Chapter 3

Materials and Methods

The nature & scope of methodology is to identify HBsAg, HBcAb, ID- NAT and HBV genotyping. For this in depth research work was done in laboratory of blood bank and also the laboratories of Artemis Health Institute, Sector 51, Gurgaon, Haryana & Department of Biotechnology, Faculty of Engineering and Technology, Manav Rachna International University, Faridabad, Haryana. The details of data collection, sample collection, use of required materials and methods are discussed below:

3.1 STUDY DESIGN

3.1.1 Prospective cross- linking study.

3.2 SAMPLE

3.2.1 611 HBcAb positive blood donors (both males and females) between 18-60 years of age group.

3.3 INCLUSION CRITERIA

3.3.1 Male and female selected donor (as per FDCA guideline)

3.3.2 Age between 18-60 years (as per FDCA guideline)

3.4 EXCLUSION CRITERIA

3.4.1 All deferral donors (as per FDCA guideline)

3.5 DESIGN

3.5.1 Prospective cross- linking study design.

3.6 DEPENDENT VARIABLE

Following variables will be investigated:

3.6.1 To perform HBsAg by Chemiluminance Technique from ECi vitros

3.6.2 To perform HBcAb by Chemiluminance Technique from ECi vitros

3.6.3 To perform HBV by ID NAT DNA testing by TMA Technique from Procleix Ultrio

3.6.4 To perform HBV Genotyping by RFLP Technique

3.7 NON CONSUMABLE ITEMS AND CONSUMABLE ITEMS

3.7. 1 List of Non consumables

1. Barcode Scanner
2. Bio safety cabinet type II
3. Centrifuge
4. Centrifuge (Swing out)
5. Computer
6. Deep freezer (-150 C to - 350C)
7. Digital water bath
8. Dustbins
9. Electronic pipette
10. Electrophoresis with assembly
11. Gel doc with camera
12. HC+ Luminometer
13. High speed centrifuge
14. Lab coat
15. Micro pipette
16. MS Office Software
17. Mug
18. Multi channel Pipette
19. Operating Software
20. Plastic discarding box
21. Plastic trough (35X30X8)cm

22. Printer
23. Refrigerator (20 C -8 0C)
24. Repeat pipette
25. Shoe covers
26. Squirt bottle
27. Target capture system
28. Test tube rack (for sample)
29. Thermocycler
30. Thermometer
31. Timer
32. TTU rack
33. UPS
34. Vacuum pump
35. Vacuum trap
36. Vitros ECi Immunodiagnostic system
37. Vortex

3.7.2 List of Consumables

1. Alcohol
2. Antibody Calibrator
3. Antibody Reagent Pack
4. Barcode Label
5. Blotting paper
6. Combitips 2.5 ml
7. Combitips 5 ml
8. Conical bottom containers (sterile) for reagent aliquoting
9. DI water(Temporarily using RO water)
10. Floppy
11. K2 EDTA Vacutainer (6 mL Draw)
12. Powdered non sterile gloves
13. Printer paper
14. Sealing cards
15. Sodium hypochlorite / Bleach
16. Sterile blue tips

17. Sterile non powdered gloves
18. Tissue paper
19. Tissue roll
20. TTC
21. TTU
22. Vitros ECi - Anti HB core Reagent Pack
23. Vitros ECi - Anti HB core Calibrator
24. Vitros ECi - Anti HBc Control
25. Vitros ECi - Signal Reagent
26. Vitros ECi – Universal
27. Wash Reagent

3.7.3 Materials Required

1. Serum samples
2. Vitros ECi – HBsAg Reagent Pack
3. Vitros ECi - HBsAg Calibrator
4. Vitros ECi – HbsAg Control
5. Vitros ECi - Signal Reagent
6. Vitros ECi - Universal Wash Reagent
7. HBV primer
8. DNA extraction kit
9. Master mix
10. Buffers- phosphate and Tris
11. Agarose 1%
12. Ethidium bromide etc.
13. Aluminum foil
14. Sterile gloves
15. Distilled Water etc.

