CHAPTER III

MATERIALS
AND
METHODS
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The present study was conducted on the End-stage renal disease [ESRD] patients on maintenance dialysis who were immunized with hepatitis B vaccine. A preliminary study was conducted on 5 patients to study the feasibility protocol and make necessary changes in consultation with the Muljibhai Patel Urological Hospital, Nadiad for additions and subtraction to make it appropriate to be approved by the Institutional Ethics Committee of the Hospital.

GROUPS UNDER STUDY:

Experimental Subjects:
One hundred and nineteen End-stage renal disease patients on maintenance dialysis in Muljibhai Patel Urological Hospital, Nadiad, Gujarat, India were selected as experimental group.

Control Subjects:
One hundred and thirty-six, apparently healthy doctors and the staff members of the dialysis unit were selected as Control group.

Selection criteria:
- For Control group: All control subjects were immunized by hepatitis B vaccine at least once by a recommended dose. They were all HIV (I+II) antibody negative.
For ESRD patients [experimental subjects]: One hundred and nineteen End-stage renal disease patients on maintenance dialysis in Muljibhai Patel Urological Hospital, Nadiad, Gujarat, India, during the period April 2003 to September 2004 were included in the present study. The criteria for their selection as subjects for the present study was that

- All of them were on maintenance dialysis and
- All had received immunization with hepatitis B vaccine.
- All were negative for HIV (I + II) antibodies.

The number of hemodialysis, these subjects had undergone, varied from one to one to more than one thousand.

The dialysis unit of Muljibhai Patel Urological Hospital, Nadiad, Gujarat has sixteen dialysis machines out of which six dialysis machines are used exclusively for the segregated patients positive either for HBsAg or Anti-HCV antibodies.

Sample collection:
Blood from all the subjects in the present study was collected by venipuncture and separated serum samples were preserved at -20°C until they were tested for the viral markers.

Protocol followed: Uniform protocol was followed for all the subjects.

- Control Subjects: Serum samples were tested for HBsAg and other serological markers for HBV and HCV infection (Anti Hbc [Total] and Anti HCV antibodies respectively) and for HIV [I + II] antibodies. The quantitative determination of Anti- HBs antibody titer was also carried out.
**Experimental Subjects:** According to the protocol only Hepatitis B immunized End-stage renal disease patients on maintenance hemodialysis were included in the present study. At Muljibhai hospital all the ESRD patients are immunized by hepatitis B vaccine. Anti-HBs titer of these subjects is estimated periodically and those having antibody levels less than 10 mIU/mL, despite previous immunization, are given booster dose of Hepatitis B Vaccine. Whenever the ESRD patients require blood transfusions at Muljibhai Hospital the blood units are stringently screened for HBsAg, HIV [I + II] Antibodies and Anti-HCV antibodies. Some of the patients had received blood transfusion at different centers before they had come to Muljibhai hospital. It was not feasible to acquire exact information regarding the number and the place of blood transfusion in these patients.

Serum samples of the ESRD patients [Experimental group] were tested for HBsAg, Anti-HBc [Total], Anti-HCV and for HIV [I + II] antibodies. The Anti-HBs titers of all the samples were measured.

**Reagents and Kits used:**

The following kits were used for the estimation of various parameters under study

- For HBsAg: Sandwich enzyme Immunoassay kit from Hepanostika HBsAg Uni-Form II, BioMerieux, The Netherlands.
• For anti-HCV: third generation sandwich enzyme immunoassay kit [SP-NANBASE C-96 3.0] from General Biologicals Copro. Taiwan.

• For Qualitative estimation of Antibody to Hepatitis B Core Antigen [Anti HBc, Total] in human serum: enzyme immunoassay kit, ANTICORASE B-96 [TMB] from General Biologicals Corp., Taiwan.

• For quantitative determination of Anti HBS: Enzyme immunoassay kit, ANTISURASE B-96 [TMB] from General Biologicals Corp., Taiwan.

• Enzyme ImmunoAssay kit, GENEDIA HIV 1 / 2 ELISA 3.0 from Greencross life science Copro., Korea was used to detect HIV [I+ II] antibodies.

