4. MATERIALS AND METHODS

This case control study was carried out in the department of microbiology at Chettinad Hospital and Research Institute from August 2015 to July 2018.

Study subjects

This study comprises three groups of individuals; which were patient group and healthy control group.

Patient group

Patients positive for HBV seromarker (HBsAg) with or without a history of Hepatitis B infection were enrolled in case group

Healthy control group

People who show negative for HBV seromarkers, no liver disease, and no previous history of hepatitis were enrolled. The selection of the healthy controls was matched 1:1 with HBV infected patients on the basis of age and sex.

Sample Source

Clinical sample

Five ml of blood was collected from cubital venous puncture in plain clot activator tube and K3 (liquid form) EDTA tube with appropriate precaution for serological and genomic study.

Patients, who attend Inpatient and Outpatient departments in General medicine, Gastroenterology at Chettinad Hospital and Research Institute, Kelambakkam, Chennai.

Sample Size: 182 HBV infected patients; 182 Healthy controls
**Statistical Methods:** The formula used for the sample size estimation based on Odds ratio for case control study

\[ n = \frac{(r+1) \ p \ (1-p) \ (z_\beta+z_\alpha/2)^2}{r \ (p_1-p_2)^2} \]

P1 = (OR)*P2/ P2 (OR-1) +1

P= (P1+P2)/ 2

r = ratio of cases to controls

P1= expected proportion of cases of interest in the study group.

P2= proportion of cases of interest in the control group.

α and β are Type I and Type II errors.

(1-β)= power of the test.

\[ Z_\alpha^2 \text{ and } Z_\beta \text{ – Standard Normal Table Values} \]

Odds ratio of DRB1*07= 3.76; P2= 0.25; r= 1

P1= 0.55; P= 0.45; α = 0.05; Z_\alpha^2 = 1.96; β = 0.20; z_\beta = 0.84

n = 182 samples (each for case and control).

**Criteria**

**Inclusion criteria**

Patients who shows positive for HBV seromarkers along with a history of Hepatitis B infection.

**Exclusion criteria**

Samples from the age groups below 18 years and above 65 years were excluded. Patients with Hepatitis of all other causes and samples showed reactive for Hepatitis C virus (HCV), Human Immunodeficiency virus (HIV) infection. Patient who were not willing to participate.
**Clinical Trial Registration & Human Ethics**

The study is registered in Clinical Trial Registration (CTRI/2018/01/011460). The study was approved by Institutional Human Ethics Committee (12/IHEC/3-18) on 18th April 2016 before the commencement of the study.

**Serological method**

Serum was separated from the sample after subjecting for centrifuge at 3000 rpm for 5 minutes. The serum samples were used for screening the viral markers by ELISA.
ELISA HBsAg [Transasia Bio-Medicals LTD, Daman, India]

Erba Lisa SEN HBsAg ELISA test is based on the sandwich principle. This technique allows qualitative detection of as low as 0.1ng/ml of HBsAg in patient serum/plasma. Presence of HBsAg in the specimen will bind to the polyclonal antibodies coated on the wells. Then horseradish peroxidase labelled monoclonal anti-HBsAg (conjugate) is added to the well which in turn binds to the HBsAg captured on the solid phase. Addition of substrate identifies antigen- antibody complex. The absorbance values are read when the reaction stopped. The colour developed is proportional to the amount of the HBsAg in the sample.

Test Procedure:

- An aliquot of 100µL of the sample diluent was dispensed in well A1 (blank). Then 25µL of the sample diluent was added to the rest of the wells.
- An aliquot of 75µl of HBsAg negative control (B1, C1 and D1), HBsAg positive control (E1) were dispensed into assigned wells. Then 75µL of the first sample was added in well F1, second sample in well G1 and so on….
- 50µL conjugate was added into all the wells including blank. After covering the plate with an adhesive seal, the plate was incubated at 37°C for 60 min., and then washed 5 times with washing buffer.
- Thereafter, 50µl of colour reagent was dispensed into each well including blank well; then the plate was incubated at dark room for 15 minutes at 25°C.
- The reaction was stopped by adding 100µl of 1 mol / L of H₂SO₄. The blue colour of the substrate turns to yellow (for positive samples) or remains colourless (for negative samples).
- The photometric reader was set at 450 nm.