3.8 INSTRUMENTATION: (PHOTOGRAPHS)

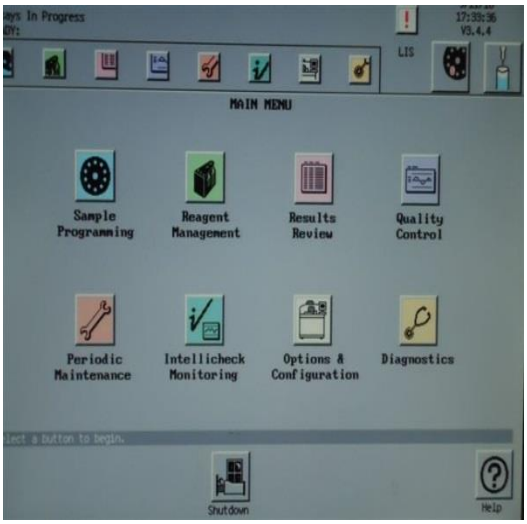
3.8.1 Blood collection monitor

3.8.2 Reagent Refrigerator

3.8.3 Sample loading in sample well

3.8.4 Refrigerator centrifuge machine

3.8.5 ECi Fully Automated Immunoassay Analyser



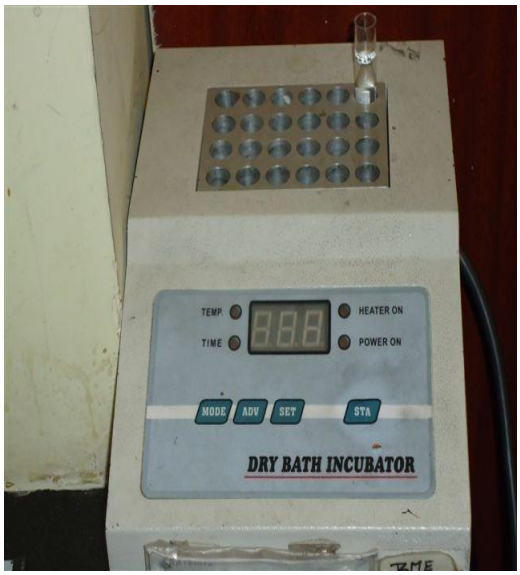
3.8.6 Sample monitor window

3.8.7 DNA Analyser

3.8.8 Sample load area in NAT Lab

3.8.9 DNA Transcriptase area

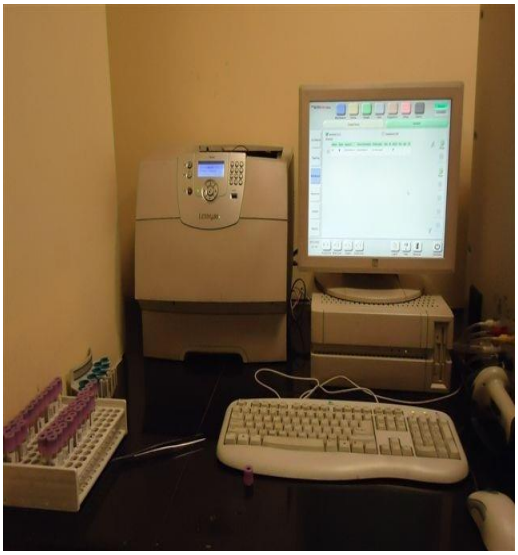
3.8.10 Sample Incubation area



3.8.11 Dry bath Incubator for DNA Extraction

3.8.12 Defreezer -80^{0}C

3.8.13 Blood Group Centrifuge



3.8.14 Automated Blood Group System

3.8.15 Sample processing for Blood Group

3.8.16 Centrifuge Machine

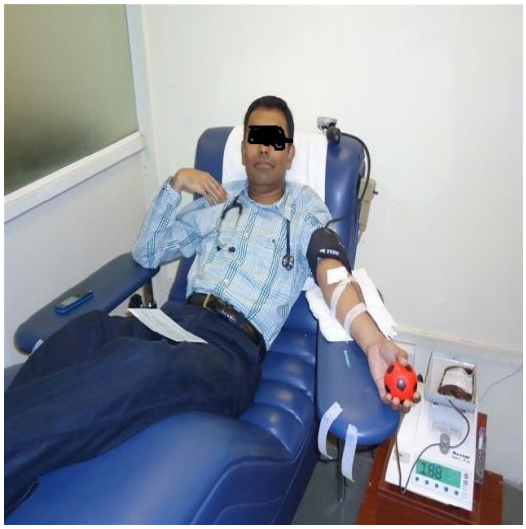
3.8.17 TTUs & samples bar coding

3.8.18 The Procleix Ultrio Kit

3.8.19 Luminometer

3.8.20 Weighing Scale

3.8.21 Digital Blood Pressure Monitor



3.8.22 Blood Collection from Donor

3.9 SCREENING OF HEPATITIS B SURFACE ANTIGEN (HBsAg) BY CHEMILUMINANCE TECHNIQUE FROM ECI VITROS

3.9.1 Test Description

To screen the donor blood samples for the presence of Hepatitis B Surface Antigen using VITROS ECi HBsAg Reagent Pack.

