Frequency of infection by hepatitis B virus and its surface mutants in a northern Indian population

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Introduction: The reported prevalence of hepatitis B virus (HBV) infection in the Indian general population varies from 2% to 11%. Epidemiological studies conducted so far have selection biases, since these included populations of defined age group, gender, social class, high-risk group, etc. The present study was designed to look for the molecular epidemiology of HBV infection in the rural and urban general populations in India. Methods: Sera obtained from healthy volunteers during college and social service camps from parts of northern India were tested for HBsAg and anti-HBC using enzyme immunoassays and for HBV DNA using polymerase chain reaction and Southern blot hybridization. The amplification products were cloned and sequenced, and nucleotide and deduced amino acid sequences of the surface and polymerase genes were analyzed for mutations. Results: Of the 730 subjects (rural 543, urban 187), 15 (2.1%) tested positive for HBsAg and 143 (19.5%) for anti-HBc; 10 were positive for both. The overall HBV exposure rate in the population was 20.3% (148/730). The HBsAg carrier rate was similar in the urban and rural populations (1.5% and 2.3%; p=ns). History of parenteral interventions or blood transfusion was associated with markers of exposure to HBV (10.2% vs. 4.6%; p=0.01). Among the 220 representative samples tested for HBV DNA, 14 (6.4%) were positive; of these, only four were positive for HBsAg or anti-HBc. Sequencing of a 388 nt segment of the S-gene from three individuals (two adw and one ayw subtype) revealed four mutations. Two and three of these led to amino acid changes in the HBV surface and polymerase genes, respectively; alterations in known cytotoxic T cell epitopes of HBV surface and polymerase proteins were observed in one individual each. None had the G587A mutation, which is known to be associated with loss of the 'a' determinant of HBsAg. Conclusion: Our study shows a high frequency of exposure to HBV infection in the Indian general population; a proportion of HBV infected persons were detectable only by molecular methods. The positivity rate was higher in the rural population. [Indian J Gastroenterol 2003;22:132-137] Key words: Epidemiology, variants

The reported hepatitis B surface antigen (HBsAg) carrier rate in India ranges from 1% to 13%, with a national average of 4.7%. However, these figures are based mostly on prevalence studies in blood bank donors. In India, most of the donors are not true voluntary donors but are replacement donors, and are often young men. Also, these data have an urban bias since most blood banks are located in urban areas. Some studies have looked at prevalence of hepatitis B virus (HBV) infection in tribal populations and in some select groups such as pregnant women, children and high-risk groups. Only one community-based study has looked at the prevalence of HBV infection in a rural community.

It is important to understand the dynamics of HBV transmission. Perinatal transmission is believed to be the most important mode in regions with intermediate and high HBV prevalence rates; in contrast, sexual transmission is the predominant route among adolescents in low-prevalence, developed countries. Studies from India have also shown horizontal transmission in the first few months of life as important. However, the route of transmission in a large majority remains unknown.

Variants of HBV have been described for all the four gene products; of these, surface ('S') gene variants are of particular interest. They were originally described as vaccine escape mutants in children who had developed HBV infection despite having received immunophylaxis at birth. Recently, surface gene variants have been found in unvaccinated random population too. This emphasizes the need for constant epidemiological surveillance as these variants could pose a threat to HBV control programs.

The present study was designed to generate community-based data on the prevalence of HBV infection in both rural and urban populations using serology and HBV DNA detection using polymerase chain reaction (PCR). Further, we looked at the frequency of naturally occurring mutations in the 'S' gene in this population.

Methods
Subjects
Venous blood was collected from 730 healthy volunteers
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(median age 28 years, range 4 mo to 80 y; 353 male) attending college or social service camps from in and around the northern Indian cities of Lucknow, Saharanpur (Uttar Pradesh) and Jalandhar (Punjab). Of these, 626 (298 men) were adults and 104 (55 male) were children (<18 y of age). All adult volunteers gave informed consent; in case of children, consent was obtained from a parent or guardian. Volunteers were asked about any previous episodes of jaundice and potential risk factors for exposure to HBV, such as close household contact with a patient suffering from jaundice, intravenous infusion of blood or other fluids, and alcoholism. None of the volunteers had received HBV vaccination.

