Appendix: II

List of Publications:


Hepatitis C Virus Infection in the General Population: A Community-Based Study in West Bengal, India

Abhijit Chowdhury,1 Amal Saurstra,1 Susmita Chaudhuri,2 Gopal Krishna Dhall,1 Sujit Chaudhuri,1 Satya Gopal Maity,1 Trailoky Nandi Naik,2 Sujil Kumar Bhattacharya,2 and Debendra Nath Guha Mazumder1

Limited information is available about the prevalence and genotype distribution of hepatitis C virus (HCV) in the general population of India. A community-based epidemiologic study was carried out in a district in West Bengal, India. By a 1:3 sampling method, 3,579 individuals were preselected from 10,737 inhabitants of 9 villages of the district, of whom 2,973 (83.1%) agreed to participate. Twenty-six subjects (0.87%) were HCV antibody positive. The prevalence increased from 0.31% in subjects <10 years of age to 1.85% in those ≥60 years. No difference in prevalence between men and women was observed. Serum alanine aminotransferase (ALT) levels were elevated in 30.8% (8 of 26) of anti-HCV-positive subjects compared with 3.2% (94 of 2,947) anti-HCV-negative subjects (P < .001). HCV RNA was detectable in 80.8% (95% CI, 65.6%-95.91%) of the anti-HCV–positive subjects by reverse transcription–primed polymerase chain reaction (RT-PCR). The participants were HCV types 1b in 2 (9.5%), 3a in 8 (38.1%), 3b in 6 (28.6%), and unclassified in 5 (23.8%). Nucleotide sequencing and phylogenetic analysis assigned the unclassified type to genotype 3c. In conclusion, this study provides general population-based estimates of HCV prevalence, including genotypes, from a South Asian country. Although the prevalence of HCV infection in this population was lower than that reported from industrialized countries of the west, the total reservoir of infection is significant and calls for public health measures, including health education to limit the magnitude of the problem. (Hepatology 2003;37:802-809.)
South Asian countries, including India, has been derived mostly from voluntary and paid blood donors. The population-based studies have been limited to small samples of arbitrarily selected random cross-sections of the population. Because such samples lack the demographic heterogeneity of the general population, they are prone to selection bias and do not represent the general population.

We report here a population-based epidemiologic study of HCV infection in a rural population in West Bengal in Eastern India. The prevalence, associated risk factors of HCV infection, and the descriptive epidemiology of HCV genotypes are described. HBV markers were also determined in the same population to look for comparative significance of HBV and HCV in an area of intermediate HBV prevalence. To the best of our knowledge this is the largest community-based epidemiologic study of HCV infection from the Indian subcontinent.

**Patients and Methods**

**Study Site and Population Description.** The population sample was systematically obtained from the inhabitants of 9 villages of Birbhum district, which is one of the 18 administrative units (equivalent to counties) comprising the state of West Bengal (Fig. 1) in the eastern part of India. The Birbhum district consists of 19 peripheral administrative units called community development (CD) blocks. The majority of the population of this district (91.02%) live in rural areas and are engaged in agriculture-related occupations. The CD blocks consist of villages with mean populations of 1,193, residing an average of 207 households. A local self-governing authority, called the Panchayat runs the local administration of each village and maintains a population register constructed on a house-to-house basis by listing the names of all inhabitants of each household in the order of decreasing age. Because of poor financial ability and inadequate health care facilities, these rural people often depend on health care providers without formal medical training.

**Community Sampling.** For population sampling, one village was randomly selected from each of alternate CD blocks of the district. We used the population register that had been updated last in March 1999. The sample was selected at the end of July 1999, and the data and blood samples were collected between August and December 1999. The study population was selected by a systematic 1:3 sampling procedure. Every third person from the village population register was selected and approached for participation. Thus, 3,579 subjects were eligible out of a total of 10,737 inhabitants in the 9 selected villages. No willful participation was allowed and if any eligible candidate declined to participate, his or her name was dropped from the list of participants.

The investigative team performing the field work consisted of physicians who performed clinical examination, paramedics who collected blood samples, and trained social workers who conducted house visits to explain the objectives of the study and to interview each participant. The field work was performed with active help of the village elders and the Panchayats. Of the 3,579 eligible subjects, 2,973 (83.1%) agreed to participate. The response rate among women (73.88%) was lower than that among men (91.66%). Informed consent was obtained from the participating subjects and, in the case of the minors, the parents or guardians.

Each subject was administered a structured questionnaire that was designed to ask the sociodemographic details, present and past health status, history of jaundice, and potential risk factors for HCV transmission. Local customs and rituals, available health care facilities, as well as trends in health care utilization of the people were all taken into account for framing the questionnaire that was pretested and validated. Auditing of the generated data was done by readministration of the questionnaires to 20% of the participants. Monthly household income was used as the parameter for determining the socioeconomic status. Households with monthly income less than Indian Rs. 1,000 (~$20 at current exchange rate) were defined as below poverty level. The level of education was defined in terms of years spent in school. At the end of the interview and clinical examination, the participants were invited to donate 5 to 6 mL blood for assessment of HCV and HBV status and serum alanine aminotransferase (ALT) levels. All study procedures and the questionnaire were reviewed and approved by the ethical committee of the Institute of
Laboratory Procedures: Serum was separated within 2 hours of collection and was transported in small aliquots on dry ice to the laboratory where the samples were preserved at -70°C until use. All samples were tested for antibodies to HCV (anti-HCV) by a third generation enzyme immunoassay kit (United Biomedical Co. Ltd., Beijing, PR China) according to the manufacturer's instructions. Positive samples were confirmed by retesting in duplicate. Sera were also tested for antibodies to hepatitis B core antigen (anti HBc) and hepatitis B surface antigen (HBsAg) using commercially available enzyme immunoassay kits (Organon Teknika bv, RM Boxtel, The Netherlands). Serum ALT levels were determined from one of the serum aliquots the day after sample collection, using a commercial kit (Boehringer Mannheim, Mannheim, Germany) according to the instruction of the manufacturer. ALT levels that were 1.5-fold of the normal upper normal limit (40 IU/L at 37°C) or higher were considered elevated.