3.9.2 Principle of the Assay

The method is based on Immunometric assay, using enhanced chemiluminescence technology. This immunometric is involved in the

simultaneous reaction of HBsAg in the sample with mouse monoclonal anti HBs antibody coated on the wells and a horse radish peroxidase (HRP) labeled mouse monoclonal anti HBs antibody in the conjugate. The unbound conjugate is removed by washing the wells. The bound HRP conjugate is measured by a luminescent reaction by adding the signal reagent which contains luminogenic substrate (Luminol and peracid salt) and an electron transfer agent. The HRP in the bound conjugate catalyzes the oxidation of the luminol derivative, producing light. The light signals are measured by the Luminometer in the Vitros ECi system

3.9.3 Materials Required

- Serum samples
- Vitros ECi – HBsAg Reagent Pack
- Vitros ECi - HBsAg Calibrator
- Vitros ECi – HbsAg Control
- Vitros ECi - Signal Reagent
- Vitros ECi - Universal Wash Reagent

3.9.4 Equipments Required

- Vitros ECi Immunodiagnostic system.

3.9.5 Procedure

The HBsAg Reagent pack was loaded in the Vitros ECi system and scanned the protocol magnetic card and Lot calibration card. The assay was calibrated using HBsAg calibrator as per the calibration protocol. After successful calibration, HBsAg controls were processed. The obtained values (Signal / Cut-off value) for the controls remained within assigned value ±2 SD.

The serum samples were segregated by centrifuge and then they were transferred in to the sample cups. These sample cups and disposable tips were loaded in the sample tray. Defined sample programs were processed using the sample programming screen. Sampling operation was started by clicking on the start icon. All samples followed the processing steps and results were analyzed automatically.

3.9.6 Result Interpretation:

The results were automatically calculated by the Vitros ECi system and represented in terms of unit based on “Signal for test sample / Cut-off value”. The result of < 0.9 was considered as non-reactive sample with the possible presence of HBsAg. The result of <0.9 and < 0.99 was considered the borderline or grey zone sample where as the result of less than 1.0 indicated a non-reactive sample negative for Hepatitis Surface antigen.

3.10 SCREENING OF HBcAb (TOTAL) BY CHEMILUMINANCE TECHNIQUE FROM ECi VITROS.

3.10.1 Test Description

To screen the donor blood samples for the presence of Anti HB core antibody using VITROS ECi Anti-HB Core Antibody Reagent Pack

3.10.2 Principle

This method is based on competitive immunoassay, using enhanced chemiluminescence technology. This competitive immunoassay is involved involves a two stage reaction. In the first stage, the anti HBc antibody present in the test sample binds with the hepatitis B core antigen coated on the wells. The unbound serum sample is removed by washing the wells.

In the second stage, the horse radish peroxidase (HRP) labeled anti HBc mouse monoclonal antibody was allowed to react with the remaining exposed HBc Ag on the well surface. The unbound conjugate is removed by washing the wells. The bound HRP conjugate is measured by a luminescent reaction by adding the signal reagent which contains luminogenic substrate (Luminol and peracid salt) and an electron transfer agent. The HRP in the bound conjugate catalyzes the oxidation of the luminol derivative, producing light. The light signals are measured by the luminometer in the Vitros ECi system.

3.10.3 Materials Required:

- Serum samples
- Vitros ECi - Anti HB core Antibody Reagent Pack
- Vitros ECi - Anti HB core Antibody Calibrator
- Vitros ECi - Anti HBc Control
- Vitros ECi - Signal Reagent
- Vitros ECi - Universal Wash Reagent

3.10.4 Equipments Required

- Vitros ECi Immunodiagnostic system.
- Centrifuge

3.10.5 Procedure

The Anti HBcore Antibody Reagent pack was loaded in the Vitros ECi system and scanned the protocol magnetic card and Lot calibration card. The assay was calibrated using anti HBcore antibody calibrator as per the calibration protocol. After successful calibration, anti HBc controls were processed. The obtained values (Signal / Cut-off value) for the controls remained within assigned value ± 2 SD.

The serum samples transferred in sample cups. These sample cups and disposable tips loaded in the sample tray. Defined sample programs were processed using the sample programming screen. The sampling operation was by clicking on the icon. All samples followed the processing steps automatically and results were analyzed.

3.10.6 Result Interpretation

The results were automatically calculated by the Vitros ECi system and got expressed in terms of unit based on “Signal for test sample / Cut-off value”. A result of < 1.0 was considered as reactive sample with the possible presence of anti HBc antibody. A result of 1.0 and 1.2 was considered the borderline or grey zone sample. A result of 1.2 indicated a non-reactive sample negative for anti HBc antibody.

3.11 NUCLEIC ACID TESTING OF BLOOD FOR HBV

3.11.1 Test Description

The Procleix Ultrio Assay is a qualitative in vitro nucleic acid amplification test for the detection of human immunodeficiency virus type 1 (HIV-1) RNA, hepatitis C virus (HCV) RNA, and hepatitis B virus (HBV) DNA in human plasma for screening of infectious markers in donated blood.

