MATERIALS AND METHODS

3.1 STUDY AREA

The present cross sectional study on immunological and biochemical aspects of dengue fever was carried out in Salem, Tamilnadu, India (Fig 4) from 2005 to 2007. Salem is the district head quarters of Salem district of Tamil Nadu. It is the fifth largest city of Tamil Nadu. It has a total population of 7, 51,433 comprising of Salem municipal corporation (6, 96,760), Kondalampatti (16,808), Kanankurichi (14, 994) Neykkarappatti (9,869), Mallamooppampatti (6,783) and Dalavaipatti (6,224). Males constitute 51 % and females constitute 49 % of the total population.

Fig 4 Salem Map

3.2 STUDY POPULATION

Patients who attended clinics and private hospitals with suspected dengue infection in and around Salem were included for the study. Both out patients and in patients were considered. Irrespective of age and sex, all the
patients with clinically suspected dengue infection were included. Patients were enrolled for the study after obtaining consent from the individual. A detailed history of each patient was collected on a specifically designed performa (Appendix 1). Patients with obesity, diabetes mellitus or parenteral history of diabetes mellitus, lipid disorder or any previous exposure to viral infection were excluded from the study. Alcoholic patients were also excluded from the study. Finally, a total of 254 clinically suspected dengue patients were enrolled for the study.

A second set of study population was 50 patients with other febrile illness for whom the diagnosis was already confirmed. They had malaria, typhoid and leptospirosis.

The third set of population was 50 healthy individuals who did not have a past history of dengue infection or any other related viral infection, bacterial infection and hereditary disorders.

### 3.3 SAMPLE COLLECTION

Blood samples were collected aseptically from the study population by routine vein puncture method. Two ml of blood was collected in a sterile tube containing 3 mgs of anticoagulant (EDTA) for haematological experiments and 5 ml blood without anticoagulant was collected for biochemical and immunological assay. Immediately after the collection, blood samples were transported to the laboratory. Serum was separated by centrifugation and store at -20 ° C for further use. All the serum samples were subjected to various assays such as immunological, haematological and biochemical. The principle and the details of the reagents are given in appendix 2.
3.4 CONFIRMATION OF DENGUE INFECTION BY SEROLOGY

Sera samples of all the individuals included in the study were subjected to IgM antibody capture ELISA (Panbio, Australia) (Xu et al., 1997).

10 µl of dengue antigen was added to 2.5 ml of antigen diluents (Phosphate buffer 7.4) and mixed well. Equal volume of diluted Ag (1:250) and monoclonal antibody (MAb) tracer were mixed in separate glass vial and incubated for 1 hour at 20 – 25 ºC. Patients serum was diluted to 1:100 with serum diluents (Tris buffer Saline pH 7.2-7.6). 100 µl of diluted samples, positive and negative controls were added to respective wells of assay plates coated with antihuman IgM antibodies. The plate was incubated at 37 ºC for 1 hour and washed 6 times with wash buffer. 100 µl of TMB (tetramethyl benzidine ) was added and incubated at 20 -25 ºC for 10 min and the reaction was stopped by adding 100 µl of stop solution ( 1 molar phosphoric acid ) and the absorbance was read within 30 min at 450 nm with reference filter of 600 – 650 nm.

**Calculation:**

Panbio units can be calculated by multiplying Index Value by 10

Index value = \frac{\text{Sample absorbance}}{\text{Mean cut off value}}

Mean cut off value = Average of absorbance of the triplicates of calibrator x Calibration factor

**Interpretation**

ELISA UNITS: Above 11 - positive

9 - 11 - Eqivocal

Below 9 - negative

Based on the ELISA results the different populations were defined as follows
3.5 CASE DEFINITION

**Dengue probable patients:** Documented fever of ≥ 38 °C and two or more of the following symptoms or signs: headache, Myalgia, arthralgia, nausea, vomiting, abdominal pain, skin rash, retero orbital pain and hepatomegaly

**Dengue confirmed:** clinically suspected dengue infection with single anti IgM Positive.

**Laboratory negative dengue:** A clinically suspected dengue but negative for anti IgM.

**Patients with OFI:** Positive for known causes of illness such as Malaria, but negative for dengue IgM.

**Control group:** Healthy individuals, laboratory negative for any infection including dengue IgM.

3.6 SEROLOGICAL ASSAYS.

All the groups were subjected to laboratory tests namely Widal test for typhoid, Blood smear analysis for malaria, Dridot Test for leptospirosis

3.6.1 WIDAL Test (Tube Agglutination test)

Patients serum was diluted in a series of test tubes and to these the antigen suspension was added. Four rows of such dilutions were prepared and to these were added the suspension of S typhi O antigen, H antigen, S paratyphi AH and BH. Agglutination was checked by examining the bottom of the tube.