Serum was separated using precautions to avoid cross contamination, and stored at -20°C in several aliquots. All the 730 specimens obtained were tested for HBsAg and anti-HBc antibody. In addition, a subgroup of 220 subjects representing both genders and all age groups was chosen for HBV DNA testing.

Serological assays
HBV serological markers (HBsAg, anti-HBc) were assayed using commercial enzyme immunoassay (Organon Teknika, Boxtel, Holland), as per the manufacturer's instructions.

PCR for HBV DNA
Serum DNA was extracted using a standard protocol. In brief, 250 µL of serum was digested with 250 µg/mL proteinase K (Sigma, St. Louis, MO, USA) in 150 mM NaCl, 10 mM EDTA, 10 mM Tris-Cl (pH 7.6), 2% sodium dodecyl sulfate and 5 µg/mL calf thymus DNA (Sigma, St. Louis, MO, USA) overnight at 37°C. This was followed by phenol and chloroform/isoamyl alcohol extraction, and DNA precipitation with one-tenth volume of 3M sodium acetate and 2 volumes of ethanol overnight at -20°C. After washing with cold 70% ethanol, the DNA pellet was resuspended in 50 µL distilled water.

Extracted DNA (5 µL) was added to a reaction mixture containing 20 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 0.25 mM deoxynucleotide triphosphates (Bangalore Genei, Bangalore), primers for the 'S' gene (forward: nucleotide [nt] 412-433, 5' CCT GCT GCT ATG CCT CAT CTC C 3' and reverse: nt 779-778, 5' CAG GGG TAT AAA GGG ACT CAC G 3' [GenBank accession numbers AJ428524 and AJ428525]) and 0.6 unit of Taq polymerase (Bangalore Genei, Bangalore) in a final volume of 50 µL: 35 cycles of amplification (94°C, 55°C and 72°C for 1 min each) were carried out followed by a final extension at 72°C for 10 min. The expected 385-basepair PCR product was detected by 1.5% agarose gel electrophoresis followed by Southern hybridization using a digoxigenin-labeled full-length HBV DNA probe and DIG detection system (Roche, Indianapolis, USA). The sensitivity of the assay was 2 x 10^4 genome equivalents of HBV DNA per mL of serum.

Serum obtained from a healthy person negative for all serological markers of HBV was included as a negative control, and serum from a patient with proven chronic hepatitis B, elevated serum ALT and detectable serum HBV DNA was used as a positive control in each assay run. Standard precautions to prevent DNA contamination, including physical separation of pre-PCR and post-PCR steps, aliquoting of reagents and use of barrier tips, were observed.

DNA sequencing
The PCR products were purified and cloned at Sal site of a pUC18 vector using Sure Clone Ligation Kit (Amersham, Buckinghamshire, UK). The cloned DNA was purified using polyethylene glycol and subjected to automated sequencing (ABI Prism™ 377 DNA Sequencer, Perkin Elmer, Cetus, CT, USA) using the M13 universal primer (5'-TGT AAA ACG ACC GCT AGT-3'; Bangalore Genei, Bangalore). Nucleotide and deduced amino acid sequences for the 'S' and the overlapping polymerase proteins were compared with sequences available in the GenBank for HBV subtypes ayw (GenBank accession numbers U55551, V01460/J02203, X68292, Y07587, X72702), adw (V00366/02201/X00715, X51970, D00330), adw (V00867) and ayw (X04615). Mutations were defined as sequences that differed from the consensus sequence of all the GenBank sequences. The deduced amino acid sequences were screened for mutations in the putative cytotoxic T cell (CTL) epitopes of the HBV 'S' and the polymerase proteins.

Statistical analysis was done using the χ² test.