Epidemiological studies have identified human immunodeficiency virus type 1 (HIV-1) as the etiological agent of acquired immunodeficiency syndrome (AIDS), hepatitis C virus (HCV) as the etiological agent for most blood-borne non-A, non-B hepatitis (NANBH), and hepatitis B virus (HBV) as the etiological agent for infectious serum hepatitis. HIV-1, HCV, and HBV could be spread by exposure to infected blood or blood products, certain body fluids or tissues, and from mother to fetus or child.

To prevent transmission of HIV, HBV and HCV infection through blood, all blood banks in our country are bound by law to test samples of donated blood for anti HIV I and II, anti HCV and HBsAg by ELISA and only blood units negative / non-reactive for these are allowed for transfusion to patients.

The recent addition of nucleic acid-based amplification tests in western countries has narrowed the window period of detection, for HIV 11 days, for HCV 23 days and for HBV 34 days. Considering these the hospital transfusion committee at Indraprastha Apollo Hospital has decided to reduce the rate of transmission of blood born infection further by introduction of Nucleic Acid Testing of all units of blood by using an internationally accepted blood screening for nucleic acid test.

3.11.2 Principle of the Technology

The Procleix Ultrio Assay involves three main steps which take place in a single tube: (i) sample preparation (ii) HIV-1 RNA, HCV RNA and HBV DNA target amplification by Transcription-Mediated Amplification (TMA) and (iii) detection of the amplified products (amplicon) by the Hybridization Protection Assay (HPA).

3.11.3 Materials Required

The following requirement is mentioned in clause number 3.8.1 and 3.8.2

3.11.4 Extraction of DNA

During sample preparation, viral DNA was isolated from plasma specimens via the use of target capture. Plasma was treated with a detergent to solubilize the viral envelope, denature proteins and release viral genomic RNA and/or DNA. Oligonucleotides ("capture oligonucleotides") that were homologous to highly conserved regions of HIV-1, HCV, and HBV were hybridized to the HIV-1 or HCV RNA or HBV DNA target, if present, in the test specimen. The hybridized target is then captured onto magnetic microparticles that are separated from plasma in a magnetic field. Wash steps are utilized to remove extraneous plasma components from the reaction tube. Magnetic separation and wash steps are performed in a Target Capture System.

3.11.5 Target amplification

Target amplification occurs via TMA, which was a transcription-based nucleic acid amplification method that utilizes two enzymes, MMLV reverse transcriptase and T7 RNA polymerase.

The reverse transcriptase was used to generate a DNA copy (containing a promoter sequence for T7 RNA polymerase) of the target sequence. T7 RNA polymerase produces multiple copies of RNA amplicon from the DNA copy template. The Procleix ultrio Assay utilizes the TMA method to amplify regions of HIV-1 RNA, HCV RNA, and/or HBV DNA.

3.11.6 HPA (Hybridization Protection Assay)

Using single stranded nucleic acid probes with chemiluminescent labels that are complementary to the amplicon. The labeled nucleic acid probes hybridize specifically to the amplicon. The selection reagent differentiates between hybridized and unhybridized probes by inactivating the label on unhybridized probes. During the detection step, the chemiluminescent signal produced by the hybridized probe is measured in a luminometer and is reported as Relative Light Units (RLU).

Internal Control was added to each test specimen, external control, or assay calibrator tube via the working Target Capture Reagent that contains the Internal Control. The Internal Control in this reagent controls for specimen processing, amplification, and detection steps. Internal Control signal in each tube or assay reaction is discriminated from the HIV-1/HCV/HBV signal by the differential kinetics of light emission from probes with different labels. Internal Control specific amplicon is detected using a probe with rapid emission of light (termed flasher signal). Amplicon specific to HIV-1/HCV/HBV is detected using probes with relatively slower kinetics of light emission (termed glower signal). The Dual Kinetic Assay (DKA) is a method used to differentiate between the signals from flasher and glower labels.

The simultaneous detection of HIV-1, HCV, and HBV, the Procleix Ultrio Assay differentiates between Internal Control and combined HIV-1/HCV/HBV signals but does not discriminate between individual HIV-1, HCV, and HBV signals. Specimens found to be reactive in the Procleix Ultrio Assay may be run in individual HIV-1, HCV, and/or HBV Discriminatory Assays to determine if they are reactive for HIV-1, HCV, HBV or any combination of the three.

The Procleix HIV-1, HCV, and HBV Discriminatory Assays utilized the same three main steps as the Procleix ul trio Assay (target capture, TMA and HPA); the same assay procedure is followed with one difference: HIV-1-specific, HCV-specific, or HBV-specific probe reagents are used in place of the Procleix ultrio Assay Probe Reagent.

Reagent Preparation

- Using the Procleix® Reagent Calculation Sheet, volume of the reagent aliquots required, were calculated for the number of samples to be tested.