3.6.2 Blood Smear for Malaria

Blood samples drawn from the patients were subjected to standard diagnostic method in malaria. A thin smear was prepared from finger prick and it was stained with Giemsa stain after fixing the smear in ethanol for 3-5 minutes and
the smear was observed under microscope for the presence of different stages of
the parasites.

3.6.3 DRIDOT Test for Leptospirosis

DRIDOT Test for Leptospirosis was done by method described by Farr, (1995)

Serum samples were tested for leptospirosis by Leto tex Dri – dot method. Briefly 10 µl of the serum sample was added to the dri dot chord which consisted of coloured latex particles activated with a broadly reactive leptospira Ag. The antigen in the blue dot was suspended with the serum and observed for the agglutination.

3.7 HAEMATOLOGICAL ASSAY

The blood and sera collected from all the individuals were examined for haematological and biochemical parameters as per the standard procedures. The haematological parameters assayed were Platelet count, Haemoglobin, ESR, WBC count and Haematocrit.

3.7.1 Platelet Count

Platelet count was done by the method described by Dacie et al., (2006). Blood obtained from the patients was transferred to test tube and the diluent was added. The contents were mixed well and a drop of the mixed samples was placed on a haemocytometer and the platelets were counted

3.7.2 Haemoglobin

Estimation of haemoglobin was done as per the method described by Drabkin and Austin (1932)

20 µl of blood was taken and added to 40 ml of ferricyanide –cyanide reagent and the colour was read against a reagent blank at 540 nm. The standards
were diluted in ferricyanide-cyanide solution to obtain a range of concentrations in the same manner.

Blood haemoglobin value was expressed as g/dl.

3.7.3 ESR

ESR was performed as per the method described by Westergen’s (1957)

1.6 ml of venous blood was added to a tube containing 0.4 ml of 3.8 % sodium citrate. The tube was inverted 2 to 3 times to mix the blood with the citrate solution. Westergen’s ESR tube was filled exactly to the 0 mark and was placed in the stand. Reading was taken at 30 minutes and 1 hour.

3.7.4 WBC Count

WBC count was done as per the method described by Drabkin and Austin (1932)

Draw the blood upto 0.5 marks in WBC pipette and was diluted upto the mark 11 using WBC diluting fluid and the counting chamber was filled with the fluid and was allowed for 3 minutes for the cells to settle and the cells in the four corners were counted.

3.7.5 Haematocrit/ Packed Cell Volume

Packed Cell Volume or Haematocrit was done by Wintrobes (1929) method.

The whole blood after adequate mixing was filled in the Wintrobe macro hematocrit tube. The level of the blood was noticed and the tubes were centrifuged at 2500 g for 30 minutes. The result was calculated using the following formula.

$$\text{Haematocrit (%) = 100 \times \frac{L1}{L2}}$$

Where L1 – Height of red cell volume in mm

L2 – Height of the whole blood specimen in mm.
The grayish white layer of leucocytes and platelets above the erythrocytes was not included in L1.

3.8 BIOCHEMICAL ASSAY

3.8.1 Enzymes

Determination of AST.

AST assay was done by kinetic method described Karmen et al., (1955).

To 0.2 ml of serum, 1.7 ml of phosphate buffer (pH 7.4), 0.3ml of NADH₂ and 0.1 ml of malate dehydrogenase was added as test in a cuvette with 1 cm path length. For blank 0.2 ml serum, 2ml buffer, 0.5 ml of aspartate and 0.1 ml of malate dehydrogenase was taken in another cuvette. Both the cuvettes were incubated until the fall in reading due to partial oxidation of NADH₂ level off. 0.2 ml of α-oxoglutarate was added to the test alone and mixed well and read against blank at a time interval of 10 minutes and the temperature of the text mixture was also noted.

Determination of ALT.