Results
Sero-prevalence
Of the 730 serum specimens, 15 (2.1%) tested positive for HBsAg and 143 (19.5%) for anti-HBc; 10 specimens

![Fig 1: Prevalence of HBsAg and anti-HBc in the population. Difference between prevalence in second and third decades was significant (p<0.001). Number of subjects in each group is given in parenthesis and number of seropositive subjects is given above bars.](image-url)
Table 1: Relationship of risk factors for HBV exposure with HBV status

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>HBV positive (n=148)</th>
<th>HBV negative (n=582)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jaundice</td>
<td>21 (14.2%)</td>
<td>37 (9.8%)</td>
</tr>
<tr>
<td>Close contact</td>
<td>12 (8.1%)</td>
<td>88 (15.1%)</td>
</tr>
<tr>
<td>Parenteral interventions</td>
<td>16 (10.8%)</td>
<td>28 (4.8%)</td>
</tr>
<tr>
<td>Alcoholism</td>
<td>5 (3.4%)</td>
<td>12 (2.1%)</td>
</tr>
<tr>
<td>No risk factor</td>
<td>102 (68.9%)</td>
<td>415 (71.3%)</td>
</tr>
</tbody>
</table>

*Some individuals had more than one risk factor.

A larger proportion of persons with past history of intravenous infusions had markers of HBV exposure than those without (10.8% vs. 4.8%; p=0.01), whereas fewer persons with history of close contact had markers of HBV infection than those who did not have such history (8.1% and 15.1%; p<0.05; Table 1).

**HBV DNA positivity**

A subset of 220 specimens was tested for HBV DNA. Of these, 48 (21.8%) were positive for one or more serological markers of HBV: 4 for HBsAg alone, 41 for anti-HBc alone, and three for both HBsAg and anti-HBc. HBV DNA was detected in 14 (6.4%) specimens, including four that were positive by serology. Thus, after the addition of HBV DNA test results, the prevalence of those with evidence of HBV infection increased from 48/220 (21.8%) to 58/220 (26.4%).

**Sequencing**

Nucleotide and deduced amino acid sequences for a 388-nt segment of the 'S' gene of HBV from three DNA-positive individuals (GenBank accession numbers AF472603 to AF472605) were compared with the published HBV sequences. Among these three, two were of the adw subtype and one of the ayw subtype of HBV. Mutations were observed at four positions (Table 2); two of these mutations were silent and two were associated with amino acid substitutions. Of the two mutations that led to amino acid changes (Ser136Pro), one was located in the immunodominant 'a' determinant region of the 'S' gene and the other (Leu94Phe) outside it (Table 2).

In the polymerase gene, which overlaps with the 'S' gene of HBV but is in a different frame, four mutations were observed; of these, 3 were associated with amino acid substitutions and one was silent (Table 2). One of the mutations (Val438Phe) was found in a region upstream of domain B of the polymerase gene; the domain B overlaps with the 'a' determinant region of the 'S' gene.

These observed amino acid changes correspond to the putative CTL epitopes of both the 'S' and polymerase proteins of HBV. Mutation at amino acid residue 94 (Leu94Phe) of the HBV 'S' protein was located within a previously described HLA class I restricted CTL epitope,
Table 2: Mutations in the 388-base-pair long HBV DNA amplification product from three HBV DNA-positive individuals and their effect on amino acid sequence of viral surface and polymerase proteins