Table 3.1 Reagent Calculation Chart / Preparation of reagent aliquots Reagent aliquots

Reagents	ml. tube	Tubes per test	Maxm volume	Required Volume	Combitip used in ml
wTCR	0.44x	= +	2.0 ml	=	5.0
AMP	0.075x	= +	0.3 mL	=	2.50
OIL	0.200x	= +	0.8 mL	=	5.0
Enzyme	0.025x	= +	0.2 mL	=	2.50
Probe	0.100x	= +	1.0 mL	=	5.0
Selection	0.250x	= +	1.0 mL	=	5.0

Preparation of reagents according to the given standard protocol, the reagents were thawed and brought to room temperature. Slowly mixed the Target Capture Reagent/ working Target Capture Reagent every 5 to 10 minutes. Kept the probe reagent in dark during thawing and after thawing, aliquoted the reagents for each assay run.

Procedure

STEP 1

The blood samples were collected from the donation room and centrifuged at 3000 rpm for 10 minutes. Arranged the samples according to the barcode number and created work list.

Table 3.2 Calibrator Positioning Chart

Position #	ultrio Assay	dHIV Assay	dHCV Assay	dHBV Assay
Position # 1	Neg	Neg	Neg	Neg
Position # 2	Neg	Neg	Neg	Neg
Position # 3	Neg	Neg	Neg	Neg
Position # 4	HIV-1	HIV-1	HIV-1	HIV-1
Position # 5	HIV-1	HIV-1	HIV-1	HIV-1
Position # 6	HCV	HIV-1	HCV	HCV
Position # 7	HCV	HCV	HCV	HCV
Position # 8	HBV	HCV	HCV	HBV
Position # 9	HBV	HBV	HBV	HBV
Position # 10	HBV	HBV	HBV	

Sample no. 1 was taken from the K2 EDTA base and by using the tissue wiped the cap and slowly lifted the cap without creating any sound and placed the cap immediately on the opened tube and discarded the tissue in the discarding box. Then placed the K2 EDTA tube whose cap was lifted in the rack leaving one tube space for the next sample. After placing all the samples in the rack kept it away from the sample decapping area. By referring to the work list follow the STEP (1) and STEP (2) till the entire sample tube caps are lifted. Kept all the racks away from each other and waited for 10 minutes before the wTCR was pipetted.

Positioning of the ten tubes units TTUs in the TTU Rack

Place the ten tube units (TTUs) by leaving 2 rows gap after the calibrators and one row gap after each and every TTU. (You need two TTU racks if the run size is more than 50)Place the first TTU in the A row (9 Calibrators+ 1 Sample). Leave two rows gap after the first TTU and place the second TTU in the D row and place the next TTU in the F row and follow the same procedure of leaving the one row gap after the previous TTU is placed. Cover the whole rack with A4 size paper. Keep all the reagents, calibrators, scanned TTUs or TTU racks away from the sample.

Manual Pipetting of wTCR

Performed at work area 2 in room no 2 assigned by quality manager in Nucleic acid Lab.

3.11.7 Materials Required

1. 5 ml Combitip.
2. Eppendorf Repeat Pipettor.
3. Sterile blue tips.
4. Micropipettor.
5. Sealing Cards.
6. Conical bottom container (For aliquoting of the WTCR)
7. Discarding Box containing freshly prepared 0.5% bleach. (1 part 5% Hypochlorite + 9parts of water).
8. Blotting papers.
9. TTC Covers/Caps.

3.11.8 Procedure of pipetting

A4 size paper was removed and placed on the TTU rack. Required amount of wTCR was aliquot into the conical bottom container by using a 5 ml comb tip. Now by using a Repeat Pipettor 400 ul of WTCR was dispensed into all tubes. Covered the individual TTUs with covers of the TTC’s that were prepared and kept sterile the previous day.

Sample Pipetting

(Performed at work area 2 in room no 2)

500 ul of sample was dispensed in no. 1 to the tube position no. 10 by referring to the work list TTU should be covered with the TTC Cover. Again 500 ul of sample was dispensed in the corresponding tube positions in the TTUs and again covered the individual TTUs with their appropriate TTC cover. Repeated STEP No. (2) until all the TTUs are filled with the appropriate samples and that all the TTUs are covered with their appropriate TTC cover. Calibrated pipette at work area 2 in room no 2, adjusted the TTC Covered such that only the first 3 tubes are exposed for dispensing out negative calibrators. Now by using a micropipettor dispense out 500ul of negative calibrator in the first three tube positions according to the calibrator positioning chart and immediately cover with the TTC cover.

Then dispensed out 500 ul of HIV-1, HCV, and HBV positive calibrators according to the calibrator positioning chart from tube position no. 4 to tube position no. 9 and cover with the appropriate TTC cover. Rearranged the TTUs by leaved two row spaces after the first TTU and placed other TTUs one after the other and locked the TTUs. Removed the TTC cover from all individual TTUs and covered the TTUs with the sealing cards. Vortex 20X 3 sec and rotated rack each time, tubes may remain at room temperature for up to 75 min prior to proceeding to 60°C incubation, Incubated water bath (60 ± 1°C for 20 ± 1 min) than left at room temp (15 min), incubated in TCS (10 min) and removed sealing cards.

