibodies to it. We presume that it is a virus, but its filterable nature has not yet been established. However, it is unlikely that such an agent is geographically restricted to India and evidence (by serologic exclusion) for its existence elsewhere should be sought. The data from the Doroshenko *et al.* (19) study of a Siberian epidemic, although limited, are consistent with results obtained in the present study. Further studies will be required to determine whether the putative new agent is a second serotype of HEV or an entirely different virus.

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