• The serum samples of control group were tested by rapid qualitative 4th generation HCV Tri-Dot method [J. Mitra & Co. Ltd., India] for the detection of Anti-HCV antibodies. The reactive serum samples were retested for confirmation using third generation enzyme immunoassay method (General Biological corpo., Taiwan).

• Detection for HBV-DNA by qualitative PCR technique was also carried out, at reference laboratory, on ten serum samples strongly positive for Anti HBc antibodies. The method used for HBV DNA qualitative assay is a single step DNA amplification procedure performed using Qiagen DNA extraction kit. Target is the highly conserved precore / core region of the HBV virus. The amplified product is detected by gel documentation system.
Schematic representation of assay procedure for HBsAg

Specimen 100μl
Negative control 3 X 100μl
Positive control 1 X 100μl

↓
Shake [to dissolve the anti-HBsAb-HRP conjugate in the sphere on microwell wall] and incubate at 37 °C for 60 ± 5 minutes.

↓
Wash with buffer 4 times

↓
Add TMB 100μl

↓
Incubate at 30 °C for 30 ± 2 minutes.

↓
Add H2SO4 100μl

↓
Read absorbance at 450 nm
All the serum samples were brought to room temperature before the assays were carried out.

**Procedure followed for tests:**

**HBsAg**

**Principle:** The test is based on one-step “Sandwich” Elisa principle. Antibody to HBsAg (Anti-HBs) coupled to horseradish peroxidase (HRP) serves as conjugate with tetramethylbenzidine (TMB) and peroxide as substrate. Microwells are coated with anti-HBs antibodies. Each microwell contains an HRP labeled anti-HBs conjugate sphere. When incubated with test or control, conjugate sphere dissolves in the sample and a solid phase antibody-HBsAg-Enzyme labeled Antibody complex is formed on the wall of the well.

**Assay procedure:**

1. Pipette 100 µl (undiluted) sample or control [three negative and one positive] into assigned wells.
2. Shake and mix then incubate at 37 °C for 60 ± 5 minutes.
3. Wash and soak each well four times with phosphate buffer.
4. Pipette 100 µl TMB substrate into each well.
5. Incubate at 15° C – 30 °C for 30 ± 2 minutes.
6. Stop the reaction by adding 100 µl 1 mol/l sulfuric acid to each well. Read within 15 minutes.
7. Read the absorbance at 450 nm.

**Calculation:**

NC = Absorbance of the negative control.
PC = Absorbance of the positive control.
NCx = Mean value of the negative control.
Schematic representation of assay procedure for Anti-HBc antibodies

Take into well
- Specimen: 50μl
- Negative control: 3 X 50μl
- Positive control: 2 X 50μl

And add 50μl Anti HBc Peroxidase Solution in to each well

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Shake and incubate at 37 ± 1°C for 1 hour.

↓
Wash with buffer 6 cycles with 350μl buffer

↓
Mix equal volumes of TMB Solutions A & B
Add TMB 100μl to each well

↓
Incubate at room temperature. For 30 minutes.

↓
Add H₂SO₄ 100μl into each well

↓
Read absorbance at 450 nm
Calculation of Cutoff value:
Cutoff value = NCx + 0.050

Anti HBc Antibodies
Principle: The test is based on "Competitive principle" assay in which the horseradish peroxiidase (HRPO) labeled anti HBc (Anti HBc-HRPO) competes with antibody in the specimen for hepatitis B core antigen (HbcAg) coated on the plate.

Assay procedure:
1. Pipette 50μl (undiluted) sample or control [three negative and two positive] into assigned wells
2. Add 50μl of Anti HBc peroxidase solution to each well. Gently tap the plate.
3. Seal the plate with the help of Adhesive slip.
4. Incubate at 37 °C for 1 hour.
5. Wash the wells with buffer 6 cycles with 350μl per wash. Soak for 10 seconds.
6. Add 100μl of mixed solution of TMB substrate (A & B equal volume).
7. Cover the plate with black cover and incubate at room temperature for 30 minutes.
8. Stop the reaction by adding 100μl of 2 N H₂SO₄ to each well.
9. Take absorbance of specimens and control within 15 minutes at 450 nm.