Aspirated fluid from TTU, visually confirmed presence of pellets on side of tube Add 1.0 ± 0.1 ml wash solution, covered with sealing cards and Vortex X 3 and rotate rack each time, Incubate in TCS (8 min) and removed sealing cards. Aspirated, add 1.0 ± 0.1 ml wash solution and Vortex 3X, rotate rack each time), Vortex X 3 rotate rack each time Incubated in TCS (8 min) removed sealing cards, aspirated, covered with sealing cards.

3.11.9 Amplification

Removed sealing cards, pipette 75 mL Amp Reagent (straight, 2.5mL Combi-tip, 1 row), confirmed color changes to red/ dark pink, pipette 200 ul oil (angle, 5.0mL Combi-tip), covered with sealing cards, vortex (X 3 rotate rack each time), Incubated in 60 ± 1°C water bath for 10±1 min, Incubated in 41.5 ± 1°C water bath for 10 min, removed sealing cards. After above incubation, while rack is in 41.5 ± 1°C water bath, pipette 25 ul Enzyme Reagent straight, 2.5 mL Combi-tip. Confirmed color changes to orange, covered with sealing cards, tap sides of rack to ensure drops are not lodged on sides of tube. Incubated in 41.5 ± 1°C water bath for 60 ± 5 min.

Hybridization Protection Assay (HPA) – Post Amp

While replaced gloves and lab coat, removed sealing cards Pipette 100ul Probe Reagent (angle, 5 mL Combi-tip) Confirmed color changes to yellow covered with sealing cards, vortex 3 times rotate rack each time, incubated at 62°C ± 1°C water bath for 15 ± 1 min. removed and sealed cards, pipetted 250 ul selection reagent (angle, 5.0 mL Combi-tip), confirmed color changes to pink covered with sealing cards, vortex (x3 rotate rack each time), incubated at 62°C ± 1°C water bath for 10 ± 1 min, incubate in 23 ± 4°C water bath for a minimum of 10 min. Removed from water bath and proceed to process on the luminometer

Detection Results

Selected ‘New Run’ and appropriate protocol, followed prompts on computer and removed sealing cards, remove TTUs from rack and wipe sides and bottom of TTU with DI water dampened tissue. Place TTUs in the luminometer with barcode acing forward and close the lid. Read TTUs and print results, take a copy of all results and saved to pre amp room computer’s result folder**.**

3.12. HBV GENOTYPING BY RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

3.12.1. Materials

Kits and Reagents

High Pure Viral Nucleic Acid HBV Kit from 3B Blackbio Biotech India Ltd.

1. Binding buffer: 6 M guanidine HCl, 10 mM urea, 10 mM Tris-HCl, 20% Triton X-100(v/v), pH 4.4.
2. Poly (A) carrier RNA: 0.2 mg/40 ul (after reconstitution).
3. Proteinase K: 20 mg/ml (after reconstitution).
4. Wash buffer: 20 mM NaCl, 2 mM Tris-HCl, pH 7.5.
5. Elution buffer: Nuclease-free redistilled H_2O.
6. High pure filter tubes: Polypropylene tubes have two layers of glass fiber fleece and can hold up to 700 ul of sample volume.
7. Collection tubes: 2 ml polypropylene tubes.

- ***Reagents, Enzymes, and Buffers for PCR***

1. 10X PCR buffer: 200 mM Tris-HCl, pH 8.4, 500 mM KCl.
2. 10X dNTPs: 100 mM of each of dATP, dCTP, dGTP, dTTP.
3. TaqStart antibody, 1.1g/L in storage buffer (50 mM KCl, 10 mM Tris-HCl, pH 7.0,50% glycerol).
4. Taq Start dilution buffer: 50 mM KCl, 10 mM Tris- HCl, pH 7.0.
5. 10X TBE: 890 mM Tris, pH 8.0, 890 mM boric acid, 10 mM ethylenediaminetetraacetic acid (EDTA).
6. 10X agarose gel-loading buffer: 1X TBE, 1% sodium dodecyl sulfate (SDS), 50%
7. Sucrose, 1 mg/ml bromophenol blue.
8. Taq DNA polymerase, 3B Blackbio Biotech India Ltd**.**

- ***DNA Purification kit from 3B Blackbio Biotech India Ltd.***

1. NaI, 125 mL of a 6 M sodium iodide solution.
2. TBE modifier, 15 mL of a proprietary mixture concentrated salts.

3. New concentrate: 14 mL of a concentrated solution of ethanol (prepared by adding to 280 ml distilled water and 310 ml ethanol).

