MATERIAL AND METHODS

SPECIMENS: blood serum,

Type of study: Study is prospective study

Field of study: This study will be conducted in the Department of Microbiology of Rama Medical College & Research Centre.

Study period: This study will be conducted for 18 month

Study subject: Serum sample will be collected from the clinically suspected cases of Dengue fever.

Sample collection: This sample will be collected from the Department of Microbiology of Rama Medical College & Research Centre.

Sample size: Sample size has been calculated by following formula

\[ n \geq \frac{z_{1-\alpha/2}^2 \times p \times q}{d^2} \]

\[ n = \text{sample size} \]
\[ z_{1-\alpha/2} = 1.96 \]
\[ p = \text{Prevalence} = .3738 \]
\[ q = 1 - p \]
\[ d = \text{length of 95\% confidence interval} = .075 \]

Using the above formula sample size should be \( \geq 160 \)
**Inclusion criteria**: Clinically suspected patient with febrile illness with one or more of the following manifestation:

- Headache
- Muscle pain
- Hemorrhagic manifestation
- Rashes
- Retro orbital pain

**Exclusion criteria**: Patient having febrile illness due to Typhoid and Malaria infection will be excluded from the study.

**Laboratory test**

A - Non specific tests

B - Specific tests

**A - Non –specific**

1 Complete blood count
2 Platelet count
3 TLC
4 DLC

**B-Specific test**

1 Rapid card test
2 ELISA Test
3 PCR
Sample collection: - About 3-5 ml blood will be collect in a sterile vial with all aseptic precautions [67]

1) Half of the blood will be allow to clot at room temperature for half an hour, after which the clot will be dislodged to separate the serum. This will centrifuged at 3000rpm for five minutes.
2) To the other half of blood, EDTA will be added and this anticoagulated blood will send for hematocrit and platelet count estimation.

Complete Blood Cell Count:-
Leukopenia, frequently with lymphopenia, is observed near the end of the febrile phase of illness. Lymphocytosis, with atypical lymphocytes, normally develops before defervescence or shock. A systematic review found that patients with dengue had significantly low total WBC, neutrophil, and platelet counts than patients with other febrile illnesses in dengue-endemic populations.[68]

A hematocrit level increase greater than 20% is a sign of hemoco oncentration and precedes shock. The hematocrit level should be monitored at least every 24 hours to facilitate early recognition of dengue hemorrhagic fever and every 3-4 hours in more severe cases of dengue hemorrhagic fever or dengue shock syndrome.

Thrombocytopenia has been demonstrated in up to 50% of dengue fever cases. Platelet counts less than 100,000cells/μl are generally seen in dengue hemorrhagic fever or dengue shock syndrome and occur before defervescence and the onset of shock. The platelet count should be monitored at least every 22-24 hours to facilitate early recognition of dengue hemorrhagic fever. [68]
**Specific test: - Dengue Day 1 Test**

Dengue Day 1 Test is a rapid solid phase immuno-chromatographic test for the qualitative detection of Dengue virus NS1 Antigen and differential detection of IgM and IgG antibodies to Dengue virus in Human serum/plasma. This rapid test is for in vitro diagnostic use only and is intended as an aid in the earlier diagnosis of Dengue infection & presumptive diagnosis between primary and secondary Dengue infection[^67]

**Rapid card method**

Dengue day 1 test kit consist two device one device ;one device for detection of dengue NS1 antigen and second device for the differential detection of dengue IgM/IgG antibodies in human serum /plasma . Dengue NS1 antigen contain two line; “C” & “T”. Test line is coated with antibodies, anti-dengue NS1 Ag. Then sample is added to this device, Dengue NS1 antigen, if present in the sample will bind to the anti-dengue NS1 gold colloid conjugation making antigen–antibody complex. This complex migrates along the membrane to the test region and forms the visible pink line at T as antibody–antigen–antibody gold colloid forms[^67]
Test procedure:-

**Dengue NS 1 Antigen device**

1) Add 3 drops of sample using Dengue antigen test sample dropper to the sample well of antigen device.
2) Allow reaction to occur for 20 minutes.
3) Read result at 20 minutes. Positive result may appear as early as 2-10 minutes. However, false results must be confirmed after 20 minutes only.

