4.1 Patient selection:

To investigate the issues related to the proposed objectives, a prospective study was undertaken including patients with chronic HBV infection screened at the Medicine and Gastroenterology unit, Central hospital, NF railway, Maligaon, Guwahati, Assam, India, between August 2007 and July 2011. Patients were seen every 3 to 6 months, 1 year or more often if clinically indicated. At each visit, liver biochemistry and HBV serology (HBsAg, HBeAg, Anti-HBe etc) was monitored. HBV DNA quantification was performed at presentation and at a minimum interval of 6 months to a maximum interval of 1 year as follow-up.

Serum was stored at -20°C for HBV DNA testing. Any patient with co-infection with other hepatitis viruses and HIV, or having an alcoholic background as well as users of hepatotoxic drugs were excluded from the study. A total of 523 HBsAg-positive for ≥6 months, anti-HBc-total positive patients from different states and corners of Northeast India (i.e. Assam, Arunachal Pradesh, Meghalaya, Manipur, Mizoram, Nagaland and Tripura) was enrolled for the present study, with complete clinical, biochemical and serological profile at presentation.

4.1A Inclusion criteria:

Patients with chronic hepatitis B infection who were (i) HBsAg positive for at least six months or more, (ii) HBeAg positive 'or' HBeAg negative/anti-HBe positive, (iii) having elevated transaminases (>1.5 times upper limit of normal), (iv) HBV DNA positive by a quantitative assay (Dot blot hybridization or b-DNA, Bayer Diagnostics Ltd., NY, USA, or Digene Co., USA).

4.1B Exclusion criteria:

These included (i) positive serology for HCV or HIV, (ii) prior treatment with an antiviral or immunomodulatory agent in the past 6 months, (iii) associated autoimmune or metabolic liver disease, (iv) severe systemic diseases such as renal failure. (v) pregnancy (vi) absence of informed written consent.

In parallel to this, family screening was performed for 2182 voluntary candidates for the above enrolled chronic HBV patients, with demographical, clinical, biochemical and serological profile. Initially, 316 asymptomatic HBsAg-positive cases at presentation were identified, and were further followed up for six months or more. A total of 172 asymptomatic patients who were found to be HBsAg after 6 months, Anti-
HBs negative, Anti-HBc-total positive and HBV DNA positive were enrolled in the present study with informed consent. The rest 144 out of the 316 patients who were found to be HBsAg-negative, and anti-HBc-total positive after 6 months were excluded from the study. All the patients enrolled for the current study (N=523+172=695) were clinically co-related with severity viz; chronic hepatitis, cirrhosis, by virtue of endoscopic and ultrasound based examination, with routine histopathological examination of liver biopsies for end-stage cirrhosis.

4.2 Terminologies:

The patients enrolled in the present study were diagnosed and treated for the hepatitis B virus infection and were assigned the appropriate terminologies for the diagnosis and outcome of the therapy.

**Chronic Hepatitis B (CHB):**

An individual testing positive for HBsAg on two occasions spaced six months apart and clinical data showing features of chronic hepatitis (Raimondo et al., 2003). Chronic hepatitis B patients were further categorized as:

(a) **Hepatitis B e antigen positive HBV infection:**

An individual having clinically proven features of chronic liver disease and were HBsAg positive for more than 6 months with positive HBeAg and HBV DNA (Raimondo et al., 2003).

(b) **Hepatitis B e antibody positive HBV infection:**

Individuals having clinically proven features of CLD, HBsAg positive for 6 months with HBeAg negative, anti-HBe positive and HBV DNA positive status (Raimondo et al., 2003).

4.3 Biochemical and Serologic Tests

Biochemical tests were performed using semi-automated analyzers. The upper limit of normal for serum ALT was 40 IU/L. HBsAg, HBeAg, and anti-HBe were assayed with commercially available enzyme-linked immunosorbent assay (ELISA) kits. HBsAg status was checked at least twice after a minimum interval of 6 months for all the HBsAg positive cases at presentation. HBeAg and Anti-HBe status was monitored for baseline samples as well as follow-ups.

4.3.1 Detection of Hepatitis B surface antigen (HBsAg):
Principle: HBsAg detection is based on Enzyme linked immunosorbant assay (ELISA). Antibody to HBsAg (anti-HBs) coupled to horse raddish peroxidase (HRP) serves as the conjugate with tetramethyl benzidine (TMB) and peroxidase as the substrate. Development of the colour after completion of the test, indicates the presence of HBsAg.