Calculation and Interpretation of Results:

- **Blank value:** Absorbance value of blank should be less than 0.2.
- **Positive control:** Absorbance value of the positive control should be greater than 1.0
- **Negative control:** Absorbance values of the individual negative controls should be less than 0.1.
Calculation of the Cut-Off Value (COV) = 0.15+NCx.

Non-Reactive: Samples with an optical density less than the cut-off value are considered Non-Reactive.

Reactive: Samples with an optical density equal to or greater than the cut-off value are considered Reactive. These samples should be retested in duplicate.

If the optical density of the duplicates is less than the cut-off value, the specimen is considered Non-Reactive.

If the retested result of the duplicates is found reactive, the specimen is considered Repeatedly Reactive.

ELISA Anti-HBc total [General Biologicals Corporation, Science- Park, Taiwan]

AntiHBc total seroprevalence was confirmed by ELISA method, for in vitro qualitative detection of total antibody to hepatitis B virus core antigen (Anti-HBc total) in human serum or plasma (heparin, EDTA or citrate). The assay is based on a competitive principle between Anti- HBc present in the serum specimen and human peroxidase conjugated Anti-HBc when simultaneously incubated in a well coated with recombinant HBcAg. After incubation substrate solution containing a chromogen is added. Blue colour develops, if the sample is negative. The blue colour turns to yellow after blocking the reaction with sulphuric acid.

Test Procedure:

- All reagents and specimens were placed at room temperature (+20 to +30°C) before assay. Incubator was adjusted to +37±1°C.
- Two wells were reserved for blanks. 50μl of each control or specimen were added to appropriate wells of reaction plate (3 Negative Controls and 2 Positive Controls).
- And then 50μl of Anti-HBc Peroxidase solution was added to each well except the 2 blanks.
- The plate was gently tapped. The reaction plate was incubated at +37±1°C in an incubator for 1 hour.
- At the end of the incubation period, the adhesive slip was removed and discarded and then the plate was washed.
- After that, the plate was covered with black cover and incubated at room temperature for 30 minutes.
• Then 100µl of 2N sulphuric acid was added to each well including the two blanks to stop the reaction.
• The absorbance of controls and test specimens was determined within 30 minutes with a precision photometer at 450nm.

**Calculation & Interpretations:**

Calculation of the Cut off Value

\[
\text{Cut off Value} = \frac{(\text{NC} + \text{PC})}{5}
\]

• Specimens with absorbance values <0.9, 0.9-1.1 and >1.1 cut off value were considered as negative, equivocal and positive for Anti-HBc Total respectively.

**ELISA Anti- HBc IgM [General Biologicals Corporation, Taiwan]**

ANTICORASE MB-96 (TMB) is a solid-phase enzyme immunoassay for in vitro qualitative detection of IgM antibody to hepatitis B virus core antigen (Anti-HBc IgM) in human serum or plasma. The wells of microtiter plate have been coated with anti-human IgM. Serum specimen containing Anti-HBc IgM incubated in such well, all IgM-class Abs will bind to the solid phase, upon the addition of HBcAg antiHBs labelled with Horse Radish Peroxidase conjugate. After incubation and washing, an enzyme substrate solution containing a chromogen is added. If the sample is positive, the blue colour produced turn to yellow when the reaction is blocked.