Calculation and determinations:
NCx = Negative control mean absorbance.
Absorption of Negative Control sample 1 + 2 + 3 / 3
Schematic representation of assay procedure for Anti-HBs antibodies

Take into well 50μl specimen samples and Standards of anti-HBs antibodies (0, 10, 100, 250, 500, 1000 mIU/ml) in the respective wells.

↓

And add 50μl conjugate in to each well

↓

Shake

↓

Incubate for 1 hour at 37 °C

↓

Add 50μl substrate A + 50μl substrate B to each well

↓

Incubate at room temperature. For 30 minutes.

↓

Add H₂SO₄ 100μl into each well

↓

Read absorbance at 450 nm
PC<sub>x</sub> = Positive control mean absorbance.

Absorption of Positive control sample \( \frac{1+2}{2} \)

**Calculation of Cutoff value:**

Cutoff value = 0.4 NC<sub>x</sub> + 0.6 PC<sub>x</sub>

Retest range = Cutoff ± 10 %

**Anti HBs Antibodies**

**Principle:** The test is based on “Sandwich principle”.

The wells of polystyrene microplate have been coated with HBsAg. When the test sample containing Anti HBs is incubated with HBsAg- Peroxidase solution in the wells. HBsAg-AntiHBs HBsAg- Peroxidase complexes are formed on the wells. After washing to remove the unbound materials, peroxidase substrate is added and color develops in proportion to the amount of Anti HBs bound. The level color is greatest in the presence of Anti HBs and false from its level with decreasing concentrations of Anti HBs in the sample.

**Assay procedure:**

1. Pipette 50μl (undiluted) sample and different standards (0, 10, 100, 250, 500, 100 mIU/ml) to microwells.
2. Add 50μl of conjugate to each well.
3. Mix and shake.
4. Seal the plate with the help of Adhesive slip.
5. Incubate at 37 °C for 1 hour.
6. Wash the wells with buffer 6 cycles with 350μl per wash. Soak for 10 seconds.
7. Add 50μl of Substrate solution A and 50μl Substrate solution B to each well.
8 Cover the wells and incubate at room temperature for 30 minutes at room temperature.

9 Stop the reaction by adding 100μl of stop solution. (2 N H₂SO₄) to each well.

10. Take absorbance of specimens and control within 15 minutes at 450 nm.

**Calculation and determinations:**
For a quantitative analysis a standard curve is made. The readings of absorptions plotted on the Y-axis and standard concentration on the X-axis. Anti-HBs titers are calculated by using this graph.

**Anti HCV Antibodies**

**Principle:** The test is based on second antibody “Sandwich principle”.

The wells of polystyrene microplate have been coated with synthetic HCV peptides (core and NS4 antigens) and recombinant (NS3 and NS5 antigens) for the detection of antibodies to HCV in human serum or plasma. When human serum is added to the well, the HCV antigens and anti-HCV will form complexes on the walls, if Anti-HCV is present in the specimen. The wells are washed to remove the unbound materials. The concentrate Anti human IgG· HRPO conjugate is added to the well results in the formation of (HCV)· (Anti HCV)· (Anti human IgG· HRPO) complex. After washing out the unbound conjugate, TMB substrate solution is added for color development. The intensity of color development is proportional to the amount of antibodies present in the specimen.
Anti-HCV antibodies

(Sampling: Make a 1:21 dilution of each control and specimen.)

200μl specimen diluent +10μl of (2 negative & 3 positive controls) and specimens into assigned wells

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Transfer 100μl of each diluted control and specimen to corresponding well in HCV coated plate

↓

Shake and incubate at 37 ± 1 °C for 1 hour.

↓

Add 100μl Diluted conjugate in each well

+ 

Incubate at room temperature for 30 minutes.

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Wash with buffer 6 cycles with 400μl buffer

↓

Mix equal volumes of TMB Solutions A & B

Add TMB 100μl to each well

↓

Incubate at room temperature for 30 minutes.

↓

Stop the reaction by adding 2N H₂SO₄ 100μl into each well

↓

Read absorbance at 450 nm within 30 minutes.
Assay Procedure:

Sampling: Make a 1:21 dilution of each control and specimen with specimen diluent.