Procedure:

100 µl of serum sample was pipetted into 96 wells plate, each coated with lyophilized conjugate (HRP- labeled anti-HBs). Two negative controls and one positive control were used in each strip. Strips were incubated at 37°C for 1 hour, followed by thorough washing by soaking four times with phosphate buffer. Next, 100 µl of TMB substrate was pipetted into each well. Again strips were incubated at 18-25°C for half an hour. Reaction was stopped by adding 100 µl of 1 N sulfuric acid to each well. The ELISA reader was then blanked on air (without stripholder and strips) then absorbance of the solution was read in each well at 450 ± 5 nm (single wavelength).

Cut off value was calculated using the formula 0.5 (P +N).

P = mean absorbance of positive Controls.

N = mean absorbance of negative Controls.

4.3.2 Detection of hepatitis B precore antigen (HBeAg):

Principle: Bioelisa HBeAg is a sandwich type immuno enzymatic method in which the wells of a microplate are coated with anti-HBe. The specimen to be analysed is incubated in one of the microfilter plate wells. If the sample contains HBeAg, it will not bind to the anti-HBe on one plate well. After washing in order to eliminate any unbound material, anti-HBe conjugated with peroxidase is added, this reacts with possible antigen-antibody complex formed during the first incubation. After this second incubation and second washing, an enzyme substrate solution containing a chromogen is added which will develop a blue colour, if the sample is positive. The blue colour changes to yellow after blocking the reaction with sulphuric acid. The intensity of the colour is proportional to the concentration of HBeAg in the specimen.
100 µl of HBeAg positive controls two in number and three negative controls were transferred to wells. 100 µl each of these specimens were also transferred to the marked wells. Next, 50 µl of conjugate was added to each well except in blanks. The microplate was then covered with an adhesive seal and shaken gently then incubated for 3 hours at 37°C. During the last 5-10 minutes of this incubation period, chromogen solution was diluted with substrate buffer. Adhesive seal was removed; contents of the wells were aspirated and filled with diluted washing solution. This process was repeated four times. After final washing, microtiter plate was blotted on absorbent tissue to remove any excess liquid from the wells, and incubated uncovered at room temperature (20-25°C) for 30 minutes. Thereafter, 100 ul of stopping solution was added to stop the reaction. Spectrophotometer was used at 450nm, first for blank then for reading the absorbance of each well. The cut off value was calculated using a formula 0.5 (N+P).

4.3.4 Detection of HBe Antibody:

Principle: It is a sandwich type immnoenzymatic method in which wells are coated with anti-HBe using anti-HBe marked with peroxidase as conjugate. This test is based on the principle of the neutralization of anti-HBe by a solution containing HBeAg. The greater the concentration of anti-HBe present in the specimen, the smaller the amount of HBeAg which will bind to the well and which will react with anti-HBe conjugated with peroxidase.

Procedure:
First, 50 µl of anti-HBe positive control to 2 wells and 50 µl of negative control to 3 wells were transferred. Next 50 µl each of the specimens was also transferred to wells. 100 µl of neutralization buffer was added to each well except the blanks. 50 µl of conjugate was also added to each well except blanks. The plate was covered with an adhesive seal and incubated for 3 hours at 37°C. After 3 hours incubation, contents of the wells were aspirated and filled completely with diluted washing solution. This process was repeated 4 times. Then, 100 µl of substrate TMB solutions was added to the wells, including the blanks. Incubated uncovered at room temperature for 30 minutes. Reaction was stopped by adding 100 µl of stopping solution. Then absorbance was read for each well at 450 nm in the spectrophotometer. The cut off value was calculated using a formula 0.5 (N+P).

4.4 Biochemical analysis:
Complete panel of liver function tests including levels of Serum Bilirubin, Aspartate Amino Transferase (AST), Alanine Amino Transferase (ALT), Serum Alkaline Phosphatase (SAP), serum Proteins were determined in a semi-auto analyzer. The values obtained were compared with the normal values chart. Raised levels of serum ALT were taken as an indication of necro inflammatory condition.

4.5 Clinical examination:

Each patient was carefully examined by me and/or a qualified gastroenterologist to determine signs of chronic liver disease. Endoscopic and CT based examination was performed for assessing the severity of liver disease in patients.