**Test Procedure**

• All reagents and specimens were brought to room temperature (+20 to +30°C) before assay.
• For each specimen 1+100 dilution was made with exception of the controls. 5µl of each specimen and 500µl of specimen diluent were added to each tube respectively.
• One well was reserved for Blank. An aliquot of 100µl of samples (Negative control Positive control, Specimen Diluent) was dispensed into assigned wells.
• Then 5µl of each diluted specimen was added to each well containing Specimen Diluent, respectively. And incubated at +37°C for 1 hour, then washing was carried out 5 times with washing buffer.
Thereafter, 50µl of HBcAg reagent was dispensed into each well except the blank followed by 50µl of Anti-HBc-Peroxidase solution was added. After covering and mixing gently, the plate was incubated at 37 °C for 1 hour and then the plate was washed 5 more times.

Then 100µl of substrate solution was added into each well including the blank, the plate was incubated at room temperature for 30 minutes and the reaction was blocked with 100µl of 2N H₂SO₄.

The photometric reader was set at 450 nm and air blanked.

**Calculation and Interpretations**

**Calculation of the Cutoff Value**

Cutoff Value = NCx+ (PCx)/4

- Specimens with signal/cut-off ratio ≤0.9 and ≥ 1.1 were considered non-reactive and reactive for Anti-HBc IgM.

**ELISA HBeAg [DIA.PRO, Diagnostic Bioprobes Srl, Italy]**

Enzyme ImmunoAssay (ELISA) test for the determination of Hepatitis B Virus "e" Antigen and Antibody in human plasma and sera. This assay is based on a sandwich principle; the wells are coated with specific monoclonal antibodies to HBeAg. A conjugate contained two specific anti HBeAg monoclonal antibodies, labelled with peroxidase (HRP) is added to the wells to detect captured HBeAg. Presence of HBeAg in the sample is determined by means of a cut-off value, which allows the semi quantitative detection of the antigen.

**Test Procedure**

- The required number of strips was placed in the plastic holder and carefully identifies the wells for controls, calibrator and samples. A1 well was left empty for blanking purposes.
- Then 100µl of the antigen positive control was added in single and then 100µl of samples was dispensed in the proper wells.
- Then the micro plates were incubated for 60 min at +37°C. After incubation plates were washed.
- Enzyme conjugate (100 µl ) was added in to all wells, except for A1, used for blanking operations and then the microplates were incubated for 60 min at +37°C.
• 100μl Chromogen/Substrate was added into all the wells, including A1 and then the micro plate were protected from light and incubated at room temperature (18-24°C) for 20 minutes.
• The wells were dispensed with positive control and a positive samples turn from clear to blue.
• Then100μl Sulphuric acid was added into all the wells and when the stop solution was added, the positive control and positive samples turns from blue to yellow.
• The colour intensity of the solution in each well was measured using a 450nm.

**Calculation and Interpretations**

Cut-Off (Co) = NC + 0.100

• Specimens with absorbance values < 0.9, 0.9-1.1 and > 1.1 cutoff values were considered negative, equivocal and positive for HBeAg.

**ELISA HBsAb [Dia. Pro, Diagnostic Bioprobes Srl, Italy]**

Enzyme Immunoassay [ELISA] was done based on the indirect immunoenzymatic principle for qualitative determination of antibodies to the Surface Antigen of Hepatitis B Virus in human plasma and sera.

**Test Procedure**

• The required number of strips was placed in the micro plate holder. A1 and B1 wells were left empty as a blank.
• 50μl of the sample diluent was added to all the wells except for A1 and B1.
• Then 100μl of all the Calibrators, 100μl of Control Serum in duplicate and then 100μl of samples was added.
• Then the micro plate was incubated at +37°C for 60 min. After washing, 100μl enzyme conjugate was added to all the wells except A1 and B1.
• Then the microplate was incubated at +37°C for 60 minutes.
• After washing microplate, 100μl TMB/H2O2 mixture was added in to each well including the blank.
• After then the microplate was incubated at room temperature for 20 minutes.
Sulphuric acid (100µl) was added into each well to stop the enzymatic reaction. The colour intensity of the solution in each well was measured using a 450nm.

