MATERIALS AND METHODS
The present study was conducted in the Department of Pathology and Blood Bank, G.S.V.M. Medical College, Kanpur. A Total of 20000 blood donors of either sex and different age groups were included in the study. Apparently healthy Blood donors visiting the blood bank were tested for the presence of Hepatitis B Surface Antigen(HBsAg) and antibodies to Hepatitis C Virus(HCV).

1.5 ml of venous blood was drawn from anticubital vein of each individual of the study group using disposable needles and syringe. The blood was collected in plain, sterile vials under all aseptic precautions and the other relevant details including the age and sex were recorded. The whole blood was used for blood grouping and sera separated from the samples was subjected to tests, for HBsAg and anti-HCV antibodies detection.

**Combined ABO and Rh-d Typing**

For ABO typing whole blood was used. Anti-A and Anti-B sera provided in the kit agglutinate with the corresponding antigen present in blood. Rh blood group of a person was determined by the slide agglutination test using the person’s red blood cells and prepared known anti-Rh sera.

**Materials**

1. Slides

2. Anti-A, Anti-B and Anti-D typing sera, applicators or toothpicks 70% alcohol and cotton.
Photograph 1. Donor samples for HBsAg and anti-HCV detection

Photograph 2. Blood Grouping
Methods

1. Three small drops of blood from each sample were placed on the slide.
2. A drop of Anti-D, Anti-A and Anti-B sera was added to each drop of blood separately. Sera and blood were mixed with toothpick.
3. Mixtures on the slide were slowly agitated for two minutes.
4. All the drops were carefully examined for agglutination:
   a) agglutination in drop containing Anti-A sera showed blood group A.
   b) agglutination in drop containing Anti-B sera showed blood group B.
   c) agglutination in both the drops showed AB blood group.
   d) Blood group O showed no agglutination in the drops containing Anti-A and Anti-B sera.
   e) agglutination in drop containing Anti-D sera showed Rh-positive blood group.

Detection of Hepatitis B surface antigen

Tests for the detection of hepatitis B surface antigen were performed with Enzyme Linked Immunosorbent Assay(ELISA) using commercially available kit Microscreen (Span Diagnostics Ltd., SURAT, INDIA).
**Principle**

**Microscreen** is a direct, noncompetitive solid phase enzyme immunoassay.

1. If HBV antigen was present in patient’s serum, it combined with anti HBV antibody coated to the microtitre plate wells.
2. HBV antigen complexed with HBV antibody on solid phase further combined with peroxidase conjugate of anti HBV antibody.
3. On addition of substrate chromogen, the enzyme action produced a coloured end product.

**Contents of the kit**

**Reagent 1: Sample Diluent**

Contains Tris buffer and detergent

**Reagent 2: Conjugate**

Monoclonal anti HBsAg antibodies conjugated with Peroxidase containing protein stabilizers. (Ready to use)

**Reagent 2A: Conjugate Stabilizer**

Tris buffer containing stabilizer and preservatives. (Ready to use)

**Reagent 3: Washing Buffer(10x)**

Concentrated (10x) Tris buffer containing Tween-20 and Thimerosal (0.01%) as a preservative. Before use, dilute by adding one volume of concentrate to 9 volumes of distilled or reagent grade water.
Microwell coated with anti-HBV antibody

Test serum containing HBsAg antigens

Incubate
Wash

Add Enzyme conjugate

Incubate
Wash

Add chromogenic substrate
Add stop solution
Measure OD of coloured end product using ELISA reader

Figure 10: Principle of ELISA for detecting HBsAg
Reagent 4: Negative Control
HBsAg Negative Human serum containing preservative.(Ready to use)

Reagent 5: Positive Control
HBsAg Positive Human serum containing preservative(Ready to use)

Reagent 6: Colour Reagent
Citrate Acetate buffer containing peroxide and 3,3' 5,5' Tetramethylbenzidine(TMB) solution.(Ready to use)

Reagent 7: Stopping solution
Mineral Acid

Reagent 8
Microwell Strips coated with anti-HBs antibodies.