4.6 Histopathological studies:

Many of the enrolled patients scoring positive for HBsAg and HBV DNA with evidence of hepatitis (ALT > 1.5 times that of normal) with clinical or radiological evidence of advance chronic liver disease including cirrhosis were advised to go for liver biopsy (in and outside the hospital), which was performed with prior informed consent. Liver sections of 5-micron thickness were stained with eosin and hematoxylin. All the liver biopsies were examined by a qualified and experienced pathologist to assess the Histological Activity Index (HAI, calculated according to Knodell et al, 1981) and fibrosis score, and confirm for the presence of cirrhosis.

4.7 Extraction of HBV DNA from sera samples:

The DNA was extracted form 100 µl of patient serum with the help of standard phenol/chloroform extraction method. Briefly, 100 µl sera samples were incubated with 5 µl proteinase K (15mg/ml), 10 µl of 10X sera lysis buffer and 5 µl of 20% SDS at 37°C for 3 hours. Subsequently, the supernatant obtained after phenol, chloroform and chloroform Iso-amyl alcohol (24:1) steps were left for overnight DNA precipitation in the presence of 3M Sodium Acetate pH 5.2, and absolute alcohol. After centrifugation, the obtained pellet was washed with 70% alcohol, dried, and dissolved in 30µl of TE (10mM Tris and 1 mM EDTA) buffer.

4.8 Quantification of Serum HBV DNA Levels

Serum HBV DNA levels were quantified commercially using ultrasensitive hybrid capture assay by Digene Co. (Gaithersburg, MD) with a lower limit of detection being 4700 copies/ml (940 IU/ml).
Procedure:

30 µl of each calibrator, control and specimen was pipetted into the bottom of the designated Microplate well. The clear plastic 96 well round bottom plate was used. Immediately before pipetting into microplate wells, the serum specimen was vortexed for standard test thoroughly for 10 to 15 seconds.

Denaturation:

The denaturation reagent with indicator dye was transferred into reagent reservoir. With multichannel pipette, 30 µl of denaturation reagent was pipetted into the bottom of each well. The microplate was covered tightly with a plate sealer by pressing down firmly and the plate was placed on rotary shaker set at 1100 rpm for 1 minute for mixing thoroughly. The controls, calibrators and specimens should be purple. Then incubated at 65°C ± 2°C for 30 minutes in the microplate heater.

Hybridization:

The plate was removed from the incubator immediately and plate sealer was carefully peeled off to avoid condensation and splashing. Each well was inspected and noted for any location where evaporation has occurred; specimen in that well was re-tested. The probe mix was transferred into reagent reservoir and then with a multi channel pipette, 30 µl of probe mix was pipetted into the bottom of each well. Then, microplates were placed onto a rotary shaker set at 1100 ± 100 rpm for 1 minute to mix thoroughly. The controls, calibrators and specimen turned yellow. Then again incubated at 65°C ± 2°C for 60 ± 5 minutes in the microplate heater.

Hybrid Capture:

After removing plate from the incubator, peeling off by the sealer was done immediately to avoid condensation and splashing. Using multi channel pipette, carefully transferred 75 µl from each microplate well to the corresponding well of the capture microplate. After covering the microplate tightly with a new plate sealer by pressing down firmly, it was placed on the rotary shaker set at 1100±100 rpm for 60 minutes at room temperature. Wash buffer was prepared during this incubation and the liquid was removed form the capture microplate by aspirating it out with a multi channel pipette set at 100 to 150 µl. The plate was tilted to about 45 degrees to capture maximum volume without touching the bottom of the well. Extreme care was taken at this step to avoid cross contamination and scratches at the bottom of the capture microplate. To remove maximum amount of liquid from the capture microplate after aspiration, blot plate was placed on adsorbent blotting pad available from Digene, with enough force to create imprints of individual wells.
**Hybrid Detection:**

An appropriate amount of Detection Reagent 1 was aliquoted into a reagent reservoir then 75 µl of Detection Reagent 1 was carefully pipetted into each well of the capture microplate using the multi channel pipette. Then plates were covered with clean parafilm and incubated at 20-25°C for 30 ± 3 minutes.