Anti-HCV-positive subjects were recalled for further evaluation at a subsequent date. A detailed clinical examination was performed and blood samples were drawn for repeat serum ALT estimation and HCV RNA determination. The presence of HCV RNA in the anti-HCV-positive samples was assessed by nested reverse transcription-polymerase chain reaction (RT-PCR), using RNA extracted from 140 µL of serum (viral RNA extraction kit; QIAGEN GmbH, Hilden, Germany) and amplimers corresponding to the 5' untranslated region.

HCV RNA was genotyped by multiplex PCR with genotype-specific primers, which allowed for the determination of at least 6 major types and a series of subtypes as described by Ohto et al.10 Ten randomly chosen HCV-RNA-positive samples out of 21 HCV-RNA-positive samples were partially sequenced from the amplicon of the core gene for confirmation. The nucleotide sequences of the amplimers of the 5' untranslated region and the core gene were determined directly from both directions using the ABI Prism Big Dye terminator cycle-sequencing ready reaction kit (Perkin-Elmer Biosystems, Foster City, CA) and an automatic DNA sequencer (ABI Prism Model 310; PE Applied Biosystems, Foster City, CA). The results were concordant with genotype-specific PCR in all the cases.

The basic Local Alignment Search Tool (BLAST version 2.0) was used to search the public domain nucleotide database maintained by National Centre for Biotechnology Information (NCBI). Nucleotide sequences of HCV isolates were analyzed for the nucleotide substitution pattern to choose an appropriate model for phylogenetic analysis. Nucleotide sequences were multiply aligned using CLUSTAL X version 2.0. The resulting alignment was used to construct an unrooted phylogenetic tree using the neighbor-joining method. The neighbor-joining tree was bootstrapped 1,000 times with the SEQBOOT program to obtain final phylogenetic tree.

Statistical Analysis. Participants were stratified by age and gender, and the prevalence of HCV infection was calculated. To allow comparison without distortion by age, the prevalence was directly standardized to the age distribution of all participants of the same sex in the study population. The crude odds ratios (OR), which estimate the relative risk factors for HCV transmission, were calculated by univariate analysis. Confidence intervals (CI) of 95% for OR estimates were also calculated.

Results

Demographic characteristics of the participants, of inhabitants of the selected villages, people of Birbhum district, and the population of the entire state of West Bengal are shown in Table 1. The age and sex distribution and the level of literacy in the study area were comparable with the general population of Birbhum district as well as West Bengal as a whole. The participation rate among women (73.88%) was lower than that among men (91.66%).

Prevalence of HCV Infection. Among the 2,973 subjects studied, 26 were anti-HCV positive by EIA, representing an overall prevalence of 0.87%. The prevalence increased from 0.31% in children below 10 years of age to 1.85% in those ≥60 years of age (Table 2). No gender difference was observed for anti-HCV prevalence among the participants. The youngest positive subject was 4 years old while the oldest was 80 years. All of the anti-HCV-positive subjects were clinically asymptomatic and had no clinical stigma of liver disease.

The overall prevalence of HBsAg and anti-HBc in this study population was 1.61% and 17.00%, respectively. The prevalence of anti-HBc positivity, which indicates exposure to HBV, in this community increased from 13.93% in children less than 10 years of age to 33.33% in those ≥60 years of age. Anti-HCV was detected in 8 (1.57%) of the 508 anti-HBc-positive subjects and in 18 (0.73%) of the 2,465 subjects who were negative for anti-HBc. None of the HBsAg-positive subjects were anti-HCV positive. The prevalence of HBsAg and anti-HCV are shown in Fig. 2. The age-related prevalence of HBsAg showed a significant progressive decrease after 50 years, so that among participants who were older than 60 years, the prevalence of anti-HCV was higher than that of HBsAg.

Elevated serum ALT concentration (≥50 IU/L) was found in 8 (30.76%) of the 26 anti-HCV-positive subjects and in 94 (3.18%) of the 2,947 anti-HCV negative
Hepatology, Vol. 37, No. 4, 2003

**Table 1. Comparative Analysis of Demographic Characteristics of (A) Participants of the Study, (B) Total Population of the Selected Villages, (C) Entire Birbhum District In Which the Selected Villages Belonged to, and (D) the State West Bengal**

<table>
<thead>
<tr>
<th>Demographic Characteristics</th>
<th>Study Participants (n = 2,973)</th>
<th>In 9 Selected Villages (n = 10,737)</th>
<th>In Birbhum District</th>
<th>In West Bengal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age groups in y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td>21.72%</td>
<td>24.00%</td>
<td>26.2%</td>
<td>24.76%</td>
</tr>
<tr>
<td>10-19</td>
<td>24.32%</td>
<td>23.30%</td>
<td>23.8%</td>
<td>21.11%</td>
</tr>
<tr>
<td>20-39</td>
<td>31.45%</td>
<td>28.75%</td>
<td>29.3%</td>
<td>37.48%</td>
</tr>
<tr>
<td>40-59</td>
<td>17.05%</td>
<td>18.60%</td>
<td>15.6%</td>
<td>15.74%</td>
</tr>
<tr>
<td>&gt;60</td>
<td>5.44%</td>
<td>5.78%</td>
<td>4.5%</td>
<td>6.05%</td>
</tr>
<tr>
<td>Sex ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of women per 1,000 men</td>
<td>755*</td>
<td>937</td>
<td>946</td>
<td>917</td>
</tr>
<tr>
<td>(per 1,000)</td>
<td>66.8</td>
<td>64.6</td>
<td>62.96</td>
<td>60.90</td>
</tr>
</tbody>
</table>

*Sex ratio among the participants was low due to low participation rate among the women (73.88%) compared with the participation rate of men (91.66%) in the study.

