3. MATERIALS AND METHODS

The present work entitled that “Prevalence and Genotyping of Hepatitis B Virus surface antigen among pregnant women attending maternity hospital in Krishnagiri district, Tamil Nadu” carried out in the Department of Microbiology, M.G.R. College (Arts & Science), Hosur, from June, 2006 to September, 2011.

3.1. Study area

The study was conducted in Krishnagiri district which is located in north Tamil Nadu. All pregnant women attending antenatal clinics in General Hospital and primary health centers, Krishnagiri district (Hosur, Krishnagiri, Kelamangalam, and Kaveripattanam taluk) were included for study. Participation was voluntary and each one involved in the study has given a written consent. The selection was by simple random sampling method from January 2006 to March 2007. The information regarding age, occupation, marital status, previous history of surgery, blood transfusion, dental therapy and vaccination for jaundice were requested from the participants in the structural questionnaire.

3.2. Collection of sample

Five ml of blood sample was collected by venopuncture and allowed to clot naturally and the serum was separated by centrifugation at 1500 rpm for 15 minutes. The serum collected was tested for HBsAg using one step HBsAg test (Intec products, INC., India). The positive samples were stored at -20°C and further confirmed for HBsAg, HBeAg, and HBeAb using commercially available Enzyme Linked Immunosorbant Assay kit, (Span Diagnostics, India).

3.3. One step HBsAg test

One step HBsAg card test was performed to screen the samples positive for HBsAg. 100 µl of test serum sample was added to the card by using sample dropper. The result was
observed between 5-10 minutes for 5ng/ml and at 30 minutes for 1ng/ml. Development of purplish red colour in the test strip region shows positive result. The results were observed within 30 minutes.

3.4. Detection of HBsAg by ELISA

The prevalence of HBsAg was analyzed by ELISA using Span diagnostic Ltd, Surat, India. Required number of microwellstrips and reagents was brought to room temperature before performing test. 100 µl of sample diluents was added to the test and control well of the microwell strips, except blank well i.e., 1A. 100 µl of negative control was added to 1B, 1C, & 1D and 100 µl of positive control to 1E&1F and to rest of the wells 100 µl of serum or plasma was added as shown in the Table -3. The above sample mixture was mixed well and adhesive strip was covered over the wells. Incubated the reaction wells at room temperature for 30 minutes. After incubation the adhesive strip cover was removed and the well content was discarded. 350 µl of wash buffer was added to the wells and allowed to soak for 30 seconds per well and same procedure was repeated for 5 times. Then 50 µl of conjugate stabilizer and 100 µl of conjugate were added to the wells except 1A i.e., blank and incubated at room temperature for 30 minutes. After incubation the contents were discarded and washed 5 times with 350 µl of wash buffer. The moisture content of the micro well plate was removed by fresh disposable absorbent pad. 100 µl of TMB was added to all the wells and incubated in dark at room temperature for 30 minutes. Then 100 µl of stop solution was added to all the wells to stop the reaction. Then the content was mixed gently for 5-10 seconds and absorbance at 450 and 630nm were taken using ELISA reader (ECIL-MS5608A). The cut-off value was calculated based on the mean absorbance of three negative controls and addition of factor (0.10). Cut-off = NCX+0.10
### Table - 3 Plan and programme for ELISA test

<table>
<thead>
<tr>
<th>1A</th>
<th>Blank</th>
<th>2A</th>
<th>Test sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1B</td>
<td>Negative control</td>
<td>2B</td>
<td>Test sample</td>
</tr>
<tr>
<td>1C</td>
<td>Negative control</td>
<td>2C</td>
<td>Test sample</td>
</tr>
<tr>
<td>1D</td>
<td>Negative control</td>
<td>2D</td>
<td>Test sample</td>
</tr>
<tr>
<td>1E</td>
<td>Positive control</td>
<td>2E</td>
<td>Test sample</td>
</tr>
<tr>
<td>1F</td>
<td>Positive control</td>
<td>2F</td>
<td>Test sample</td>
</tr>
<tr>
<td>1G</td>
<td>Test sample</td>
<td>2G</td>
<td>Test sample</td>
</tr>
<tr>
<td>1H</td>
<td>Test sample</td>
<td>2H</td>
<td>Test sample</td>
</tr>
</tbody>
</table>

