Antigen and Analytical Techniques
During Hepatitis B virus infection, at least four antigen-antibody systems are observed: (1) Hepatitis B surface antigen (HBsAg) and its antibody (anti-HBs); (2) The preS antigens associated with HBsAg particles and their antibodies; (3) The particulate nucleocapsid antigen (HBeAg) and anti-HBc-Ag antibodies and (4) An antigen structurally related to HBcAg namely HBeAg and its antibody (anti-HBe).

The specific serological marker of HBV infection is HBsAg, which is present both in the intact virion and as excess free circulating filamentous and spherical 22nm subviral particles. HBsAg is a complex antigen and is composed of a major polypeptide, P25 and its glycosylated form GP28. Additional polypeptides of higher molecular weight (P39, L and GP33, M) are also components of the viral envelope. The relative proportions of P25 and GP33 are similar in the three morphologic forms of HBsAg. The nucleocapsid of HBV is a 30-32nm particle composed of multiple copies of a single polypeptide (P21) and intact structure exhibits hepatitis B core antigenicity (HBeAg). A non-particulate form of HBeAg is secreted into the serum during HBV infection.

Fig. 2.1: Antigen and polypeptide composition of envelope proteins of intact hepatitis B virus L:Large; M: Middle; S:S-region
In human vaccine trials HBsAg/P25 derived from the serum of HBV chronic carriers or produced by recombinant DNA technology has proved to be relatively effective in inducing anti-HBs and protecting against HBV infection.

**ELISA (ENZYME LINKED IMMUNO-SORBENT ASSAY)**

Enzyme linked immunosorbent assay is one of the widely acceptable techniques used for assaying of IgG and interleukin levels in serum after administration of an antigen.

Enzyme Linked Immuno-Sorbent Assay, commonly known as ELISA or EIA works on the principle of binding of enzyme linked antibody to antigen. An enzyme conjugated to an antibody reacts with a colourless substrate to generate a coloured reaction product. A number of enzymes have been employed for ELISA, including alkaline phosphatase, horseradish peroxidase, and p-nitrophenyl phosphate. When mixed with suitable substrate, each of these enzymes generate a coloured reaction product, which is measured using an ELISA plate reader.

A number of variations of ELISA have been developed, allowing one to detect and quantify either the antigen or antibody.

**Indirect ELISA**

Perhaps the simplest form of ELISA is the indirect or sandwich assay, commonly referred to as the 'dirty plate' assay. Antigen is bound passively by incubation with the microtitre plate, hence the name 'dirty plate'. The antigen solid phase is then used to bind specific antibodies in the test sample. Unbound material is removed by washing and bound antibody is detected using enzyme-labelled anti-immunoglobulin. If the enzyme-labelled antibody is specific for a particular class of immunoglobulin then the class specificity of antibody can be determined.
Two-Site ELISA
The concentration of antigen can be determined using a two-site ELISA. In this assay antibody bound to the microtitre wells is used to capture the corresponding antigen in the test sample. The bound antigen is subsequently detected using a second enzyme-labelled antibody. This provides a rapid, easy method for antigen detection.

Class Capture Assays
In certain circumstances it is desirable to separate a particular class of immunoglobulin from a sample of serum or secretion prior to measurement of antibody activity. The main reason for this is that the presence of a much larger quantity of antibody of different immunoglobulin class may interfere with the detection of a clinically relevant one. Class capture assays for the detection of specific IgM antibodies to viruses and bacteria, for IgG and IgE antibodies to allergens have been developed. In this assay microtitre plate is coated with class specific anti-immunoglobulin and used to capture the immunoglobulin in sample. The antibody activity of the bound immunoglobulin is identified by subsequent addition of enzyme labelled antigen or unlabelled antigen followed by labelled specific antibody.

Alternatively, antigen can be labelled with a hapten such as TNP or avidin. Class capture assays of this kind have considerable advantages over conventional indirect ELISA using class specific labelled detector antibodies principally because they avoid competitive inhibition caused by IgG antibodies of the same specific normally present in higher concentration in the body fluid.

Competitive ELISA
Competitive assays utilize limited concentration of both antigen and antibody as the amount bound to the solid phase is critical. Competition for binding to antigen can be
carried out using an antigen coated microtitre plate, where a fixed level of enzyme-labeled antibody competes with varying levels of unlabeled antibody in the sample. The relative concentration, epitope specificity and affinity of test and reagent antibodies is crucial to achieve sensitive, specific and reliable assays for antibody. Sensitivity can be increased if the unlabeled antibody is allowed to bind in a sequential fashion before the labeled reagent is added. The signal in this and in all competitive assays is inversely related to the concentration in the sample.