ALT assay was done by kinetic method described by Wrobleski and Ladue,1956

To 0.2 ml of serum 1.7 ml of phosphate buffer (pH 7.4), 0.3 ml of NADH₂ and 0.1 ml of Lactate dehydrogenase was added as test in a cuvette with 1 cm path length. For blank 0.2 ml serum, 2ml buffer, 0.5 ml of alanine and 0.1 ml of malate dehydrogenase was taken in a cuvette. Both the cuvettes were incubated until the fall in reading due to partial oxidation of NADH₂ level off. 0.2 ml of α-oxoglutarate was added to the test alone and mixed well and was read against blank at a time interval of 10 minutes and the temperature of the text mixture was also noted.
**Determination of ALP.**

ALP assay was done by as per the method described by Bessey *et al.*, (1946)

The reagent was brought to incubation temperature. 1 ml of reagent 1 and 250 µl of reagent 2 was added to a test tube and incubated at 37 °C and then 0.02 ml of serum sample was added to the reagent. The tube was mixed by inversion and it was incubated for 1 minute at reaction temperature (37 °C) and was read the absorbance reading at 30 sec at 340 nm with the instrument adjusted to zero absorbance with the water blank and determined the ∆ A/ minute from the linear part of the assay.

**Determination of LDH**

LDH determination was done by Colorimetric method described by Varley, 2005

0.2 ml of serum was diluted with 0.8 ml of 0.9 % saline. 1ml of buffered substrate and 0.2 ml of NAD solution was added two tubes labeled as T and C and was placed in a water bath, at 37 °C for 5 minutes. 0.1 ml of serum was added in T alone and kept at 37 °C for 15 minutes. 1.0 ml of 2, 4 DNPH was added to tube T and C. 0.1 ml of serum was added to tube C and was incubated for 15 minutes at 37 °C. 10 ml of sodium hydroxide was added to both the tubes. The brown colour developed was read at 540 nm.

**Determination of CPK.** (Varley, 2005).

To 1ml fresh serum, 0.7 ml of the NADH₂-AJP-PEP reagent and 0.05 ml of the LD-PK mixture was added. Mix and place in water bath at 25 ° C for 10 minutes. Add 1.75 ml of the glycine buffer to tube T, mixed well and was read in spectrophotometer which was adjusted with blank. After 10 minutes the spectrophotometer was adjusted again and reading was taken.
3.8.2 LIPID PROFILE

**Estimation of Cholesterol**

Cholesterol was estimated using CHOD–POD method described by Allain 1978.

In a set of 3 clean dry test tubes, 1 ml of cholesterol reagent and 10 µl of serum were added to tube 1, standard cholesterol and water was added to tube 2 and 3 respectively. The tubes were mixed well and were incubated for 20 minutes at room temperature and then absorbance was measured at 505 nm.

**Estimation of Triglycerides**

Triglyceride was estimated using GPO-POD method described by Fossati and Prencipe, (1982).

In a set of 3 clean dry test tubes, 1 ml of cholesterol reagent and 10 µl of serum was added to tube 1, standard and water was added to tube 2 and 3 respectively. The tubes were mixed well and incubated for 10 minutes at 37 ºC and then absorbance was measured at 505 nm.

**Estimation of HDL**

HDL cholesterol was estimated by Phosphotungstate / Magnesium precipitation method. (Demacker et al., 1997)

The precipitating reagent was added to the test tube and was centrifuged at 4000 rpm for 10 minutes. The clear supernatant was taken in a clean dry test tube and the cholesterol was estimated and the HDL was calculated by multiplying with dilution factor and standard concentration.

**Estimation of VLDL AND LDL**

VLDL cholesterol was found by dividing the triglyceride concentration by five and LDL was also calculated by using the following Friedwald’s formula.

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LDL \text{ Cholesterol (mg/dl)} = TC – HDL - VLDL.
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3.8.3 MINERALS

Estimation of sodium and potassium

Sodium and potassium was estimated by flame photometry described by Varley, 2005.

Three test tubes were taken and labeled as blank, standard and test and filled with 5 ml of diluting solution. 50 µl of standard was added to standard tube and 50 µl of serum was added to test tube labeled as test. The blank was sipped and flame photometer was set to zero. The instrument was standardised with standard solution and test serum was measured.

Estimation of calcium

Calcium was estimated by titrimetry method described by Alport, (1924). Calcium is precipitated from serum as calcium oxalate by addition of ammonium oxalate. 2 ml of 1 N sulphuric acid was added to the precipitate and heated till it dissolved and was titrated against standardised potassium permanganate. Blank was performed by titrating 2 ml of sulphuric acid. The potassium permanganate was standardized by titrating with standard oxalic acid.