**Washing:**

The plate was decanted and was shaken 2-3 times with a downward motion above the absorbing blotting pad. The plate was hand washed 6 times with the help of Digene's wash apparatus.

**Signal Amplification:**

The Detection Reagent 2 was aliquoted into a new reagent reservoir then carefully with multi channel pipette, 75 µl of detection reagent 2 was pipetted into each well of the capture microplate. The microplate was covered with clean parafilm and incubated for 15 minutes. Finally reading of the microplate was taken on the DML 2000TM Illuminometer immediately after 15 minutes of incubation period.

**4.9 Genotype Determination by Multiplex PCR**

HBV genotypes were determined by multiplex PCR method designed by Oliver Krischberg et al. Briefly 3µL of extracted HBV DNA was subjected to PCR using primers used by Oliver et al, PCR amplification with the help of set of twelve primers was carried out with initial denaturation at 94°C for 10 min., followed by 35 cycles of 94°C for 1 min., 60°C for 1 min., and 72°C for 2 min. A single cycle for primer extension was done at 72°C for 7 min. Ten µl of amplified product was analyzed on 2.5% agarose gel in 1X TAE.

The size of the expected genotype specific amplification was: genotype A 370 bp, genotype B 190 bp, genotype C 701 bp, genotype D 147 bp, genotype E 787 bp, genotype F 481 bp. The samples showing multiple band patterns, i.e. same sample showing different amplicon size corresponding to the sizes of the PCR product for specific genotype by multiplex PCR genotyping method were categorized as mixed genotype. To avoid false positive results, 20% of the cases were re-genotyped and 10% of such representative cases including mixed HBV genotype were subjected to direct sequencing and compared with the standard gene bank isolates for respective HBV genotypes, to further validate our results.

The sequences of the primers used for HBV genotyping were:

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<tr>
<th>Primer</th>
<th>Sequence</th>
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<thead>
<tr>
<th></th>
<th>Primer Sequence</th>
<th>Size</th>
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<tbody>
<tr>
<td>As</td>
<td>5’CGGAAACTACTGTGTTAGACGACGGGAC-3’</td>
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<tr>
<td>Aas</td>
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<tr>
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<td>5’AGAGGCAATAGTCGGAGCCAGGTCTG3’</td>
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### 4.10 Pre-core and core-gene amplification:

The amplification of Core promoter, Pre-core and core genes was done by using outer primers; PC1: 5’GTTGCAATGGACGACCACCGTGAAC3’ (nt. 1605-1627) and PC2: 5’CTTCTGCGACGCGATGGGA3’ (nt. 2410-2432) and the inner primers; PC3: 5’CATAAGAGAAGCTCTTGGACAT3’ (nt. 1655-1674) PC4: 5’GGCGAGGGAGTTCTTCTC3’ (nt. 2378-2396). First round PCR with the help of outer primers PC 16 and PC 17 was carried out with initial de-naturation at 94°C for 3 min., followed by 30 cycles of 94°C for 1 min., 60°C for 1 min., and 72°C for 1.30 min. A single cycle for primer extension was done at 72°C for 7 min. Ten ul of amplified product was analyzed on 1.2% agarose gel in 1X TBE. Samples that did not show amplification were directly used for nested amplification. Where amplifications were seen on the gel, nested PCR was done after appropriate dilution. Three ul of the first round PCR product (directly or after dilution depending on the band intensity visualized on the gel) was used as a template in a second amplification step. Second round PCR with the help of inner primers PC 70 and PC 74 was performed with initial denaturation at 94°C for 3 min., followed by 30 cycles of 94°C for 1 min., 55°C for 1 min., and 72°C for 1.30 min. A single cycle for primer extension was
done at 72°C for 7 min. Outer primers (PC1 and PC12) showed the amplification of 828bp and inner primers (PC3 and PC4) generated the fragment of 742bp.

For all the above described sets of PCRs, all negative controls from the first round of amplification were included in the second amplification step. DNA extracted from sera samples of healthy controls and water were used as negative control. The HBV DNA positive cases which couldn’t be genotyped by multiplex PCR method were subjected to semi-nested PCR for the surface and the overlapping polymerase region of HBV along with the PCR amplification of the BCP, precore and core region of HBV. The PCR amplified products were purified by gel-extraction method following the manufacturer’s protocol (*Qiagen, Germany*) and subjected to direct sequencing commercially.