**Interpretations**

- Samples with a concentration lower than 10 WHO m lU/ ml are considered negative for anti HBs.
- Samples with a concentration higher than 10 WHO ml U/ ml are considered positive for anti HBs

**Molecular analysis**

Blood (2ml) in the K3 EDTA tube was used for genomic DNA by commercially available Nucleospin Blood column kit method. The quantity and the purity of DNA were confirmed by UV spectrophotometer. Each HLA gene presence was confirmed by uniplex PCR program, thereby HLA association was found out by Multiplex PCR for various HLA class II genes.

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Hepatitis B virus infected patients & Healthy controls

Blood was collected in K3 EDTA Vacutainer

Genomic DNA extraction (Nucleospin Blood Column Kit)

Uniplex PCR

PCR amplification and Primer designing (Inhouse)

Gel documentation and identification of HLA genes

Multiplex PCR

Confirmation of HLA association
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Genomic DNA Extraction from blood [NucleoSpin Blood, MACHEREY-NAGEL GmbH& Co. KG, Germany]

Principle

In Nucleospin Blood method, genomic DNA is prepared from whole blood, cultured cells, serum, plasma, or other body fluids. Incubation of whole blood in a solution containing large amounts of chaotropic ions in the presence of Proteinase K. Addition of ethanol to the Nucleospin Blood Columns was done for binding of DNA to the silica membrane. The binding process is reversible and specific to nucleic acids. Washing steps efficiently remove contaminations.

Preparation of working solution Wash

Buffer B5

Ethanol (200ml) - 100% was added to Wash Buffer B5 Concentrate for 50 preparations. The bottle was labelled to indicate that ethanol was added. Wash Buffer B5 was stored at room temperature (18-25°C).

Proteinase K

30mg of lyophilized Proteinase K was added to 1.35ml of Proteinase Buffer PB. This Proteinase K solution was stored at -20°C for up to 6 months.

Kit specifications

The kits allow purification of highly pure genomic DNA with an \( A_{260}/A_{280} \) ratio between 1.60 - 1.90 and a typical concentration of 40-60 ng per \( \mu \)L for the NucleoSpin Blood Kit

Protocols Lyse

blood sample

- Proteinase K (25 \( \mu \)L) and 200\( \mu \)L blood (equilibrated to room temperature) was added into 1.5ml micro centrifuge tubes.
- Then 200\( \mu \)L Buffer B3 was added to the samples and vortex the mixture vigorously (10-20s).
- Incubated samples at 70°C for 10-15 min.
Adjust DNA binding conditions

- Ethanol (210µL) -100% was added to each sample and vortex again.

Bind DNA

For each preparation, one Nucleospin Blood Column was placed in a Collection tube and the samples were loaded. Centrifugation was done for 1 min at 11,000 rpm. Collection tube with flow- through was discarded.

Wash silica membrane

1st wash

- The Nucleospin Blood Column was placed into a new collection tube (2 ml) and 500µL Buffer BW was added. Centrifugation was done for 1 min at 11,000 rpm. Collection tube with flow- through was discarded.

2nd wash

- The Nucleospin Blood Column was placed into a new collection tube (2 ml) and 600µL Buffer B5 was added. Centrifugation was done for 1 min at 11,000 rpm. Flow- through was discarded and the collection tube was reused.

Dry silica membrane

- The Nucleospin Blood Column was placed back into the collection tube and centrifugation was done for 1 min at 11,000 rpm.

Elute highly pure DNA

- The Nucleospin Blood Column was placed in a 1.5 ml microcentrifuge tube and 100µL preheated Buffer BE (70°C) was added. Buffer was dispensed directly onto the silica membrane. Incubation was done at room temperature for 1 min. Then after centrifugation was done for 1 min at 11,000 rpm.