Accessories
Adhesive strip covers

Materials and Equipment used
1. Distilled water
2. Multichannel pipette (100µl), Micropipettes(100 & 1000µl) and disposable tips(0.1ml & 1.0 ml)
3. Measuring cylinder-100ml
4. Timer
5. Disposable absorbent pad or towels
6. Bleach(5% Sodium hypochlorite)
7. Disposable gloves
8. Microplate ELISA reader
Materials and Methods

Procedure

1. 100µl of sample diluent (Reagent 1) was added to test and control wells of the microwell strips (Reagent 8). 100µl of Negative control (Reagent 4) and Positive control (Reagent 5) were added to the respective wells. First well was kept as reagent blank.

2. Wells were covered with adhesive strip covers and incubated at room temperature (25°C to 40°C) for 1 hour.

3. Strip covers were discarded and the content of the wells were aspirated. 50µl of conjugate stabilizer (Reagent 2A) was added first followed by 100µl of conjugate (Reagent 2) to each well.

4. Wells were incubated at room temperature for 30 minutes.

5. Wells were washed 5 times with 350 µl of washing buffer (Reagent 3).

6. 100µl of colour reagent was then added (Reagent 6).

7. Wells were incubated at room temperature for 30 minutes in dark

8. 100 µl of stopping solution (Reagent 7) was added.

9. Absorbance was read at 450 nm.

Interpretation of the test was done within 30 minutes. The colour of each test sample well was compared with the positive and negative controls. The strip holder with the strips was placed in top of a light box.

The negative control was colourless whereas the positive control had Yellow-orange colour.
Photograph 3. Microwell ELISA plate for detecting HBsAg

Photograph 4. ELISA Reader (Labsystems)
A negative result was read when the colour of the test sample matched that of the negative control and the result was positive if it matched that of a positive control.

**Calculation**

**Cut off Value:** Cut off value was calculated by adding 0.1 to the mean O.D. of the negative control replicates.

**Interpretation of Results**

1. Samples giving absorbance less than the cut off value were considered negative or "Non-reactive" in the test.
2. Samples with the absorbance equal or more than the cut off value were considered "Reactive" in the test.

**Sensitivity and Specificity**

Microscreen has sensitivity of 0.25ng/ml of HBsAg and specificity of 99%.

**Detection of Antibodies against Hepatitis C virus**

Antibodies to HCV were detected with ELISA, using commercially available kit **INNOVA (Span Diagnostics Ltd., SURAT, INDIA).**

**Principle:** Anti-HCV version III is an ELISA test for the detection of antibodies to HCV in human sera or plasma. It contains Recombinant antigens from putative core (structural), protease / helicase (NS3, nonstructural), NS4 (nonstructural) and replicase(NS5, nonstructural) regions of the HCV genome. It has been observed that recombinant antigen obtained from
Microwell coated with recombinant antigens

Test serum containing antibodies to HCV

Incubate
Wash

Add Enzyme conjugate

Incubate
Wash

Add Chromogenic substrate
Add stop solution
Measure OD of coloured end products using ELISA reader

Antigen
Ab to HCV

Enzyme conjugate
Chromogenic substrate

Figure 11: Principle of ELISA for detecting anti-HCV antibodies
a single genotype of the virus is not sufficient to detect the anti-HCV antibodies in all cases. In this assay diluted serum sample were incubated in micro wells coated with a cock tail of recombinant antigens. After washing, the captured anti-HCV antibodies were incubated with peroxidase-conjugated anti-human immunoglobulin(IgG) and the bound enzyme was detected by adding chromogen(3,3' 5,5'- tetramethylbenzidine TMB) and substrate (hydrogen peroxide). A bluish colour developed in the wells indicating the presence of anti-HCV antibodies (positive). The enzyme reaction was stopped by adding mineral acid, resulting in a yellow colour which was then measured at a wavelength of 450nm.