**4.11 Amplification of the surface and overlapping polymerase regions:**

The part of the HBV genome encoding the Major Hydrophilic Region (which also includes the ‘a’ determinant) up to the last amino acid (aa 226) of surface gene and overlapping catalytic domains of Polymerase gene (nucleotide position 425 to 840) was amplified by nested polymerase chain reaction (PCR). Three ul of extracted DNA from sera samples was amplified in a 25ul reaction mixture containing dNTP, MgCl₂, PCR buffer and Taq DNA polymerase (Perkin Elmer) in the appropriate quantity. The amplification step was performed for 30 cycles with the outer primers SA1: 5’- AT-CGCTGGATGTGTCTGCGG-3’ and SA2: 5’-GGCAACGGGGTAAAGGTTCA-3’ (position 369 to 388 and 1136 to 1155 respectively) in the HBV genome. Ten ul of amplified product was analyzed on 1.2% agarose gel in 1X TBE. Samples, which did not show amplification, were directly used for nested amplification. Where 787 bp fragment was generated, nested PCR was done after appropriate dilution. Three ul of the first round PCR product (directly or after dilution depending on the band intensity visualized on the gel) was used as a template in a second amplification step performed for 30 cycles with inner primers SB1: 5’-TTAGGGTTTAAATGT,ATACCC-3’ (position 822 to 842) and SB2: 5’-CATCTTCTTGTTGTTCTCTGT-3’ (position 427 to 448). PCR was carried out using thermal cycler (Eppendorf) with denaturation at 94°C for 1 min., annealing at 55°C for 1 min., primer extension at 72°C for 2 min. for total of 30 cycles and finally a single cycle for primer extension was performed at 72°C for 7 min. Ten ul of nested PCR product (416 bp) was analyzed by agarose gel electrophoresis on 1.2% gel in 1X TAE buffer.

**4.12 Automated DNA sequencing:**

Purified PCR products were sequenced commercially with respective forward and reverse primers in an automated DNA sequencer (ABI 377-18 sequencing system, Perkin Elmer). Briefly, 200-250 ng of purified template in 5ul vol. was mixed with 4ul of sequencing reaction mixture (supplied) and 1ul each of
respective forward and reverse primers of 5 pmole/ul concentration in two different sets of reactions.
Sequencing reaction was carried out under the cyclic conditions of 96°C for 30 sec., 55°C for 30 sec. and
60°C for 4 min. After completion of 30 cycles, the sequencing reaction product was precipitated by adding
90 ul H₂O, 10 ul 3M NaOAc (pH 4.6) and 250 ul absolute alcohol at room temperature for 10 min., and
centrifuged at 12,000 rpm for 15 min. Subsequently, 70% alcohol wash was given to the pellet at the same
speed for 10 min. and the dried pellet at 37°C was dissolved in 3.5 ul of gel loading dye (Formamide and
Dextran Blue). Samples were denatured for 10 min. at 90°C, and then quenched on ice for 5 min., prior to
loading on polyacrylamide gel in the automated DNA sequencer.

4.13 Sequence and phylogenetic analysis

Each electrophorogram was carefully read to repair the appearance of 'N' in the sequence and to ensure
that the laser reader has correctly read the peaks in the electrophorogram. The corrected electrophorograms
thus generated were carefully analyzed using the bioinformatics tools such as multalin and expasy; and the
genotype was determined by phylogenetic tree analysis using the MEGA4 software. The standard sequence
available from NCBI genbank database for HBV A-H (genotype A, X70185; genotype B, D00331; genotype C,
X01587; genotype D, X72702; genotype E, X75664; genotype F, X75663; genotype G [Stuyver et al., 2000])
as well as recently reported isolates from Northeast India and adjoining areas were considered for the
preparation of the phylogenetic tree. The results were also cross-checked by PCR-direct sequencing of the
BCP, precore and core region of randomly selected cases.

4.14 Statistical Evaluation

Data were expressed as means ± standard deviations (SD). Statistical analyses were performed using a Chi
square test and Fisher’s exact test for categorical variables and Mann–Whitney’s U-test or one-way analysis
of variance for continuous variables, as appropriate. Differences were considered significant for P values
less than 0.05. The statistical analysis software that was used was SPSS, version 13.0. Logistic regression
analysis was used to assess the influence of each viral factor on the risk of cirrhosis development.