1. Dispense 10μl of each control or specimen into the well of predilution plate.
2. Add 200μl specimen diluent to each well and mix.
3. Transfer 100μl of each diluted control or specimen to the corresponding well in HCV antigen coated plate.
4. Add 100μl of the diluted conjugate in each well and incubate at 37 ± 1° C for 60 minutes.
5. Wash the wells with buffer 6 cycles with 400μl per wash. Soak for 10 seconds.
6. Add 100μl of mixed solution of TMB substrate (A & B equal volume).
7. Cover the plate with black cover and incubate at room temperature for 30 minutes.
8. Stop the reaction by adding 100μl of 2 N H₂SO₄ to each well.
9. Take absorbance of specimens and control within 15 minutes at 450 nm.

Calculation and determinations:

NCᵢ = Negative control mean absorbance.
Absorption of Negative Control sample 1 + 2 / 2

PCᵢ = Positive control mean absorbance.
Absorption of Positive control sample 1 + 2 + 3 / 3

Calculation of Cutoff value:
Cutoff value = NCᵢ + 0.25 X PCᵢ
Retest range = Cutoff ± 10 %
Schematic representation of assay procedure for Anti-HIV [I & 2] antibodies

Add 100μl of sample diluent to each well

Pipette
- Specimen: 50μl
- Negative control: 3 x 50μl
- Positive control: 2 x 50μl

Shake and incubate at 37 ± 1°C for 60 minutes

Wash with buffer 5 times and soak

Add conjugate 100μl [diluted 1:51]

Incubate at 37 ± 1°C for 30 minutes

Wash with buffer 5 times and soak

Add 100μl [diluted 1:101] substrate [TMB] to each well and
Incubate at room temperature for 30 minutes

Add 100μl 1.6 N H₂SO₄

Read absorbance at 450 nm

Schematic representation of assay procedure for
**HIV [I & II] antibodies**

**Principle:** The test is based on direct “Sandwich” Elisa principle.

The walls of polystyrene microplate have been coated with HIV antigens. When the sample containing anti-HIV antibodies is incubated with recombinant HIV [1and2] Ag-peroxide conjugatesolution. On the wall [HIV-Ag]-[anti-HIV antibody]-[recombinant HIV 1 and 2 antigen-peroxide conjugate] complexes are formed. After washing to remove unbound material substrate is added and the color development takes place if HIV Antibodies are present in the test sample.

**Assay procedure:**

Pipette 100μl of sample diluent into all wells and add 50μl sample or control [three negative and one positive] into assigned wells.

1. Shake and mix then incubate at 37 ± 1° C for 60 minutes with cover sealer.
2. Aspirate the contents from all the wells wash 5 times with 300μl diluted washing solution every time. Soak each well.
3. Pipette 100μl diluted conjugate into each well. [1:51]
4. Incubate at 37 ± 1° C for 30 minutes.
5. Aspirate the contents from all the wells wash 5 times with 300μl diluted washing solution every time. Soak each well.
6. Pipette 100μl of diluted substrate in each well [1:101] and incubate for 30 minutes at room temperature.
7. Stop the reaction by adding 100μl 1.6 N sulfuric acid to each well.
8. Read the absorbance at 450 nm.

**Calculation:**

\[ NC = \text{Absorbance of the negative control.} \]
\[ PC = \text{Absorbance of the positive control.} \]
\[ NCx = \text{Mean value of the negative control.} \]

**Calculation of Cutoff value:**

Cutoff value = NCx + 0.3

Serum sample if found to fall in the retest range during the initial assay were repeated, and if the duplicate retest was found over the cutoff value, the sample was considered reactive.

To study the associated clinical factors in HBV and HCV, the coexistence of the two and hepatitis B vaccine immunogenicity and efficacy, the ESRD patients and the control subjects were classified as follows:

1. HBsAg positive and negative subjects.
2. Anti-HBc[Total] positive and negative subjects.
3. Anti-HCV positive and negative subjects.
4. Anti-HBs titers < 10mIU/ml [Vaccine Non-protected] and > 10mIU/ml [Vaccine Protected] subjects.

**Statistical Methods**

Statistical package for social science [SPSS] was used for statistical analysis. Pearson Chi-square test and t-test were used for test of association and for mean comparison respectively.