Contents of the Kit

Reagent 1: Sample Diluent

Tris Buffer pH 8.0 containing salt, protein stabilizers and preservatives.(ready to use)

Reagent 2: Conjugate

Peroxide labeled anti-human immunoglobulin containing protein stabilizers and 0.01% Thimerosal as preservative.(Ready to use)

Reagent 3: Washing Buffer(10x)

Concentrated (10x) Buffer containing detergent, salt and 0.01% Thimerosal as preservative. Before use, dilute by adding 1 volume of concentrate to 9 volume of distilled water(1:10).
Reagent 4: Negative Control

Anti-HCV negative human serum, negative for antibody to HCV and containing 0.01% Thimerosal as preservative.(ready to use)

Reagent 5: Positive control

Inactivated human serum containing anti-HCV and 0.01% Thimerosal as preservative.(ready to use)

Reagent 6: Colour Agent

Buffer containing hydrogen peroxide and 3,3',5,5' Tetramethylbenzidine (TMB) in solution.(ready to use)

Reagent 7: Stopping solution

Mineral Acid

Reagent 8: Microwell Strips

Coated with HCV Recombinant and Synthetic Peptides.

Materials and Equipment used

1. Distilled water
2. Multichannel pipette(100μl), Micropipettes(100 & 1000μl) and disposable tips(0.1ml & 1.0 ml)
3. Measuring cylinder-100ml
4. Timer
5. Disposable absorbent pad or towels
6. Bleach(5% Sodium hypochlorite)
7. Disposable gloves
8. Microplate ELISA reader
Procedure

1. All the reagents were brought to room temperature before use except colour reagent (to be store at 2-8°C ,till use). Required number of antigen coated microwells strips (Reagent 8) were removed from the packet.

2. Reaction blank well was kept empty.

3. 200µl of sample diluent (Reagent 1) was dispensed to the rest of the required wells. Three Negative (Reagent 4) and One Positive (Reagent 5) control was used in each run. 10µl of Negative, Positive and Test serum samples were added to the respective wells and properly mixed. The strips were covered with the adhesive strip cover and incubated for 30 minutes at room temperature.

4. Strip cover was removed and discarded. Contents of the well were discarded. Wells were being washed 5 times with washing buffer. Wells were drained on a disposable absorbent pad or towel to remove excess of fluid.

5. 50 µl of conjugate (Reagent 2) was added to each well, except the one used for the Reaction Blank Control.

6. Contents of the microwells were mixed by gently agitating the strips for 5-10 seconds.

7. The strips were covered with fresh adhesive strip, incubated for 30 minutes at room temperature.

8. Adhesive strip covers were removed and discarded. Strips were washed 5 times with washing buffer.
Photograph 5. Kit for detecting antibodies to HCV

Photograph 6. Microwell ELISA plate for detecting anti-HCV Antibodies

A1 - Blank
B1-D1 - Negative Control
E1 - Positive Control
F1-H3 - Test Sera
D3 - Positive Sample
9. 100 µl of colour reagent(Reagent 6) was added into each well including reaction blank control.
10. Wells were left at room temperature for 30 minutes in dark.
11. The reaction was stopped by adding 100µl of Stopping solution(Reagent 7) in all wells.
12. Absorbance was read at 450 nm.

**Calculation**

**Cut off Value:** Cut off value was calculated by adding 0.225 to the mean O.D. of the negative control replicates.

**Interpretation of Results**

3. Samples giving absorbance less than the cut off value were considered negative or “Non-reactive” in the test.
4. Samples with the absorbance more than the cut off value were considered “Reactive” in the test.

**Sensitivity and Specificity**

INNOVA has a sensitivity of 99.8% and Specificity of 100%

**Statistical Analysis**

The data has been analyzed statistically, using appropriate statistical tests like tests of proportions, chi-square tests and confidence interval wherever applicable. The p value was calculated using SPSS Software using computer. The p value less than 0.05 was considered statistically significant.

95% Confidence Interval of the proportions of HBsAg and HCV prevalence has been determined by using the formula:

\[ p \pm (\sqrt{pq/n}) \times Z_{d/2} \]