Subjects (P < 0.001). Among these 94 anti-HCV-negative subjects with high ALT, 9 were positive for HBsAg and 48 were anti-HBe positive.

**Risk Factors.** The frequency of various risk factors associated with HCV infection and the calculated crude OR, estimated by univariate analysis, are shown in Table 3. Use of reusable glass syringes was reported by 80.76% of the anti-HCV-positive subjects (OR, 3.82; 95% CI, 3.45-10.23%). Age, sex, history of using glass syringes, place of birth, blood transfusion, and dental therapy. Among the 21 HCV-RNA-positive subjects, 7 had elevated serum ALT levels (>60 IU/L); 3 of these 7 subjects carried HCV genotype 3a, 2 had genotype 3b, and 2 carried HCV of an unclassified type. For further confirmation of the genotypes, amplicons from 10 randomly chosen HCV-RNA-positive isolates were sequenced and the results matched those determined by type specific PCR.

**Phylogenetic Analysis.** A phylogenetic tree was constructed with all the partial 5' untranslated region and core sequences (from n at 90 to 680) from the 10 isolates along with published sequences of prototype strains iso-

**Table 2. Age- and Sex-Specific Prevalence of Anti-HCV in the General Population of Birbhum**

<table>
<thead>
<tr>
<th>Age Groups</th>
<th>Men</th>
<th>Women</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. Positive/ No. Tested (%)</td>
<td>No. Positive/ No. Tested (%)</td>
<td>No. Positive/ No. Tested (%)</td>
</tr>
<tr>
<td>&lt;10</td>
<td>6/339 (0.06)</td>
<td>3/307 (0.09)</td>
<td>2/646 (0.31)</td>
</tr>
<tr>
<td>10-19</td>
<td>4/453 (0.88)</td>
<td>2/270 (0.74)</td>
<td>6/723 (0.83)</td>
</tr>
<tr>
<td>20-39</td>
<td>4/505 (0.79)</td>
<td>8/430 (1.39)</td>
<td>10/935 (1.07)</td>
</tr>
<tr>
<td>40-59</td>
<td>4/296 (1.35)</td>
<td>1/211 (0.47)</td>
<td>5/507 (0.99)</td>
</tr>
<tr>
<td>&gt;60</td>
<td>3/101 (2.97)</td>
<td>0/61 (0.00)</td>
<td>3/162 (1.85)</td>
</tr>
<tr>
<td>All ages</td>
<td>15/1,694 (0.88)</td>
<td>11/1,278 (0.86)</td>
<td>26/2,972 (0.87)</td>
</tr>
</tbody>
</table>

Fig. 2. Age-specific prevalence of anti HCV and HBsAg in the study population.
Table 3. Frequency of Potential Risk Factors Associated With Anti-HCV-Positive and -Negative Subjects

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>HCV-Positive Subjects</th>
<th>HCV-Negative Subjects</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n)</td>
<td>(%)</td>
<td>(n)</td>
</tr>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;70</td>
<td>8 (30.46)</td>
<td>1.161 (40.18)</td>
<td>1</td>
</tr>
<tr>
<td>&gt;70</td>
<td>18 (69.54)</td>
<td>1.586 (53.82)</td>
<td>1.93 (0.64 4.44)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>15 (57.87)</td>
<td>1.679 (56.97)</td>
<td>1</td>
</tr>
<tr>
<td>Female</td>
<td>11 (42.13)</td>
<td>1.708 (43.03)</td>
<td>0.97 (0.71 1.37)</td>
</tr>
<tr>
<td>Years of education</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10 years</td>
<td>16 (61.54)</td>
<td>1.963 (66.61)</td>
<td>1</td>
</tr>
<tr>
<td>&gt;10 years</td>
<td>10 (38.46)</td>
<td>0.984 (33.38)</td>
<td>1.24 (0.89 1.71)</td>
</tr>
<tr>
<td>Poverty index</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Below poverty level</td>
<td>10 (69.73)</td>
<td>1.403 (47.94)</td>
<td>1</td>
</tr>
<tr>
<td>At or above poverty level</td>
<td>5 (30.27)</td>
<td>1.944 (52.39)</td>
<td>3.82 (1.45 10.23)</td>
</tr>
<tr>
<td>Use of glass syringes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>5 (19.23)</td>
<td>1.000 (34.78)</td>
<td>1</td>
</tr>
<tr>
<td>Yes</td>
<td>15 (60.77)</td>
<td>1.544 (52.39)</td>
<td>3.82 (1.45 10.23)</td>
</tr>
<tr>
<td>Shaving by community barber</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>6 (54.54)</td>
<td>6.12 (65.70)</td>
<td>1</td>
</tr>
<tr>
<td>Yes</td>
<td>5 (45.45)</td>
<td>5.30 (34.30)</td>
<td>1.59 (0.64 4.05)</td>
</tr>
<tr>
<td>Blood transfusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>26 (100.00)</td>
<td>2.917 (98.98)</td>
<td>1</td>
</tr>
<tr>
<td>Yes</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
<td>1</td>
</tr>
<tr>
<td>Tattoo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>24 (92.30)</td>
<td>2.714 (92.69)</td>
<td>1</td>
</tr>
<tr>
<td>Yes</td>
<td>2 (7.70)</td>
<td>2.33 (7.40)</td>
<td>0.67 (0.24 1.86)</td>
</tr>
<tr>
<td>Dental therapy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>23 (88.46)</td>
<td>2.590 (87.98)</td>
<td>1</td>
</tr>
<tr>
<td>Yes</td>
<td>3 (11.54)</td>
<td>3.57 (12.11)</td>
<td>0.95 (0.37 2.67)</td>
</tr>
<tr>
<td>Place of birth</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In hospital</td>
<td>8 (30.76)</td>
<td>1.406 (47.71)</td>
<td>1</td>
</tr>
<tr>
<td>At home</td>
<td>18 (69.24)</td>
<td>1.541 (52.29)</td>
<td>2.05 (0.99 4.17)</td>
</tr>
<tr>
<td>IIFsA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>26 (100.00)</td>
<td>2.899 (98.33)</td>
<td>1</td>
</tr>
<tr>
<td>Positive</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
<td>1</td>
</tr>
<tr>
<td>Anti HBC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>18 (69.23)</td>
<td>2.447 (82.08)</td>
<td>1</td>
</tr>
<tr>
<td>Positive</td>
<td>8 (30.77)</td>
<td>0.920 (16.97)</td>
<td>2.17 (0.94 5.17)</td>
</tr>
</tbody>
</table>