Purity of genomic DNA sample
• The concentration of an DNA sample was checked by the use of UV spectrophotometry
• The nitrogenous bases in nucleotides have an absorption maximum at about 260 nm.
• For quantitating DNA, readings were taken at wavelengths of 260 nm and 280 nm. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample
• 1 O.D. at 260 nm for double-stranded DNA = 50 ng/ul of dsDNA.
• Pure preparations of DNA and RNA have OD260/OD280 values of 1.8 to 2.0, respectively.
• The concentration of the DNA in the sample was calculated as follows;
  DNA concentration (ng/ml) = (OD 260) x (dilution factor) x (50 ng DNA/ml)/(1 OD260 unit)

Procedure

• One ml of TE buffer was taken in a cuvette and calibrated the spectrophotometer at 260nm as well as280nm.
• Then 10 µl of each DNA sample to 900µl TE (Tris-EDTA buffer) was added and mixed well.
• TE buffer was used as a blank in the other cuvette of the spectrophotometer.
• OD at 260 and OD at 280 values were noted on spectrophotometer.
• Calculate the OD260/OD280 ratio.
• The amount of DNA was quantified using the formula: DNA concentration (ng/ml) = (OD 260) x (dilution factor) x (50 ng DNA/ml)/(1 OD260 unit)

Inferences

• A ratio between 1.8- 2.0 denotes that the absorption in the UV range is due to nucleic acids.

Primer design

The specific PCR primers were designed for the detection of HLA-DRB1*07:01, -DQB1*03:01 and -DPB1*09:01 loci by [Olerup et al., 1993] and synthesized by Biocorporals Company, Chennai. The primers amplifying human growth hormone gene (5’primer: 5’CAGTGCCTTTCCAACCATTTCCCTTA3’,3’primer:5’ATCCACTCAGGATTCTTGTGGTTC - 3’) were synthesized by Biocorporals Company, Chennai. Buffer mix (2X) was purchased from Emerald Amp GT PCR Master Mix, Incell Technologies.
### Table 3: List of Primers used for the study (Uniplex and Multiplex PCR)

<table>
<thead>
<tr>
<th>Gene*</th>
<th>Forward and Reverse primer( 5’ - 3’)</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA DRB1*07:01</td>
<td>5’CCTGTGGCAGGGTAAGTATA3’&lt;br&gt;5’CCCGTAGTTGTGTCTGCACAC3’</td>
<td>232</td>
</tr>
<tr>
<td>HLA DQB1*0301</td>
<td>5’GAC GGA GCG CGT GCG TTA3’&lt;br&gt;5’AGT ACT CGG CGT CAG GCG3’</td>
<td>122</td>
</tr>
<tr>
<td>HLA DPB1*0901</td>
<td>5’TCC CCG CAG AGA ATT AGG TGC3’&lt;br&gt;5’TCC TTC TGG CTG TTC CAG TAG3’</td>
<td>185</td>
</tr>
<tr>
<td>HGH(Human growth hormone gene 1)</td>
<td>5’CAGTGCCTTCCCAACCATTCCCTTA3’&lt;br&gt;5’ATCCACTCACGGATTTCTGTTGTTTC3’</td>
<td>439</td>
</tr>
</tbody>
</table>

### Table 4: Thermocycler program of Uniplex PCR amplification

<table>
<thead>
<tr>
<th>Steps</th>
<th>Number of Cycles</th>
<th>Temperature (ºC)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td></td>
<td>95ºC</td>
<td>5 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>10</td>
<td>95ºC</td>
<td>30 sec.</td>
</tr>
<tr>
<td>Combined-annealing extension</td>
<td></td>
<td>66ºC</td>
<td>60 sec.</td>
</tr>
<tr>
<td>Denaturation</td>
<td>20</td>
<td>94ºC</td>
<td>10 sec.</td>
</tr>
<tr>
<td>Annealing</td>
<td></td>
<td>61ºC</td>
<td>50 sec.</td>
</tr>
<tr>
<td>Extension</td>
<td></td>
<td>72ºC</td>
<td>30 sec.</td>
</tr>
<tr>
<td>Storage of amplified product</td>
<td></td>
<td>4ºC</td>
<td>∞</td>
</tr>
</tbody>
</table>
Uniplex PCR

Uniplex PCR was performed for the detection of each HLA genes individually. For both uniplex and multiplex PCR reactions were carried out in thermo cycler (Agilent Technologies Sure Cycler 8800).