### 3.5. Elisa for detection of HBeAg

The prevalence of HBeAg was analyzed by ELISA using Smart test diagnostics kit. Required number of microwellstrips and reagents were brought to the room temperature before performing test. 100 µl negative control, antigen calibrator and antigen positive control was added to 1B, 1C, 1D and1E, 1F and 1G and 100 µl of test sample to rest of the wells respectively. The above sample mixture was mixed well and adhesive strip was covered over the wells and incubated at 37 °C for 60 minutes. After incubation the adhesive strip cover was removed and the well content was discarded and the well was washed for 5 times by using wash buffer. 100 µl of enzyme conjugate was added to all the wells except 1A and allowed to soak for 5-10 seconds, cover the wells with adhesive strips. The wells were incubated for 60 minutes at 37°C. After incubation the well content was discarded and washed with wash buffer. 100 µl of Chromogen substrate was added to all the wells including blank well 1A. The microplate was incubated at room temperature for 20 minutes. 100 µl of sulphuric acid was added to all the wells. Then the content was mixed gently for 5-10 seconds and absorbance at 450 and 630nm were taken using ELISA reader (ECIL-
MS5608A). The cut-off value was calculated based on the mean absorbance of three negative controls and addition of factor (0.100).

\[
\text{Cut-off (Co)} = \frac{\text{NCX} + 0.10}{3}
\]

### 3.6. Elisa for detection of HBeAb

The prevalence of HBeAb was analyzed by ELISA using Smart test diagnostics kit. Required number of microwell strips and reagents were brought to the room temperature before performing test. 50 µl negative control, antigen calibrator, and antigen positive control was added to 1B, 1C, 1D and 1E, 1F and 1G and 50 µl of test sample to rest of the wells respectively. 50 µl of HBeAg was added to all the wells. The above sample mixture was mixed well and adhesive strip was covered over the well and incubated at 37 °C for 60 minutes. After incubation the adhesive strip cover was removed and the well content was discarded and the well was washed for 5 times by using wash buffer. 100 µl of enzyme conjugate was added to all the wells except 1A and allowed to soak for 5-10 seconds, cover the wells with adhesive strips. The well was incubated for 60 minutes at 37°C. After incubation the well content was discarded and washed with was buffer. 100 µl of Chromogen substrate was added to all the wells including blank well 1A. The Microplate was incubated at room temperature for 20 minutes. 100 µl of sulphuric acid was added to all the wells and finally the well was taken to ELISA reader and the absorbance was recorded by using dichromatic (450-630nm) mode. The cut-off value was calculated based on the mean absorbance of three negative Controls and addition of factor (0.100).

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\text{Cut-off (Co)} = \frac{\text{NC} + \text{PC}}{3}
\]

### 3.7. Isolation of hepatitis B virus DNA

The viral DNA was isolated as per the standard procedure (Khaja et al., 2003). 100 µl of serum sample was added to sterile micro centrifuge tubes containing 400 µl of sterile triple distilled water. 500 µl Phenol (Tris-saturated, pH 8.0) was added to the tube and vortexed
gently. Then the tube was kept for incubation at 65°C for 2 hours in water bath. Then it was centrifuged at 12000 rpm for 15 minutes at 4°C. After centrifugation supernatant was transferred to the fresh tube and equal volume of chloroform and Isoamyl alcohol (24:1) was added. The content was vortexed and then centrifuged at 12,000 rpm for 15 minutes at 4°C. To the supernatant, 2/3 volume of 2M sodium acetate and 2 volume of absolute alcohol were added and it was kept for overnight incubation at -20°C. The next day tube was centrifuged at 12,000 rpm for 15 minutes at 4°C. After centrifugation the supernatant was discarded and the pellet washed with 75% ethanol. Then the pellet was dried at room temperature for 5 minutes and dissolved in 20 µl of TE buffer. This DNA samples was stored at -20°C for further analysis.