**Dengue IgM/IgG device**

*Fill* the dengue antibody lower circular part of the sample dropper with the specimen up to the mark provided on the dropper. Then add the specimen to the sample well”S” of antibody device. This will add 10µl of specimen to the device. Alternatively, add 10 µl of sample using micropipette to the sample well of the antibody device.
The Dengue virus ELISA Kit is intended for the detection of IgG and IgM antibody to Dengue virus in human serum or plasma.

**Assay Procedure:**

All specimens and kit reagents should be in to room temperature (18-26°C) and then gently mix.

1. Place the desired number of coated strips into the holder.

2. Negative control, positive control, and calibrator are ready to use. Prepare 1:21 dilution of test samples, by adding 10 µl of the sample to 200 µl of sample diluent. Mix well.

3. Dispense 100 µl of diluted sera, calibrator and controls into the appropriate wells. For the reagent blank,

   dispense 100 µl sample diluent in 1A well position. Tap the holder to remove air bubbles from the liquid
and mix well. Incubate for 20 minutes at room temperature.

4. Remove liquid from all wells. Wash wells three times with 300 µl of 1X wash buffer. Blot on absorbance paper or paper towel.

5. Dispense 100 µl of enzyme conjugate to each well and incubate for 20 minutes at room temperature.

6. Remove enzyme conjugate from all wells. Wash wells three times with 300 µl of 1X wash buffer. Blot on absorbance paper or paper towel.

7. Dispense 100 µl of TMB substrate and incubate for 10 minutes at room temperature.

8. Add 100 µL of stop solution.

9. Read O.D. at 450 nm using ELISA reader within 15 min. A dual wavelength is recommended with reference filter of 600-650 nm.
DENGUE NS1 Ag MICROLISA

Test procedure

1. Add 50 µl diluents in all wells.
2. Add 50 µl Negative Control in A-1 well.
3. Add 50 µl Calibration in B-1, C-1 & D-1 well.
4. Add 50 µl Positive Control in E-1 Well.
5. Add 50 µl samples in F-1 Well onwards.
6. Add 100 µl of working Conjugate solution in each well.
7. Ensure thorough mixing of control, sample to be tested & working conjugate to get reproducible results.
8. Apply cover seal.
9. Incubate at 37 C +_ 1C for 90 min.Z+_ 1min.
10 While the sample and working conjugate are incubating, Prepare working wash solution as specified in preparation of reagents.

11 Take out the plate from the incubator after the incubation time is over and wash the wells 6 times with working wash solution.

12 Add 150 µl of working substrate solution in each well.

13 Incubate at room temperature (20-30 C) for 30 min. in dark.

14 Add 100 µl of stop solution.

15 Read absorbance at 450 nm. Within the 30 min, (in ELISA READER.)
DENGUE IgG ELISA

**Procedure**

1. Equilibrate reagents at RT
2. Dilute samples, calibrators and controls (1:10 twice) starting with 10µL sample.
3. Add 100µL of diluted sample and incubate 30 min at 37°C
4. WASH X6
Add 100 µl HRP conjugate a human IgG and incubate 30 min 37 °C

WASH X6

Add 100 µl of TMB and incubate 10 min RT

Add 100 µl stop solution to Wells

READ 450 nm
Molecular Test

PCR TEST —

1. Collect the blood sample. (serum)
2. Extraction of RNA (with RNA kit Trizol).
4. Store –cDNA (Because RNA is not satable)
5. Generate the primer.
6. The PCR will carries out as follows: 94°C for 2 minutes followed by 35 cycles of 94°C for 30s, 52°C for 30s and 72°C for 60s and a final extension at 72°C for 10 minutes. [64]