NICED B2, B3, and B4 (accession nos. AF 520423, AF 520424, and AF 520425, respectively) clustered with the Nepalese strain (bootstrap value 99%) NE145. This Nepalese strain had been previously assigned to the genotype 3e.

Discussion

We report here a population-based epidemiologic study of HCV infection in an Indian population based on a sample representative of the local community. A multistage sampling technique was adopted among the inhabitants of a wide but structured geographical unit that forms the basis of administrative territorial functioning in India. There are only a few community-based studies of HCV prevalence worldwide, which have been reported mainly from the industrialized countries of Europe and America. There is a paucity of information on HCV prevalence in developing countries, in which the HCV pandemic is gaining momentum, especially in Asia and Pacific regions, where the largest segment of the human population resides. Therefore, the community-based data obtained in this study on the prevalence of HCV infection, characterization of prevailing HCV genotypes, and comparative prevalence of HBV in the same population, are likely to provide new insight into the magnitude of contribution of HCV in the causation of liver disease in India and, perhaps, neighboring countries.

Previous HCV prevalence data had been derived mostly from blood donors in India and other countries. However, blood donors, as an epidemiologic database, represent a skewed population sample because of the lack of participation by children and senior citizens and under-representation of women. Therefore, the available data do not effectively represent the general population. Two previous community-based studies from India on HCV prevalence involved population sam-
The overall prevalence of HCV infection in the general population of Eastern India in our study was lower (0.87%) than that reported from the United States, Italy, Egypt, and two developed countries of the Asia-Pacific Region, Japan and Australia. Age-specific prevalence of HCV in our study was lowest in children (0.31%), but increased progressively from adolescents (0.83%) to adults (1%) and older persons (1.85%). Similar low prevalence of HCV infection has been reported in children (0.2%) and adolescents (0.4%) from the United States. The increase in HCV prevalence with age, observed in our study suggests a steady cumulative increase of incidence of infection. This may be caused by sporadic transmission of infection persisting in the community through the years. This is to contrast the abrupt and unpredictable decadal changes of incidence, either increased or decreased, as reported from Italy, Japan, and Egypt, which may represent a “cohort” effect.

We have also determined the frequency of chronic HBV infection and HIV exposure in the same population. HBV is by far the single most common etiologic factor for chronic hepatitis and hepatocellular carcinoma in India and East Asia. The overall prevalence of HBV in our study population was lower than the average for the country, but it is evident that HBV prevalence and exposure rate are still much higher than those of HCV in this rural Indian population. Thus, the comparative load of the two viruses in an Indian population is the reverse of what had been found in an Italian population, in which HCV infection was over 10 times more common than chronic HBV infection (HbsAg positivity).

The low prevalence of HCV in our study population precluded extensive analysis of the risk factors associated with HCV infection. Nevertheless, univariate analysis revealed that history of medical injection using reusable glass syringes correlated with HCV infection. This finding needs to be validated in a larger sample of infected people. Because of the scarcity of trained physicians in rural parts of India, the majority of primary health care providers in the villages are individuals without standard medical training. Over half of the rural population that we studied had received some injection, mostly from untrained village physicians, who generally lack the knowledge about sterilization and often use injection therapy for trivial problems like cold and cuts, based on the prevailing belief that injections work faster and better than oral medications. Health education to the people regarding these modes of transmission of the virus may prove to be useful preventive interventions in these developing countries.

Fig. 3. Phylogram of HCV strains based on the core sequences, showing the isolates that were untypable by RT-PCR (NICEES-B2, NICEES-B3, NICEES-B4) clustering with the Nepalese strain, NE145, typed as 3e that were arbitrarily chosen for convenient sampling, without adhering to rigorous standard sampling procedures that eliminate bias in sample selection. Because 70% of the individuals reported in these studies were children or adolescents, not unexpectedly, a low HCV prevalence (0.09%-0.12%) was observed. The strength of the present investigation was the inclusion of large representative cohorts from 9 different villages of a district using a standardized systematic sampling procedure. Furthermore, our sampling method was different from that of those used in other general population-based studies from Italy and Egypt, where sampling was done from a single town or an isolated village. In contrast, we sampled the study population from each of the 9 villages that constitute geographically separated clusters of habitation of approximately 1,000 people. This sampling approach was designed to minimize bias introduced by any unidentified local social or cultural factor that could potentially influence the transmission and prevalence of HCV in a specific geographic area.

The overall prevalence of HCV infection in the general population of Eastern India in our study was lower (0.87%) than that reported from the United States, Italy, Egypt, and two developed countries of the Asia-Pacific Region, Japan and Australia. Age-specific prevalence of HCV in our study was lowest in children (0.31%), but increased progressively from adolescents (0.83%) to adults (1%) and older persons (1.85%). Similar low prevalence of HCV infection has been reported in children (0.2%) and adolescents (0.4%) from the United States. The increase in HCV prevalence with age, observed in our study suggests a steady cumulative increase of incidence of infection. This may be caused by sporadic transmission of infection persisting in the community through the years. This is in contrast to the abrupt and unpredictable decadal changes of incidence, either increased or decreased, as reported from Italy, Japan, and Egypt, which may represent a “cohort” effect.