3.8. Amplification of HBsAg gene by Polymerase chain reaction

The hepatitis B virus surface antigen gene was amplified by degenerated primers designed from the existing GenBank database and synthesized from sigma genosys, Bangalore. The PCR reactions were carried out in 0.2ml thin walled PCR tubes in Eppendorf Master Cycler. Each PCR reaction mix was prepared in separate PCR tubes with each DNA templates for experimental samples. 50 µl of reaction mixture was added in a 0.2 ml of thin walled PCR tubes. 5µl serum DNA was added to each tube containing 50 µl of master mix Table - 4. The PCR tube was capped and vortexes for a few seconds to mix evenly. Then the tube was centrifuged for few seconds. The sample was placed in the thermal cycler (Eppendorf) and 35 cycles was performed as follows. After completion of the cycle, the sample was stored at -20°C.
### Table – 4. PCR Cycling Parameter

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94ºC</td>
<td>5 Min</td>
<td>Initial Denaturation</td>
</tr>
<tr>
<td>2</td>
<td>94ºC</td>
<td>45 Sec</td>
<td>Cycle Denaturation</td>
</tr>
<tr>
<td>3</td>
<td>55ºC</td>
<td>50 sec</td>
<td>Annealing</td>
</tr>
<tr>
<td>4</td>
<td>72ºC</td>
<td>50 sec</td>
<td>Extension</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Repeat the step 2,3 and 4 for 35 Cycles</td>
</tr>
<tr>
<td>5</td>
<td>72ºC</td>
<td>10 minutes</td>
<td>Final Extension</td>
</tr>
<tr>
<td>6</td>
<td>4ºC</td>
<td>For Long Time</td>
<td>Storing</td>
</tr>
</tbody>
</table>

**3.9. Agarose gel electrophoresis for PCR amplicon**

1.2% of agarose was prepared by using 1X TAE running buffer and melted till it forms clear solutions. Then the melted agarose was cooled below 45ºC and 0.5µl (5mg/ml) of ethidium bromide was added and casted with comb. After solidification the comb was removed and 3 µl of loading buffer was added to each sample. 15-20µl of sample was loaded on each well together with lanes containing 1kb ladder DNA marker. Then electrophoresis apparatus was connected with power pack and carried out at 5 V/cm at constant voltage of 50V for 2 hours. Then the apparatus was disconnected and examined the isolated DNA bands through UV-Transilluminator.

**3.10. Purification of amplified HBsAg gene PCR Product**

The amplified PCR products were purified by Gene JET™ Gel Extraction Kit (Fermentas).

Gel was viewed on UV transilluminator (360nm). The DNA containing fragment was sliced using a clean scalp or razor blade as close to the DNA as possible to minimize the gel
volume and the gel slice was weighed and placed in pre weighed 1.5 ml tube. 1:1 volume of binding buffer was added to the gel slice (volume: weight) for every 100mg of agarose gel. The gel mixture was incubated at 50-60°C for 10 min (until the gel slice is completely dissolved). Then the tube was mixed by inversion every minute to facilitate the melting process. 800 µl of the solubilized gel solution was transferred to the Gene JET™ purification column and Centrifuged for 1 min. Discard the flow-through and place the column back into the same collection tube. 100 µl of Binding Buffer was added to the Gene JET™ purification column and centrifuged for 1 min. Discard the flow-through and place the column back into the same collection tube. 700 µl Wash Buffer was added to the Gene JET™ purification column and Centrifuged for 1 min. Discard the flow-through and place the column back into the same collection tube. Then empty GeneJET™ purification column was centrifuged for 1 min for the complete removal of residual wash buffer. The Gene JET™ purification column was discarded and the purified DNA was stored at -20°C.