Equipment

1. Water bath.
2. Vacuum centrifuge.
3. Ultraviolet transilluminator.
4. Thermal Cycler (Med Gen 20 wells).
5. Automatic sequencer.

SURFACE GENE PRIMERS OF HBV

Table 3.3 Nucleotide position numbering of Surface Gene Primers

Name of Primer sequence (5' →3')	Position
S1 CCTGCTGGTGGCTCCAGTTC	56–75
S4* GTATGTTGCCCGTTTGTCCTC	459–479
S6C GCACACG†GAATTCCCGAGGACTGGGGACCCTG	129–146
S10* TCCTATGGGAGTGGGCCTCAG	636–656
S2Na CCACAATTCKTTGACATACTTTCCA (K=G/T)	1003–979
S3* AATGGCACTAGTAAACTGAGCC	690–669
S7D GACACC ‡AAGCTTGGTTAGGGTTTAAATGTATACC	842–823
S8* AGAAGATGAGGCATAGCAGC	434–415

Source: Nucleotide position numbering is according to Okamoto et al., 1988.

3.12.2 Methods

HBV DNA Extraction

Two hundred microliters of working solution (binding buffer supplemented with poly (A) carrier RNA) and then 40 ul of 20 mg/mL proteinase K are added to 200 ul sera in a sterile Eppendorf tube, mixed, and incubated for 10 min at 72°C. After incubation, 100 ul of isopropanol is

added. The filters and collection tubes are combined and the samples are pipetted into the upper reservoir followed by centrifugation for 1 min at 8000*g*. Discard the flow through. The filter is washed twice with the wash buffer, and the flow through is discarded after each wash.

Finally, centrifuged for 10 seconds at full speed to remove the entire residual wash buffer. Collection tubes are discarded, and clean nuclease-free 1.5 ml tubes are used to collect the eluted DNA in 50 ul of elution buffer. As control for each experiment, HBV DNA positive standard at a reasonable titration (10–100 genomic equivalents/ml) and negative HBV sera should be extracted and amplified along with any specimens.

3.12.3. PCR

Preparation of Primers

The primers were designed with reference to alignments of the S gene of HBV. Primers are diluted to 500 pmol/ ul in sterile nuclease-free water, and aliquots are made and stored at -20°C. Primer concentrations are confirmed by measuring the optical density (OD) at 260 and 280 nm (where a 260/280 absorbance ratio should be around 1.8 to minimize any contamination). The PCR working dilutions are made up from these stock dilutions.

HBV DNA S Gene PCR

Hot start PCR is performed using a nested protocol and antibody to Taq polymerase to amplify the surface (S) gene of HBV. Five microliters of extracted DNA is amplified in 50 ul solution containing 1.25 U Taqpolymerase, 2.5 U TaqStart antibody, 0.25 m*M* dNTPs, 2.5 m*M* MgCl2, 10X PCR buffer, and 25 pmol of each primer (S1: sense 5'-CCTGCTGGTGGCTCCAGTTC-3' and S2Na: antisense 5'-CCACAATTCKTTGACATACTTTCCA-3'; where K=G or T). Amplified for 5 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 90 s followed by 35 cycles with the denaturation temperature reduced to 90°C. One microliter of first-round PCR product is then reamplified in the same solution as above except for nested primers (S6C: and S7D). Conditions used are 5 cycles of 95°C for 1 min, 55°C for 75 s, and 72°C for 90 s followed by 25 cycles with the denaturation temperature reduced to 90°C.

3.12.4. Agarose Gel Electrophoresis

Agarose gel electrophoresis is used to confirm the presence and correct size of amplified PCR products by visualizing the gel on an ultraviolet transilluminator. Gels are prepared by adding 1 g agarose to 100 mL 1X TBE buffer. The solution is boiled until dissolved and allowed to cool. Then, 50 ul ethidium bromide (1 mg/mL) is added before pouring the gel. One microliter of agarose gel-loading buffer is added to each sample before loading, followed by running the gel at 80–90 V in 1X TBE buffer for 30 min.

3.12.5 DNA Sequencing and Phylogenetic Analysis

Purification of the PCR Product

After an adequate amount of the PCR product (20–30 ul) is run on the gel, DNA fragments of the expected size are located by visualizing the gel on an ultraviolet transilluminator. The correct bands are excised and placed in 1.5-mL tubes with 4.5 vol of sodium iodide and 0.5 vol of TBE gel-modified buffer and incubated at 55°C for 10–15 min. After complete melting of the gel, 5 ul of GLASSMILK is added to each tube. The tubes are vortexed and incubated at room temperature for 10 min. The tubes are spun for 30 s at 6000*g* in a bench-top microfuge, and the resulting pellet is washed twice with 0.5 mL ice cold NEW wash (containing 14 mL concentrate provided with the Geneclean kit, 280 mL distilled water, and 310 mL 100% ethanol). The pellet is dried, and the DNA is eluted in 10 ul of dH^2O by incubation at 55°C for 5 min. Finally, the supernatant is collected after centrifuging the suspension at 6000*g* rpm for 2 min.