We have also determined the frequency of chronic HBV infection and HIV exposure in the same population. HBV is by far the single most common etiologic factor for chronic hepatitis and hepatocellular carcinoma in India and East Asia. The overall prevalence of HBV in our study population was lower than the average for the country, but it is evident that HBV prevalence and exposure rate are still much higher than those of HCV in this rural Indian population. Thus, the comparative load of the two viruses in an Indian population is the reverse of what had been found in an Italian population, in which HCV infection was over 10 times more common than chronic HBV infection (HbsAg positivity).
Most HCV-seropositive subjects had measurable viremia (80.7%), which is consistent with findings in studies from France (80.6%), Italy (75.9%), and the United States (73%). Although a lower incidence of viremia has been reported from Egypt (65.5%). Only one third of anti-HCV-seropositive subjects in our study had increased serum ALT levels, suggestive of necroinflammatory active liver disease and viral replication. The normality of ALT levels in two thirds of the HCV-RNA-positive subjects underscores the relative insensitivity of ALT determination in identifying active HCV infection.

HCV and other RNA viruses exhibit a high frequency of nucleotide substitution during replication, resulting in significant genetic heterogeneity among viral strains. This nucleotide sequence variability spans the entire viral genome and forms the basis of categorizing HCV into at least 6 major genotypes and several subtypes. HCV genotypes are important in predictors or response to interferon therapy. Moreover, there are geographical differences in the distribution of the predominant genotypes of HCV. It has been suggested that genotypic distribution of HCV is likely to provide information regarding the introduction, spread, and evolution of the virus in a defined human population and may be informative of human population migration. Information on the distribution of HCV genotypes in the Indian subcontinent has been derived mostly from studies of chronic liver disease patients and select population groups. Despite some inter-study differences in frequencies of the various genotypes, in general, genotype 3 predominates in northern India, Pakistan, and Nepal, whereas one report from southern India indicated a higher prevalence of genotype 1. In our study, genotype 3 was again the predominant genotype, comprising over 60% of the isolates, confirming the previously reported results from different parts of the subcontinent. Information on HCV subtypes within a major specific genotype often provides useful molecular epidemiological tools. In our study, 5 strains could not be genotyped by type-specific PCR. Determination of nucleotide sequence of 3 of these 5 nontypable strains revealed that they belonged to genotype 3 and had a 98% to 99% homology with a subtype 3c, described heretofore only from Nepal. The finding of genotype 3c in eastern India, which is geographically close to Nepal, warrants the analysis of a larger cohort from this geographical region to test the hypothesis that this genotype may represent a region-specific type that might have spread from one place to another via population migration and admixture. In Western populations, genotypes 3a and 1a had been linked to injection drug abuse. In the population that we have studied, intravenous drug abuse was nearly nonexistent, but because of the high frequency of injections used for "medical treatment" this genotype might have been acquired through injections in this community as well.

In a large country like India, it will be informative to have population-based data from different parts of the country, apart from the one from eastern India that we present here. Even with a prevalence of HCV below 1%, there is a large reservoir of over 8 million HCV-infected persons in India. This number, when added to the nearly 40 million chronically HBV-infected persons in this country, indicates that nearly 50 million people are at risk of developing chronic liver disease related to viral hepatitis. However, chronic viral hepatitis, despite the magnitude and projections, is yet to be perceived as a public health priority in India. It is hoped that the descriptive epidemiologic data presented in this communication should underscore the significance of this major threat to public health. Antiviral therapy is not affordable by the vast majority of people in developing countries. Therefore, prevention by health education is likely to be the critical intervention that might help limit the spread of these infections.

Acknowledgment. The authors are thankfully indebted to Professor Jayanta Roychowdhury of Albert Einstein College of Medicine, New York, and Professor Subhata K Achariya, All India Institute of Medical Sciences, New Delhi, for editing the manuscript. They also gratefully acknowledge the help of Prasanta Chatteljee, Pragmuth Mundaal, Santan Mondal, Debabrata Sutrallah, Sumanta Sarkat, Jindar Ahmed, Neati Kundu, Datal Chandu Roy, Shubh Dutt, Biswajit Debhat, and Anil De in conducting this epidemiologic study.

References


Molecular epidemiology of HCV infection among acute and chronic liver disease patients in Kolkata, India

S. Chaudhuri, S. Das, A. Chowdhury, A. Santra, S.K. Bhattacharyya, T.N. Naik

Abstract

Background: In recent years, hepatitis C virus (HCV) infection is gaining importance in Asian countries. Recent studies conducted in different parts of the world revealed that there is a genotypic correlation of disease severity and treatment outcome.

Objectives: A detailed study was carried out to delineate the genotypic distribution of HCV among acute and chronic liver disease patients in Kolkata, a city in eastern India.

Study design: Acute and chronic liver disease was diagnosed among patients attending hepatitis clinics in the city. Anti-HCV ELISA was performed on the blood samples of the cases and positive samples were tested for presence of HCV-RNA and genotyping of the samples were carried out by reverse transcription and polymerase chain reaction (RT-PCR) and sequencing.

Results: Seroprevalence of HCV infection among acute (11.0%) and chronic (25.3%) hepatitis patients were high and among them 97 (75.8%) and 323 (86.1%) were HCV-RNA positive for acute and chronic hepatitis patients, respectively. Genotyping by PCR showed that the predominant genotype was 3b (42.3%) followed by 3a (28.9%) among acute hepatitis group whereas among chronic hepatitis group, the most prevalent genotypes were 3a (34.7%) and 3b (47.7%). Sequence analysis of the untypeable isolates revealed the presence of a rare subtype 6b.

Conclusions: The study revealed very high prevalence of HCV among acute and chronic hepatitis patients with predominance of genotype 3. Subtype 6b was commonly found in Thailand but not in India. The detection of this rare strain of Thai origin reveals the spread of HCV infection from Thailand to other parts of Asia. This observation necessitates further intensive surveillance of HCV infection in India to unravel the distribution of genotypes in the country and to correlate disease severity and treatment outcome to the genotype prevalence.