TECHNIQUES USED IN PRESENT STUDY

ELISA for Estimation of HBsAg
HBsAg content was measured using AUZYME MONOCLONAL® Kit purchased from Abbott Laboratories, USA. Standard curve was plotted according to the instructions given by the manufacturer (Fig. 2.2 & 2.3 and Table 2.1). Recombinant HBsAg was used in concentration range from 1-10ng/ml. One positive, one negative control and one reagent blank were prepared. 200μl of +ve control, -ve control, double distilled water and different concentrations of HBsAg (1-10ng/ml) were dispensed into bottom of wells of reaction tray. 50μl of conjugate (anti-HBsAg conjugated to Horseradish peroxidase enzyme) was added to each well and tray was gently tapped. One bead coated with antibody to HBsAg was added to each well and incubated at 40±1°C for 3 hours. After incubation liquid was aspirated and beads were washed four times with double distilled water. Washed beads were transferred to properly identified assay tubes. 300μl of freshly prepared OPD (O-Phenylenediamine) substrate solution was added to each tube and incubated for 30 minutes at room temperature followed by addition of 1N H₂SO₄ to each tube. Absorbance of the resultant solutions was measured at 492nm against the reagent blank.
Fig. 2.2: Principle of AUZYME MONOCLONAL Kit (Abbott Lab, USA)

Table 2.1: Standard curve of HBsAg using AUZYME MONOCLONAL® Kit (Abbott Lab, USA) at 492nm

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<th>S.No.</th>
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**Correlation coefficient**

\[ r = 0.999486 \]

**Intercept**

\[ c = -0.0045 \]

**Slope**

\[ m = 0.029609 \]

**Equation of line**

\[ Y = 0.0296X - 0.0045 \]

Estimation of Antibodies against HBsAg

Antibodies against HBsAg were measured using AUSAB®EIA Kit procured from Abbott Laboratories, USA. The AUSAB EIA test for anti-HBs uses the "Sandwich principle" a solid phase enzyme-linked immuno assay technique (Fig. 2.4).
Fig. 2.3: Regressed standard curve for HBsAg using AUZYME MONOCLONAL Kit (Abbott Lab, USA)
Serum specimens were serially diluted using specimens dilution buffer (Negative human plasma). 200µl of serum specimen dilutions, negative, positive controls as well as blanks were taken along with human serum standards of known anti-HBs concentration provided in the kit in separate wells. HBsAg coated beads were added to each well and incubated at 40±1°C for 2 hours. 200µl of biotin-HBsAg and Anti-biotin-HRPO into each well and incubated at 40±1°C for 2 hours. The liquid was aspirated and beads were transferred to identified assay tubes and 300µl of freshly prepared OPD substrate solution was added to each tube followed by incubation of 30 minutes at room temperature. 1N H₂SO₄ was added to each tube in order to stop the reaction and absorbance of control and specimens was measured at 492nm against a reagent blank.

Fig. 2.4: Principle of AUSAB®EIA Kit (Abbott Lab, USA) for estimation of Antibodies against HBsAg

ELISPOT
Spleen cells secreting antibodies (IgG) against HBsAg were tested by ELISPOT assay. The ELISPOT (Enzyme Linked Immunospot) assay was carried out as described by Sedgwick and Holt, 1986 with little modification.
Spleen of immunized animal was harvested and disrupted. Mononuclear cells were suspended at a concentration of 1-2X10⁶ cells/ml in complete tissue culture medium RPMI-1640 containing 10% Fetal clone I serum followed by incubation for 1 hour at 37±1°C. Microtitre plate was coated with HBsAg (0.2μg/ml) overnight at 4°C. The plate was washed with PBS-Tween solution and non-specific binding was inhibited by coating with skim milk (3%) in PBS-Tween 80 for 1h at room temperature. Spleen cells were placed in wells and plate was incubated at 37±1°C with 5% CO₂ for 4h. The plate was washed with PBS-Tween to remove free cells and alkaline phosphate conjugated rabbit anti-IgG were added to wells and incubated overnight. 100μl of 5-bromo-4-chloro-3-indoyl-phosphate in 0.6% agarose was added to each well and subsequently after solidification, the developed spots were counted using an inverted microscope. Antibody Secreting Cells (ASC) per 10⁶ cells were counted from mean number of spots for six wells.

**Cytokine Estimation in Serum**

Cytokine, i.e. interleukin 2, 4, 5 & 6 were estimated using the murine ELSA kit. 50μl of kit reagent was added to each well of precoated (with monoclonal antib-mIL-2, mIL-4, mIL-5 or mIL-6) microtitre plate followed by addition of 50μl diluted samples. Plate was then incubated at room temperature for 2h with subsequent washing with double distilled water. 100μl of conjugate in diluted buffer was added to each well and incubated for 1h at room temperature followed by washing with distilled water. After this 100μl of substrate was added to each well and kept in dark for 30 minutes at room temperature. The reaction was stopped using 100μl of stop solution provided in the kit and absorbance of resultant solution was measured at 450nm against the reagent blank.