3.11. Genotyping of Hepatitis B virus

3.11.1. Ligation

About 0.3µg of the purified product of DNA was used for ligation in a 10µl reaction using InstAclone™ PCR Cloning Kit (Fermentas). The ligation reaction was performed at 16°C overnight. The ligation mixture containing about 0.3µg of the purified PCR product, 0.1µg of T/A vector, 1X Ligation buffer and 5U of T4 Ligase enzyme. The ligation mixture was used to transform competent E. coli DH5α cells and the clones were selected on an Ampicillin plate with X-gal by blue/white selection. The white colonies were screened by HBsAg gene specific primers by colony PCR.
3.11.2. Bacterial Transformation

The bacterial transformation was carried as per procedure described by (Sambrook et al., 1989). The bacteria were streaked out on a LB agar plate and allowed to grow overnight for single colony isolation. For plasmid extraction the bacteria were grown with appropriate selection antibiotic in 5 ml liquid medium for mini-prep and 50 ml for maxi prep. The cultures were grown for 12-14 hours at 37°C in an incubator shaker at a speed of 150 rpm.

3.11.3. Competent cell preparation

Bacterial transformation was carried out by using *Escherichia coli* competent cells. The competent cells were prepared by Calcium chloride method (Sambrook et al., 1989). *E.coli* was streaked on LB agar plate from a glycerol stock maintained at -70°C. The cells were grown at 37°C overnight to obtain single colonies. A single colony was inoculated into 5ml LB broth in a 50ml culture tube and was allowed to grow overnight at 37°C under vigorous shaking at 200rpm in an orbital shaker incubator. 1ml of this overnight culture was added to 50ml of 2 X YT broth and placed at 37°C in an orbital shaker incubator at 200rpm for 2-3 hours. The O.D was checked every one hour at 600nm in a spectrophotometer.

The culture was removed when the O.D$_{600\text{nm}}$ reached 0.5 and chilled at 4°C. 30ml of cells were transferred to 50ml centrifuge tubes and harvested by centrifugation at 4000rpm in refrigerated centrifuge set at 4°C. The rotors and tubes were chilled to 4°C prior to use. The medium was removed completely and the cells were gently resuspended in 100mM CaCl$_2$. The cells were incubated on ice for 30 minutes. The cells were again harvested by centrifugation at 4000 rpm at 4°C. 4ml of fresh CaCl$_2$ with 15% glycerol were added to tubes and the pellet was very gently resuspended. 100μl of cells were transferred into 1.5 ml microfuge tubes. The tubes were frozen in liquid nitrogen and stored at -70°C until further use. Transformation efficiency was calculated as No. of colonies / μg of DNA /mL of competent cells.
3.11.4. Transformation of HBsAg DNA into *E. coli*

The frozen *E. coli* competent cells stored at -70°C were thawed on ice. 0.3 µg DNA was added to the cells and placed on ice for 30 minutes. Thereafter, the cells were given a heat shock at 42°C for 90 seconds in a water bath. The cells were quickly placed back on ice and maintained for 5 minutes. 900µl of sterile LB broth was added and the mix was placed in an incubator shaker at 37°C for 1 hour. After one hour 200µl of cells were plated on LB agar plates with appropriate antibiotic selection and incubated at 37°C overnight. The transformed cells grew as colonies in the plates and were used for further analysis.

3.11.5. Screening of clones by Colony PCR

The white colonies were picked up with a sterile toothpick and were resuspended in 50µl of TE buffer. They were placed in a boiling water bath for 5 min. The cells were then centrifuged for 5 min and 1µl of it was used as template in a PCR reaction mixture. The PCR reaction mixture consists of Template DNA 1µl, dNTP mixture 2µl (final concentration 200µM), HBsAg Forward primer 2µl (0.4µM), HBsAg Reverse primer 2µl (0.4µM) 10X Taq polymerase assay buffer 2µl, Taq Polymerase enzyme 0.5µl (1.5unit) and Sterile MilliQ water to 20µl. Thermal cycling was performed at 95°C for 5min followed by 30 cycles of 95°C for 45s, 55°C for 45s and 72°C for 45s insert and a final extension of 72°C for 10min. The positive colonies gave an amplicon of appropriate sizes.