Keywords: HCV; Hepatitis, Genotype; Sequencing, Phylogenetic analysis

1. Introduction

Hepatitis C virus (HCV) infection is a leading cause of chronic hepatitis and primary hepatocellular carcinoma in most parts of the world. In the developing countries of Asia and Africa, though hepatitis B virus (HBV) infection is the commonest cause of chronic liver disease, HCV is fast evolving as an equally important infection among these populations (Poovorawan et al., 2002). Moreover, with successful immunization programme against HBV infection in most countries of Asia, there is a decrease in the rate of HBV infection. On the other hand, chronic liver disease burden due to HCV infection has increased significantly (Chang et al., 1997; Chen et al., 2000).

HCV belongs to the family *Flaviviridae* and is a positive sense single stranded RNA virus containing about 9400 nucleotides. It has a single open reading frame flanked by 5' and 3' untranslated regions (UTRs). The polyprotein precur-
Genotype was carried out by nested multiplex PCR of the core region using genotype specific primers, which allowed for the determination of at least six major types and a series of subtypes as described by Ohno et al. (1997). A total of 200 isolates chosen at random from all the genotyped samples, were sequenced partially to confirm the PCR data.

2.4 Genotyping of HCV

Genotyping was carried out by nested multiplex PCR of the core region using genotype specific primers, which allowed for the determination of at least six major types and a series of subtypes as described by Ohno et al. (1997). A total of 200 isolates chosen at random from all the genotyped samples, were sequenced partially to confirm the PCR data.

2.5 Sequencing of HCV-RNA

A few isolates selected at random representing all the genotypes were sequenced partially as described previously (Varghese et al., 2004). Amplified products from the 5'-UTR, core and envelope region were directly sequenced. The sequencing reactions were carried out from both directions using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Norwalk, CT, USA). The sequences were determined in an automated DNA sequencer (ABI Prism 310 Genetic Analyser, Applied Biosystems, Foster City, CA, USA).

2.6 Phylogenetic analysis of the sequences

The basic local alignment search tool (BLAST Version 2.0) was used to search the public domain nucleotide database maintained by National Center for Biological Information (NCBI). From the BLAST results, similar sequences were grouped together and representative sequences of each group were taken for phylogenetic analysis to ascertain genotype to the strains. Nucleotide sequences were aligned by using Clustal X (v 1.81) programme and unrooted phylogenetic trees were constructed by neighbor joining (NJ) algorithm. The NJ trees were bootstrapped 1000 times with the SEQBOOT programme to obtain the final phylogenetic trees.

2.7 Statistical analysis

Data were reported as mean (standard deviation). Frequency between groups was compared using the Chi-square test. Group means were compared using Student’s t-test and one-way analysis of variance. The level of statistical significance was $P < 0.05$. 

...
3. Results

3.1 Prevalence of HCV infection among acute and chronic liver disease patients

A total of 2640 serum samples were collected from the patients of liver diseases in Kolkata. The clinical history, particularly duration of illness, physical findings and laboratory investigations were taken into consideration for diagnosis of acute as well as chronic hepatitis. Patients, who visited the clinics with ailments like anorexia, nausea, fatigue, malaise, pain in right hypochondrium and jaundice were clinically examined for possible liver diseases. Acute liver disease was diagnosed if the patients had a short duration of illness (less than 6 months) and showed an enlarged liver. Patients complaining about the above ailments for more than 6 months, with firm hepatomegaly, were diagnosed as chronic liver disease patients. Overall, the study population consisted of symptomatic patients only. Among the study group, 1159 were acute liver disease patients and 1481 had confirmed chronic liver disease. The El A results showed that, 128 (11.0%) of the acute patients and 375 (25.3%) of the chronic liver disease patients were anti-HCV positive.

In our study, the aetiologic factors of hepatitis in the anti-HCV and HCV-RNA negative patients varied. In acute hepatitis, hepatitis E, hepatitis A, hepatitis B, viruses were the predominant causes of disease. In chronic hepatitis, hepatitis B virus was the most common cause, followed by autoimmune hepatitis and cryptogenic liver disease.

3.2 Prevalence of HCV viraemia

All the anti-HCV positive samples were tested for HCV viraemia by RT-PCR and only 97 (75.8%) isolates from the anti-HCV seropositive acute patients were positive for HCV-RNA. On the other hand, 323 (86.1%) out of 375 were HCV-RNA positive among the seropositive patients with chronic liver disease.

3.3 Hepatitis C and hepatitis B co-infection

Total number of HBV and HCV co-infected patients were three (0.1% of total number of liver disease patients). Mean ALT level of these cases was much higher (532 ± 1.3), two of them were in age group 30–45, and one was in the 45–60 age group. All had chronic liver disease and the HCV strains of these cases belonged to genotype 3a.

3.4 Risk factors for HCV infection

Risk factors for HCV infection in this population were mostly needle-prick, ear piercing, tattooing, use of unsterilised blade in the community barber’s shop, etc. These percutaneous factors resulted in 292 cases of the total infected population and 19 cases had a previous history of blood transfusion. We could not identify the exact risk factor for the rest of the cases due to lack of information provided by the patients (Table 1).

3.5 Distribution of HCV genotypes

Among 97 HCV-RNA positive acute liver disease patients, 12 (12.4%) were genotype 1b, 2 (2.1%) genotype 2a and 9 (9.3%) belonged to genotype 2b. However, the predominant genotype was 3b (42.3%) followed by 3a (28.9%). The remaining five (5.1%) samples were untypeable by RT-PCR (Table 2).