**Uniplex PCR amplification program**

PCR was performed in 25µL reaction mixture containing

- 15.5µl of 2X buffer
- 100 ng genomic DNA (2µl)
- 0.2µmol/L primer
- 0.10µM of the internal control primer and
- 1.5 µl of deionized water.

The Uniplex PCR cycling parameters of HLA DRB1*07:01, -DQB1*03:01 and –DPB1*09:01 was given in table 4.

In each PCR reaction a primer pair was included to amplify the human growth hormone gene, which functioned as an internal positive amplification control to eradicate false negative result.

Multiplex PCR

Multiple primer pairs were used for various target sequences are used to enable simultaneous analysis of more than one sequence of interest.

**Multiplex PCR amplification program**

PCR was performed in 25µL reaction mixture containing

- 15.5µl of 2X buffer
- 100 ng genomic DNA (2µl)
- 0.2µmol/L primer
- 0.10µM of the internal control primer and
- 1.5µl of deionized water.
The multiplex PCR cycling parameters of HLA DRB1*07:01, -DQB1*03:01 and –DPB1*09:01 was given in table 5.

In each PCR reaction a primer pair was included to amplify the human growth hormone gene, which functioned as an internal positive amplification control to eradicate false negative result.

**Detection of PCR products**

Agarose gel of 1.5% was prepared by adding 0.90g of agarose to 60 ml of 1x TAE (Tris-Acetate-EDTA, Electrophoresis buffer), then the mixture was mixed thoroughly until it becomes clear. Gel was allowed to cool for few minutes and then ethidium bromide (40 μL) was added. After placing the comb in electrophoresis tank, the gel was poured and allowed to settle at room temperature, later the comb was removed carefully. 1x TAE buffer was added to the chamber that covers 2nm over the gel surface. DNA marker (7 μl) mixed with 3 μl of loading buffer (6xBlue) was loaded in first well. The amplified PCR products (25μl) were loaded to each of the well. The gel was run at 100 volts for 45 minutes. The results were read on UV transilluminator. The allelic type was determined according to the presence or absence of PCR products of the desired length.

**Table 5: Thermocycler program of Multiplex PCR amplification**

<table>
<thead>
<tr>
<th>Steps</th>
<th>Number of Cycles</th>
<th>Temperature (ºC)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td></td>
<td>95ºC</td>
<td>4 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td></td>
<td>95ºC</td>
<td>30 sec.</td>
</tr>
<tr>
<td>Combined-annealing</td>
<td>10</td>
<td>95ºC, 66ºC</td>
<td>30 sec.</td>
</tr>
<tr>
<td>Extension</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>20</td>
<td>94ºC, 61ºC, 72ºC</td>
<td>10 sec.</td>
</tr>
<tr>
<td>Annealing</td>
<td></td>
<td></td>
<td>50 sec.</td>
</tr>
<tr>
<td>Extension</td>
<td></td>
<td></td>
<td>30 sec.</td>
</tr>
<tr>
<td>Storage of amplified product</td>
<td></td>
<td>4ºC</td>
<td>∞</td>
</tr>
</tbody>
</table>
Statistical Analysis:

Allele Frequency (AF) of HLA class II alleles were calculated by direct count. To assess the association of HLA class II allele with AHB, CHB infection and healthy controls, Chi-square test was applied to a two-by-two contingency table based on the allele frequencies. Odds ratios (OR) were calculated based on the 2×2 table of allele count. Demographic characteristics of the study groups were compared using Pearson’s Chi-square test. The p value <0.05 considered as statistical significant for all tests. Statistical analyses were done using SPSS 10.0 software.