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Subtype</th>
<th>Nucleotide position</th>
<th>Substitution</th>
<th>Amino acid position</th>
<th>Substitution</th>
<th>Nucleotide position</th>
<th>Substitution</th>
<th>Amino acid position</th>
<th>Substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>ayw</td>
<td>G-T (TTT -&gt; TTT)</td>
<td>94</td>
<td>Leu -&gt; Phe</td>
<td>458</td>
<td>G-T (TTT -&gt; TTT)</td>
<td>94</td>
<td>Leu -&gt; Phe</td>
<td>458</td>
</tr>
<tr>
<td>N2</td>
<td>adw</td>
<td>A-C (CCA -&gt; CCC)</td>
<td>120</td>
<td>Silent</td>
<td>516</td>
<td>A-C (ATC -&gt; CTC)</td>
<td>497</td>
<td>Ile -&gt; Leu</td>
<td></td>
</tr>
<tr>
<td>N3.2</td>
<td>adw</td>
<td>T-C (TAC -&gt; TAG)</td>
<td>196</td>
<td>Ser -&gt; Pro</td>
<td>562</td>
<td>T-C (CTC -&gt; CCG)</td>
<td>497</td>
<td>Ile -&gt; Leu</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>T-C (TAT -&gt; TAC)</td>
<td>200</td>
<td>Silent</td>
<td>756</td>
<td>T-C (TGT -&gt; CCG)</td>
<td>497</td>
<td>Silent</td>
<td></td>
</tr>
</tbody>
</table>

Nucleotide position determined from position 0 at EcoRI site in ayw
Amino acid positions numbered from start Met residue of hepatitis B surface protein
Amino acid positions numbered with respect to ayw genotype D of hepatitis B virus

namely, HBsAgL[11-1] (LLCLLLYLV). Mutation at amino acid residue 497 (I497F) in the HBV polymerase protein coincided with HLA class I restricted CTL epitope PolG6G9-496 (LLGNYSHFI).

Discussion

This study reports epidemiological data of hepatitis B prevalence in a large group of randomly selected urban and rural subjects in India. Although the overall HBsAg positivity was low, the seroprevalence (HBsAg and/or anti-HBc positivity) was high, with a significantly higher HBV exposure rate in the rural than in the urban population. No person in the first decade of life had serological markers of HBV. There was an increase in seroprevalence with increasing age, especially from 2nd to 3rd decade of life; this was particularly evident among males. Also, some of the subjects who were seronegative for HBsAg and anti-HBc tested positive for HBV DNA.

The present study reports a high exposure rate to HBV (30.9%) in a general population, whose HBsAg positivity rate was relatively low. Our data are based on an unselected sample of rural and urban communities and hence do not suffer from the biases that could have been present in previous reports, which were based on blood donors or other select population groups.

The HBV seroprevalence in our study showed a significant increase from second to third decade of life (p<0.001), suggesting that exposure frequency occurs in early adulthood and that routes other than perinatal transmission may also be important in the spread of HBV. The overall seroprevalence rate was higher in males as compared to females; this difference was more pronounced in the rural population. These data point to a higher transmission rate of HBV infection among young males, especially in rural areas. Age below 20 years and male gender have been shown to be risk factors for HBV infection in other studies. Rural societies impose strict social restrictions on young women, while men are allowed to move out freely. Sexual transmission in prepubescent age has been implicated in the West; however, close physical contact and other local cultural practices may be more important in our population.

Another significant observation in this study was the higher anti-HBc positivity in the rural than the urban population (23.4% vs. 8.6%); this trend was observed in all the age groups, reaching significance in the 11-20 y age group. A high HBsAg carrier rate of 5.3% has been reported from a rural community from West Bengal, and HBsAg positivity was reported to be higher among rural blood donors than their urban counterparts in Kashmir. Studies on rural-urban differences in HBV prevalence have shown carrier rate of 5.3% in Nairobi city as compared to 12% among rural Kenyans, and higher seroprevalence rates among South American low-income pregnant women as compared to women from higher income brackets. These rural-urban differences may be related to horizontal transmission between siblings, which is facilitated by poor and crowded living conditions. Practices such as scarification, shared razors, tattooing, and ear piercing; use of inadequately sterilized syringes and mosquito bites may be important in rural areas and have been previously implicated.

In our study group, parenteral interventions were found to be the most significant risk factor for exposure to HBV. History of injections was also found to be the most significant risk factor in the study from West Bengal. It has been estimated that up to 50% of injections may be given using re-used syringes and needles. Repeated use of syringes and needles without sterilization is known to lead to outbreaks of HBV infection.