3.12.6. Sequencing of the amplified products

After purification, sequencing of the S gene is performed directly from the purified PCR products by to the dideoxy method using a Taq Dye Deoxy Terminator cycle sequencing kit using an automated DNA sequencer). The reaction mix is prepared by adding 4 ul of the terminator ready reaction mix to 1.6–3.2 pmol of the sequencing primer and 30–90 ng purified PCR product in a total volume of 10 ul, the total volume to be adjusted by dH_2O. Cycling program for 30 cycles of 96°C for 20 s,

50°C for 20 s, and 60°C for 4 min followed by holding at 4°C. For each reaction, prepare a sterile well-labeled 1.5-ml microcentrifuge tube by adding 2 ul of 3 *M* sodium acetate, pH 4.6 (stop solution). Transferred sequencing reaction to the appropriately labelled tubes and then add 50 ul cold 95% ethanol. Immediately centrifuge at 12,000*g* at 4°C for 15 min and carefully remove the supernatant with a micropipettor.

Rinsed pellet twice with 250 µl 70% cold ethanol; for each rinse, centrifuge immediately for a minimum of 2 min. Removed supernatant after centrifugation and dry the pellet in a vacuum centrifuge. Finally, resuspend sample in a loading buffer (deionized formamide and 25 m*M* EDTA pH 8.0, containing 50 mg/ml blue dextran in a ratio of 5:1 formamide to EDTA/blue dextran) and transfer the resuspended samples to the appropriate wells. The presence of different DNA populations in a sample, detected by the appearance of double bands in sequencing gel or double peaks in a sequencing chromatogram, can be confirmed by the following procedures: sequencing the complementary DNA strand or the use of a second amplificate and/or another fresh sample. If two DNA populations are present in the sample, the number of base differences between DNA samples should be determined. The relative concentration of the DNA population can be roughly estimated by comparing the respective intensities of the double bands.

3.12.7 Result Analysis of HBV Genotype

The Sequence Navigator software program included in the software package was used to edit and align the forward and reverse sequences to ensure reliability of the generated sequences and to resolve possible ambiguous nucleotides. Sequences were then aligned, and a pairwise matrix of evolutionary distances of nucleotide sequences was generated using DNADIST Kimura's two-parameter method. Confirmed reliability of the phylogenetic analysis, bootstrap resampling and reconstruction performed 1000, or at least 100, times. If more than 75% of trees constructed from the resampled data are essentially similar to the tree generated from the original set, the topology is considered stable.

The HBV genotype for each sequenced strain was then determined on the basis of this analysis, which showed co-clustering with reference genotype strains. Comparison with the published sequences in the European Molecular Biology Laboratory (EMBL), other international databases, and local regional sequences is essential to characterize and identify the genetic variability of isolated viral sequences.

3.13 Descriptive investigation measurements

3.13.1 Blood groups and Rh typing by Microtiter test to determine the amount of anti-sheep red blood cell (RBC) antibody.

Requirements

Anti A, anti B, anti D coated microtiter plate.

Centrifuge machine

Whole blood

Procedure

In a round-bottom microtiter plate, add 50 ml of PBS to columns 2-9 in rows A-C. Leave column 1 empty. Add 100 ml of rabbit anti-sheep RBC to column 1 in rows A-C. In each row, transfer 50 ml from column 1 to column 2 and mix well. Now take 50 ml from column 2 and transfer to column 3. Repeat this process across the columns. Discard the final 50 ml taken from row 9. Add 50 ml of a 2% suspension of sheep RBC to all wells (columns 1-9, rows A-C). Make sure the RBCs stay adequately resuspended. Periodically invert the capped tube to keep them resuspended evenly. Incubate the plate for 24 hours at room temperature. Come back the next day and observe agglutination. Positive wells will exhibit a diffuse and confluent settling of the RBC and Ab, while in negative wells, all cells will roll down to the bottom and it will look more like a dot.

3.13. DATA ANALYSIS

Data was entered in MS Excel and analyzed by using SPSS version 19.0 statistical software, descriptive statistical measures (Frequency distribution, Mean and Standard deviation) were used to describe all the variables (Age, Gender, Height, Weight, Blood pressure (Diastolic and Systolic) Pulse and Hemoglobin and outcome variables viz. HBsAg, HBcAb and ID- NAT within a group comparison of mean values. Comparison of HBcAb with HBsAg, ID- NAT with HBsAg and HBcAb and ID- NAT with HBsAg variables was analyzed by using Cohen`s kappa test. Relative risks between outcome variables were analyzed by Chi- squire test. The 95% confidence intervals were calculated for difference of mean of all outcome variables. A p-value of <0.05 was considered as statistical significant.