Among 323 chronic hepatitis patients with active viral infection, 31 (9.6%) isolates were genotype 1b, three (0.9%) were genotype 2a and two (0.6%) were genotype 2b, whereas most prevalent genotypes were 3a (34.7%) and 3b (47.7%). In this study, 17 samples (5.3%) could not be typed by the present genotyping scheme and 12% was of mixed genotype (3a and 3b) (Table 2).

3.6 Correlation of genotype with other parameters

Since genotype 3a, 3b and 1b were most prevalent in the study population, age specific prevalence of these major genotypes were analyzed (Fig 1). The number of patients with active HCV infection [n = 420] belonging to each age group were as follows, 142 were in the age group <30 years, 227, 30–45 years, 39, 45–60 years and 12, >60 years. The
prevalence of genotype 1b increased with age in both the groups (P < 0.01), whereas 3a and 3b showed association with younger age groups. Serum alanine amino transferase (ALT) levels were significantly higher in acute than in the chronic hepatitis C cases (P < 0.001) (Table 2).

3.7. Comparison of sequences of the core and envelope genes

A stretch of 1791 bp from the 5'-UTR, core and envelope region of the genome was sequenced in 200 randomly selected HCV isolates representing all the genotypes detected during this study. Then BLAST search was carried out with all the sequences individually to know their affinity to other prototype HCV strains. Homology analysis by BLAST of those sequences confirmed the RT-PCR data except for one sample (NB51). Therefore, sequence analysis indicated high reliability of Ohno's genotyping scheme and confirmed the RT-PCR data. The ambiguous isolate NB51 was designated genotype 2b by RT-PCR, but interestingly showed maximum sequence homology (98.2%) with genotype 3a isolates.

Similar sequences showing maximum homology to one particular genotype were grouped together, making 2 groups of genotype 1b, 6 groups of genotype 3a and 9 groups of genotype 3b. NB149 and NB265 of genotype 2a, NB19, NB169 and NB358 of genotype 2b and one representative sequence from each of the rest of the groups were considered for construction of a phylogenetic tree along with reference HCV sequences (Fig 2a). Representative sequences were 1b—NB27, NB234, 3a—NB106, NB117, NB181, NB308, NE362, NB485, 3b—NB39, NB96, NB153, NB160, NB279, NE328, NB389, NB411. All the test sequences clustered within the expected clades.

3.8. Analysis of untypeable isolates

All the 22 untypeable isolates were sequenced to assign probable genotypes. The 5'-UTR, core and envelope regions were sequenced partially from the PCR amplified products. Phylogenetic analysis showed that eight of them (NB67, NB882, NB125, NB128, NB131, NB135, NB187, NB192) had maximum homology with genotype 3a isolates and five (NB42, NB57, NB134, NB211, NB236) showed maximum homology with genotype 3b isolates. The phylogenetic tree (Fig 2b) also revealed clustering of the three isolates with 3a reference strains and the other five clustered with prototype 3b isolates. Among the remaining nine isolates, only four could be amplified, leaving five isolates, which could not be amplified. These four isolates showed varied homology to different unique HCV isolates. One isolate (NB50) showed maximum homology with the strain Th580, which is believed to be of genotype 6b. Another isolate (NB74), showed 90.4% homology with an American strain H77, genotyped as 1a, and 2 isolates (NB179 and NB193) showed maximum homology with an Indian strain, IND308, which was closely related to a Nepalese strain NE137, genotyped as 3b (Tokita et al., 1994). All 17 HCV sequences included in this study were submitted to the GenBank database and the accession numbers are AY231582 to AY231598.

4. Discussion

Infection with HCV has been ascribed as the cause of most of the chronic liver disease cases in various parts of the world. The death toll due to HCV infection, from end-stage liver disease and its complications including hepatocellular carcinoma is increasing continuously over the years (Okada K., 1997). As a consequence, hepatitis C infection is a devastating problem for the individual patient and also a huge financial burden on the society particularly in developing countries with limited resources at their disposal for the treatment of infected cases. Moreover, there is also a paucity of information on HCV prevalence from the developing countries of the world. Recently, information on HCV prevalence in the developing countries, especially from Asia and Pacific regions are significantly increasing, where the vast majority of human population resides. It is necessary to have detailed data on the prevailing HCV genotypes in these regions. In these parts of the world, HCV epidemiological data had been derived mostly from blood donors and small segments of liver disease patients (Armanpurkar et al., 2001, Das et al., 2002; Issar et al., 1995, Mathai et al., 2002, Panyagrahui et al., 1997, Raghuraman et al., 2003, Saran et al., 1996, Sawant et al., 1999, Sood et al., 1999). Data on prevalence of HCV marker in the general population of the city of Kolkata is not available. However, a previous study carried out among multiple transfusion recipients and multiple needle stick injury cases, showed a moderate HCV prevalence (8.8%) among the latter group (Neogi et al., 1997). A recent study showed an extremely high seroprevalence (80%) of HCV infection among the intravenous drug users in the city of Kolkata (Sarkar et al., 2003). On the contrary to the above information, a recent study among rural asymptomatic population of West Bengal showed a very low prevalence (0.9%) of HCV in-
Infection (Chowdhury et al., 2003), which confirmed a similar study conducted in rural Rajasthan, Western India, where the rate of prevalence was 0.1% (Chadhya et al., 1999). Invariably, the urban populations of India are more exposed to risk factors for blood borne diseases than rural population. Data on HCV prevalence in large metropolitan cities like New Delhi, showed a significant 19% seropositivity among voluntary and replacement blood donors, which is supposed to be a reflection of the general population (Pangrahi et al., 1997). A low prevalence was reported from semi-urban areas of south India, 0.8% (Chandrashekharan et al., 2000), from western India, 0.3% (Garg et al., 2001) and moderate rate (1.8%) from central India (Jaiswal et al., 1996). A few studies were conducted among north Indian population, from acute and chronic liver disease patients with a large sample size, but no comparative genotype prevalence data were available from those studies (personal communication).