3.11.6. Isolation of plasmid DNA from *E. coli*

The plasmid extraction was performed according to the method of Birnboim and Doly (1979). A mini prep (3-5ml) was used for small scale extractions and a maxi prep (50ml) was used for large scale extractions. The *E.coli* cells were grown for 12-14 hours in 5ml medium with appropriate antibiotic selection in 50 ml culture tubes at 37°C in an incubator-shaker with a rotation speed of 150 rpm. The cells were then harvested by centrifugation in 1.5ml
micro centrifuge tubes at 12,000 rpm for 2 minutes. Tubes were completely decanted to remove even traces of the medium. The cells were resuspended in 100 μl of solution - I by vortexing or vigorous mixing. 200μl of solution - II was added, mixed thoroughly by inverting the tubes and were placed on ice for 5 minutes. 150μl of solution- III was added and the tubes were incubated on ice for 15 minutes. The mixture was centrifuged at 12000 rpm at 4°C for 5 minutes to remove the cellular debris and the clear lysate was transferred to a fresh tube. Equal volume of phenol (pH 8.0): chloroform (1:1, v/v) was added, mixed well and centrifuged at 12000rpm for 5 minutes. The upper aqueous phase was transferred to a fresh microfuge tube and an equal volume of chloroform was added, mixed well and centrifuged for 5 minutes at 12000rpm. The upper aqueous phase was transferred to a fresh microfuge tube. The DNA was precipitated by addition of equal volume of isopropanol. The pellet was dried and the DNA was dissolved in 25μl of STE buffer. RNase (5μg) was added to the tube and placed at 37°C for 10 minutes. The solution was made up to 100μl with sterile deionised water. Equal volume of phenol: chloroform mixture (1:1, v/v) was added, mixed well and centrifuged at 12000rpm for 5 minutes. The upper aqueous phase was transferred to a fresh microfuge tube and an equal volume of chloroform was added. After mixing well, the tubes were centrifuged for 5 minutes at 12000rpm. The upper aqueous phase was transferred to a fresh microfuge tube, 2.5 volumes of chilled 95% ethanol was added and was incubated at -20°C overnight for precipitation. The DNA was pelleted by centrifugation at 12000 rpm for 5minutes and was washed with 500μl of 70% ethanol and air dried. The pellet was dissolved in 50μl of 0.1X TE buffer. This plasmid DNA was directly used for further molecular analysis.
3.12. Sequencing of HBsAg gene

The extracted plasmid was reconfirmed for the presence of insert by PCR and sequenced with vector specific M13 Forward and reverse primers at Ocimum Biosolutions, Hyderabad. The sequences submitted to Gene bank database.

3.13. Sequence Analysis

The obtained sequences were analyzed using the viral genotyping tool in NCBI site (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi).

3.14. Phylogenetic Analysis

Complete coding sequence of Surface antigen protein of 21 HBV isolates were aligned with the CLUSTAL W software in MEGA4 and the alignment was confirmed by the visual inspection. The phylogenetic tree was constructed by the neighbor joining method using MEGA4 software. To confirm the reliability of the phylogenetic trees, bootstrap resembling test were performed 1000 times. The evolutionary history was inferred using the UPGMA method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.16927183 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein et al., 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 689 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2004).
3.15. Statistical analysis

Results obtained were subjected to statistical analysis using statistical package for social science (SPSS) version 10.0. Comparison of proportion was done using chi square test while p value less than 0.5 were considered significant. The mean percentage ages were calculated and compared. Multiple regression was used for HBsAg as dependent variable and the age, educational status, occupational status and other possible risk factors as independent variable P>0.5 was considered significant. The variables consist of percutaneous and non – percutaneous exposure e.g. age group, educational status, occupational status, previous pregnancies, history of jaundice, history of blood transfusion, immunization status, dental therapy and history of surgery.