Molecular detection of HBV by PCR was done in a subgroup of 220 subjects. HBV DNA was detected in 14 of them (6.4%); of those, 10 (71.4%) were negative for other serological markers (HBsAg and anti-HBc) of HBV infection. HBV DNA has been detected in HBsAg-negative blood units from Taiwan, Italy, France, and India.

In the present study, we found amino acid substitutions in putative CTL epitopes in both the surface and polymerase genes of HBV. Mutations in HLA-A2
restricted CTL epitopes of the surface gene have been previously reported.28 Such mutations may lead to failure of immune cells to recognize the proteins of the mutant virus and thus predispose to chronic infection.29 In a similar survey done in Singapore, 4% of a random population was HBsAg positive; 8.6% of subjects were HBV DNA positive and had mutations in the 'a' determinant.11 The identification of HBsAg mutants in unimmunized general population points to emergence of mutations in the absence of any immune pressure. HBV S gene mutants have been shown to cause liver disease in both chimpanzees and humans30,31 and should thus be treated with caution because they pose threat to control of HBV.

The present study highlights the high prevalence of HBV infection in both rural and urban communities in India and transmission of infection in young adults. This high transmission rate implies inadequacies in our health delivery system, such as the indiscriminate use of parenteral interventions. There is thus an urgent need for improvement of health care delivery and introduction of health education in these areas.

References


Hepatitis B and mutant prevalence in northern India


Conference report
European Association for Study of the Liver, 2003

The EASL 2003 conference was to be held in Istanbul, Turkey in March-April 2003 but, due to the war in Iraq, was shifted to Geneva, Switzerland and held from July 3-6, 2003. There were about 2000 delegates attending and 750 Abstracts submitted. I have reported here on subjects that may interest Indian gastroenterologists. The EASL recommendation on treatment of hepatitis B was presented and is available at www.easl.ch. Apart from the standard use of interferon and lamivudine, it covers recommendations on the use of adefovir, which has recently been approved for use in Europe and by the US FDA and should soon be available in India. Adefovir 10 mg/d for 2 years results in ALT normalization in 70% of patients, with undetectable HBV DNA and <2% viral resistance (Abstracts A-492, 529, 627, 628); it is effective in patients failing lamivudine therapy (A-635). New drugs likely to be approved in 2004 include entecavir (A-585). A point to note is that HIV+HBV co-infected patients may be given lamivudine if on HAART or adefovir if not on HAART. The combination of lamivudine + pegylated-interferon resulted in sustained undetectable HBV DNA in 50% of patients compared to 10% with pegylated-interferon alone (A-95); the combination of lamivudine + adefovir did not improve response (A-69). Quantitative determination of HBV DNA and pre-genomic RNA in liver biopsy by TaqMan PCR may provide evidence of residual HBV (A-384).

With regard to HCV, the number of CD8+ cells in pre-treatment liver biopsy may predict response to IFN-ribavirin treatment (A-585). HIV+HCV co-infected patients who have CD4 >200 may be given pegylated-interferon + ribavirin combination for 48 weeks (A-92).

Use of albumin to prevent renal failure during large-volume paracentesis is a standard recommendation, but paracentesis with a slow flow rate could be safe and economical (A-80).

Anti-fibrotic drugs under investigation include sirolimus / rapamycin (A-167), pentoxifylline (A-243), realxim (A-246), K-17.22 (A-258) and metadoxine (A-259). It would be interesting to know if such drugs are useful in non-cirrhotic portal fibrosis.

Despite all the fanfare, artificial liver support systems have not reduced the mortality from fulminant hepatitis and liver transplant remains the only treatment that improves survival. However, the early use of N-acetyl-cysteine was encouraged in all patients since the contribution of paracetamol can rarely be excluded.

Combination of tamoxifen, retinoic acid, angiogenesis inhibitor and histone deacetylase inhibitor has additive effects against experimental hepatoma (A-53, 318), as may sRNA directed against bel-1 xl (A-362). These could be the future approach for treatment of unresectable hepatomas.

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Reference