Materials and Methods
I PREVALENCE RATE

Baseline data collection:

Registration of Population

The census registration of the population in the city was done by corporation health inspector by house-to-house visit. All people living in each house were registered, starting with the head of the family, spouse, children and others.

The following informations were collected during the registration: Name, Age, Sex, Family relationship to the head of the house, Place of origin and duration of residency. Table III shows an example of a census registration form. At the top are recorded entries of a general nature of the family and its living conditions. This is followed by a list of the members of the household.

Night-Blood collection

For night blood surveys, the teams visited different zones, during 8.00 pm to 11.00 pm. Approximately 20 cmm of blood was collected from each person on a slide, a thick and thin smears were prepared and proper record of the Name, Age, Sex and Address of each person was maintained. The slides were taken to the laboratory and the next day, they were de-haemoglobinized, dried, fixed in acidified methyl alcohol and stained with JSB-1 and examined for the presence of microfilariae. The number of mf in each slide was recorded. Another slide was stained with Giomsa stain for the identification of mf.
Table III
Census registration form

Community No Section No. Date

Investigator

House hold No. Type of housing

Protection against mosquitos

Source of water Sewage disposal Latrines

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Sex</th>
<th>Age</th>
<th>Relation</th>
<th>Status</th>
<th>Profession</th>
<th>Migrations</th>
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</tbody>
</table>
Clinical examination

The clinical examination included inspection and palpation of the limbs, regional lymph nodes, distal lymphatic trunks and external genitalia along with pulmonary auscultation. Genitalia were examined only in males for operational reasons, The clinical disease was classified as follows.

Acute inflammation

Red streaks with tenderness (Suggestive of ascending lymphangitis) scrotal lymphangitis, acute lymphadenitis, funiculitis, epididymo orchitis.

Chronic lymphatic obstruction

Oedema of limb, thickening of scrotal skin. thickening or lymphvarix of sepermatic cord, enlargement of testes, hydrocoele.

Elephantiasis

Elephantiasis of limbs and genitalia (Scrotal, penial).

The prevalence of microfilaraemia and chronic disease cases was calculated using the following formulae.

\[
\text{mf prevalence rate} = \frac{\text{Number of cases positive for mf}}{\text{Number of cases screened}} \times 100
\]
Prevalence of chronic cases = \frac{\text{Number of persons with disease manifestation}}{\text{Number of cases examined}} \times 100

II-I IMMUNOLOGICAL STATUS AND IMMUNE RESPONSES IN BANCOFTIAN FILARIASIS PATIENTS

Study Sample

Blood samples were collected from Regional Research Institute of Unani Medicine Hospital, Royapuram and Govt. General Hospital, Saidapet, Madras for the period of three years (1989-1991).

The patients included in the study, were categorised into the following groups.

Group I - Microfilariaemic carriers (mf positive)

Group II - Chronic patients with elephantiasis of extremities (mf negative)

Group III - Endemic normals.

Group IV - Other intestinal nematode infected patients.

Group V - Non-endemic normals.

A total of twenty two (22) non-endemic normal patients' sera were obtained through the courtesy of Dr. V. K. Vinayak from Post Graduate Institute of Medical Education and Research, Chandigarh, (India).
Wuchereria bancrofti mf (wet smear)
W. bancrofti mf
(Leishman's stain)
II-1.a Studies on peripheral blood leucocytes

Peripheral blood leucocytes were evaluated at different intervals of time in chronic, microfilaraemic and endemic normals. Tests were done on the first day of the patient attending the hospital, 0 day, 20th and 60th days.

Total and differential WBC counts were done following standard procedure (De Gruchy’s Clinical Haematology in Medical practice 1990).

II-11 Cell-mediated immune response

CMI responses in different categories of patients were studied by Leucocyte migration inhibition test (LMI) as described by Soborg M (1971) and as modified by Sundararaj and Agarwal (1976). LMI tests were carried out using homologous (W.bancrofti mf) and heterologous (S.digitata ww) antigens. LMI test was carried out on different intervals of time viz 0, 20 and 60 days.

Collection of leucocytes for LMI Test

10 ml of heparinized blood was collected, centrifuged at 2000xg for 20 minutes in a swing out Remi table centrifuge. The plasma was pipetted out and the buffy coat was carefully taken in another test tube. The cells were washed thrice in cold Hank’s Blanced Salt Solution (HBSS) containing heparin 5 unit per ml.

The red blood cells were lysed with 0.83 percent ammonium chloride. It was then centrifuged and the leucocytes were collected. The cells were
washed thrice in HBSS and the final pellet was suspended in Minimum Essential Medium (MEM) containing 10% inactivated (56°C for 30 min) bovine serum and antibiotics (Penicillin 10 units/ml and streptomycin 10 mcg/ml). The cells were counted in a haemocytometer and adjusted to contain 1 x 10^7 cells per ml.

II-iii *W. bancrofti* mf separation

Night blood samples collected from microfilaraemic carrier patients were used as a source of mf antigen.

Hundred ml of night blood in anticoagulant (Heparin 25 unit/ml) was collected from patients with microfilariaemia (100 or more microfilariae per ml) and the Mf were isolated by nucleopore filtration technique (Dennis and Kean 1971).