Estimation of chloride

Chloride was estimated by titrimetry method described by Varley, 2005. 0.2 ml of serum was taken in a conical flask and 5 drops of diphenyl carbazone was added as indicator and was titrated against mercuric nitrate until a violet colour was obtained. Mercuric Chloride was standardized by titrating with standard sodium chloride.
3.8.4 OTHER BIOCHEMICAL PARAMETERS

Estimation of proteins

Protein estimation was done by Lowry’s method (Lowry et al., 1951). In a clean dry test tube 0.1 ml of the Serum sample was taken. The volumes were made up to 1.0 ml with distilled water. 5.0 ml of copper reagent was added and was mixed well and incubated for 10 minutes. 0.5 ml folin ciocalteau reagent was added and mixed well and incubated at room temperature for 30 minutes. Standard was also performed. A reagent blank was also prepared. After 30 minutes, the blue color developed was read at 660 nm.

Estimation of blood glucose

Glucose was estimated by standard GOD-POD method described by Trinder, (1969).

Estimation of bilirubin.

Bilirubin was estimated by Vandenburg method described by Danderfield (1953).

For bilirubin estimation 0.2 ml of serum was taken and 0.7 ml of diazo reagent was added to the test tube and was incubated for 10 minutes, optical density was read at 540 nm and from the graph the amount of bilirubin was calculated.

Estimation of urea

Urea was estimated by DAM–TSC method explained by Natelson et al., (1951).

In a clean test tube 0.5 ml of serum, 1.0 ml of DAM, 1.0 ml TSC and 3 ml of acid reagent was added and kept in boiling water bath for 30 minutes. A blank was also set up with water. The contents of the tube and the colour developed was read at 540 nm. The values are expressed in mg/dl.
Estimation of uric acid

Uric acid was estimated using phosphotungstic method described by Caraway (1963).

2ml of the serum was taken and 1.0 ml of phosphotungstic acid and 1 ml of 10 % sodium carbonate was added. A blank was set up with water. Standard with graded volume were also set up for the test. After 10 minutes the color was read at 610 nm. The values are expressed in mg/dl.

Estimation of creatinine

Creatinine was estimated by Jaffe’s reaction described by Oven et al., (1954).

4.0 ml of the serum supernatant was taken and to this 1.0 ml of 0.15 N sodium hydroxide and 1.0 ml of picric acid was added. The color developed was read at 470 nm. The values are expressed as mg of creatinine/dl.

3.8.5 ANTIOXIDANTS

Estimation of SOD

Assay of SOD was performed by method described by Ohkuma 1982,

Pipette 1.4 ml aliquot of the reaction mixture in a test tube. 100 ml of the sample was added followed by pre incubation at 37 °C for 5 minutes. 80µl of riboflavin was added and the tubes were exposed to 200 W Philips fluorescent lamps for 10 minutes. The control tube contained equal amount of buffer instead of sample. The sample and respective control were run together. At the end of the exposure time, 0.1 ml of Greiss reagent was added to each tube and the absorbance of the color formed was measured at 543 nm.

One unit of enzyme activity was defined as the amount of SOD capable of inhibiting 50 % of nitrite formatting under assay condition.
Estimation of Total Reduced Glutathione

Total reduced glutathione was performed by the method described by Zakowski et al., 1978,

1.0 ml of 10% serum was mixed with 4.0 ml of metaphosphoric acid. The precipitate was removed by centrifugation. To 2.0 ml of the supernatant 2.0 ml disodium hydrogen phosphate and 1.0 ml of DTNB reagent were added. The absorbance was read within 2 min at 412 nm against a reagent blank. A set of standard was also treated in the above manner. The amount of glutathione was expressed as μg/mg of protein.

Estimation of lipid peroxidation

Lipid Peroxidation was performed by a method described Buege and Aust 1978.

1.0 ml of the serum and 2.0 ml of TCA-TBA-HCl reagent was added and mixed thoroughly. The solution was heated for 15 minutes in a boiling water bath. After cooling the flocculent precipitate was removed by centrifugation at 1000 rpm for 10 minutes. The absorbance was determined at 535 nm against a blank that contained the entire reagent minus the sample. The results were expressed as nmoles of MDA formed/mg protein using an extinction coefficient of the chromophore 1.56 x 10^-5 M^-1 CM^-1.

Estimation of Total Antioxidant Status (TBARS)

TBARS was performed by standard method described Koracevic et al., (2001)

3.9 STATISTICAL ANALYSIS

Mean and standard deviation were calculated for the results obtained in this study and statistical significance of difference between the study groups was
obtained by students T test and Analysis of variance (ANOVA) for comparing
the means of control and the other study groups were obtained by using Turkey –
Krammer multiple comparison test. Fischers Exact test was also performed. The
analysis was performed by Graphad Instat Software, Inc, California.