The overall prevalence of active HCV infection among symptomatic liver disease patients in this study was significantly high (15.9%). Such high prevalence of HCV among liver disease patients were also reported from Africa (21%-33%) (Som et al., 1996, Tsega et al., 1995), Korea.
(15.4%), Indonesia (11.8%) and also from some other parts of the world (Ayoola et al., 1992, el Guneid et al., 1993, Kim and Park, 1993, Sulaiman et al., 1991) In this study, HCV and HBV co-infection were very few in number among these liver disease patients. Since this study encompasses very few cases with HCV/HBV co-infection, it is extremely difficult to draw any conclusive inference on the effect of co-infection on the disease progression.

Most of the HCV seropositive subjects were viraemic (83.5%) indicating active and possibly recent infection. Such high prevalence of viraemia was also observed in other studies conducted in several countries of the world (Ahmed et al., 1998, Garner et al., 1997, Madhava et al., 2002, Strickland et al., 2002). The seropositive acute liver disease patients group showed a comparatively lower percentage of active viral infection probably indicating the fact that in most of these cases the infection was resolved. Approximately 33% of the seropositive subjects in this study had elevated ALT levels suggesting the presence of necro-inflammatory active liver disease and vi-
ral replication. As expected, we could not detect any significant correlation between genotype preference and ALT levels. Majority of the HCV–RNA positive subjects (62%) had elevated ALT levels in acute hepatitis, but a significant proportion of the chronic liver disease patients (69%) had normal serum ALT levels, probably reflecting the fluctuations of ALT levels in chronic HCV cases. These findings are in agreement with the findings of other studies (Dincer et al., 2001), which revealed that significant liver disease might be present in chronic patients irrespective of viral load, genotype and alanine transaminase levels.

In the current study, the frequencies for HCV genotypes in different age groups were compared for the two study populations. There was a significant correlation between HCV genotype and age of patients. The frequency of genotype 1b was found to increase in older individuals in both the groups. On the other hand, genotype 3b was found to be more prevalent among younger age group. Genotype 3a showed a similar pattern of distribution as observed for genotype 3b. Since we could not detect any subtypes of genotype 1 other than 1b (only one strain of genotype 1a detected after sequencing), it is not possible to assess the age preferences of different subtypes of genotype 1 from currently available data. The association of type 1b with older age may indicate that 1b was previously the main subtype in this region and subsequently there was a shift in predominating subtype of HCV from 1b to 3a and 3b. Recent studies from some parts of the world indicated that increased rate of intravenous drug use was the cause of the shift in genotypic prevalence from subtype 1b to 3a (Bourliere et al., 2002, Kalimaa et al., 2001). But in our study population, the subjects were not exposed to intravenous drug abuse, whereas most of the exposures were due to improper sterilization of glass syringes and needles for reuse in injecting drugs during treatment of ailments. Therefore, it is difficult to hypothesize any possible reason for the genotypic shift in our study population.

According to recent studies, HCV genotype 3 was mostly prevalent in Thailand and other Southeast Asian countries (Verachai et al., 2002) whereas in Japan, China and Indonesia, 1b and 2a were predominant genotypes (Dev et al., 2002, Hotta et al., 1997, Kim and Park, 1993). Our genotypic prevalence data are in agreement with earlier reports from north India (Pangrahi et al., 1996) and south India (Amarapurkar et al., 2001, Vallammam et al., 1995), since they also reported high prevalence of genotype 3. The distinctive shift in the genotypic prevalence clearly indicates the fast changing evolutionary scenario of HCV infection and the ensuing danger caused by the virus in this geographical region.

Sequencing data of the untypeable isolates showed that eight of them had maximum homology with type 3a reference strains. However, the multiplex RT-PCR for genotyping of the isolates did not yield any product with 3a type-specific primers. When we aligned these sequences with 3a reference sequences, we detected significant changes in the 3a specific primer binding region, but overall, the sequences maintained 98% homology with reference strains. Sequence analysis of another five isolates that showed maximum homology with 3b reference strains also yielded similar results. One isolate (H77) showed maximum homology with a type 1a strain, but could not be amplified by 1a-specific primer in genotyping PCR. This isolate was the only 1a strain we could detect during this study period. Another isolate (NB56) showed maximum sequence homology with a Thai strain (Th580) typed as 6b. Since our genotyping method does not encompass 6b, this isolate was not typeable by multiplex PCR. Though this is the second report of detection of type 6 m this country, the previous being from Pune (Lole et al., 2003), it is difficult to provide any explanation for the spread of a Thai strain over to this subcontinent. But geographical proximity and frequent visits of travelers between India and Thailand suggest that HCV might have spread along with HIV, since strain similarity among these population have already been reported for HIV infection in India (Chakrabarti et al., 2000, Naik et al., 1991). It also indicates that such strains may be circulating all over South Asia, but due to lack of intensive surveillance system many of them could not be detected earlier. Recent studies conducted on Southeast Asian population support the concept that race and ethnicity are important determinants of treatment outcome in HCV infected patients (Dev et al., 2002). Therefore, further studies of molecular epidemiology of HCV infection are essential for designing treatment modalities for control of HCV infection in this part of the world.

Acknowledgements

This study was supported by financial assistance from Indian Council of Medical Research (ICMR), Government of India. We are grateful to S Sarkar for excellent technical assistance. We thank the Medical Officers of Command Hospital, B M Birla Heart Research Center, Woodlands Hospital and Research Center, ID and BG Hospital, Kolkata, for providing us with the samples for this study. S Chaudhuri and S Das were supported by Senior Research Fellowships from ICMR and Council of Scientific and Industrial Research, Government of India, respectively.

References


Ohno M, Marzouma M, Wu RR, Saleh MG, Ohba K, Orto E, et al. New hepatitis C virus (HCV) genotyping system that allows for identifi-
cation of HCV genotypes la, 1b, 2a, 2b, 3a 3b 4, 5a, and 6a J Clin Microbiol 1997,35 201–7.