Concentration of Microfilariae

Aliquots of 5 ml of blood was withdrawn in a heparinised syringe and processed. It was forced through nucleopore filter held in the holder. Later thirty ml D H₂O was forced through the same filter followed by 30 ml air.

The filters were washed in excess of phosphate buffer saline (PBS) pH 7.2 and transferred to 15 ml conical tube containing 5 ml of PBS. These tubes were agitated mechanically for a minute to release the trapped microfilariae from the filter. After removal of the filter, microfilariae were
pelleted by centrifugation (500xg) and resuspended in a small volume of PBS pH 7.2 and washed thrice.

**Preparation of mf antigen**

Finally microfilarial antigen was prepared by homogenization and ultrasonic disintegration using vibronic sonicator for 30 sec each to disrupt the microfilariae. The sonicate was centrifuged, frozen and lyophilized. The concentrated, material was dialysed against phosphate buffer (pH 7.2), filter sterilized and the protein content was estimated by Lowry method (1951). This material was used as whole cell lysate antigen (mf WCL).

**II-iv Test procedure**

The leucocytes were drawn into microcapillaries, one end of which was heat sealed and centrifuged at 1500 x g for 2 min. The capillaries were cut at cell fluid interface, the portion containing cells were fixed in locally made plastic ring migration chambers with the help of sterile silicone grease. Then the chambers were filled with enriched MEM with antigens and one without antigen. The antigens used in the test were, crude *W.bancrofti* mf antigen (20µg/ml) and crude *S.digitata* ww antigen (20µg/ml) (III.ii a). The chambers were placed horizontally in moist containers, and cells were allowed to migrate for 18-24 hours at 37°C. The area of migration was traced on a paper with the help of camera lucida and measured with planimeter and the percentage of migration inhibition was calculated using the following formula.
LEUCOCYTE MIGRATION INHIBITION TEST

Migration Without Antigen

Migration inhibition in presence of antigen
Percentage of migration inhibition = 100 - \left( \frac{\text{Mean area of migration with antigen}}{\text{Mean area of migration without antigen}} \times 100 \right)

For each patient, triplicate migration values of antigen stimulated cells were compared with the triplicate migration values of control cells without antigen. The significance was tested by actual "T" test at 5% confidence limit.

II-v T Lymphocyte count

This test was carried out using peripheral blood lymphocytes.

Procedure

Peripheral blood lymphocytes were purified by density gradient centrifugation on Ficoll-hypaque (density = 1.071 gm/ml). The purified lymphocytes were washed thrice with HBSS. The lymphocytes were suspended uniformly in HBSS containing 10% heat inactivated bovine serum so as to contain 4 x 10^{-6} lymphocytes ml^{-1}. Preparation of fresh sheep erythrocytes, mixing with equal volumes of suspension of lymphocytes and sheep erythrocytes, incubation, staining with 1% methylene blue and counting of erythrocyte rosette forming cells were done according to the method of Jondal et al (1978).
PERIPHERAL BLOOD T CELLS
III-1 ISOLATION, PURIFICATION AND CHARACTERIZATION OF HOMOLOGOUS AND HETEROLOGOUS ANTIGEN

Homologous antigens of \textit{W.bancrofti} mf were isolated by nucleopore filtration technique (Dennis and Kean 1971). \textit{W.bancrofti} mf whole cell lysate was prepared as described in Section II.iii.

III-ii Collection of \textit{Setaria digitata} adult worms.

Adult \textit{S.digitata} worms were removed from the peritoneal cavity of cattle from the local slaughter house at Perambur, Madras-600 011. The worms were transported in saline to the laboratory and subjected to repeated washing in phosphate buffer saline (PBS pH 7.2) and stored at \(-20^\circ\text{C}\) until used.

III-ii.a Preparation of Extracts of adult \textit{S.digitata} worms

(Dissanayake and Ismail, 1980)

Adult \textit{S.digitata} (50) worms were washed two times with PBS pH 7.2, and were homogenized in a glass homogenizer for 15 min at 4\(^\circ\text{C}\) in the buffer. Homogenate was sonicated in ultrasonicator for 5 min at 4\(^\circ\text{C}\), the soluble material was centrifuged at 15,000 g for 30 min and supernatent was collected and dialysed against PB pH 7.2 for 24 hours at 4\(^\circ\text{C}\). This preparation was labelled as whole worm antigen (\textit{S.digitata} ww antigen) and stored at \(-20^\circ\text{C}\) until used.
S. digitata (female and male)
III-li.b  Column chromatography

Preparation of adsorbent

Materials

1. Diethylaminoethyl (DEAE) Sephadex A50 (DEAE Sephadex A50, Ion exchange resin, 40-120µ (Phramacia Fine chemicals).
2. Glass column - 20 x 1.5 cm
3. Buffer - PB pH 8.0 (0.005m)
   - linear gradient salt concentration
   - PB (0.005 m with 0.1 mol/NaCl
   - PB (0.005 m with 0.2 mol/NaCl)
   - PB (0.005 m with 0.3 mol/NaCl.
4. Eluting buffer - 0.005 M PB pH 8.0/ 0.35 m NaCl.
5. UV spectrophotometer.

One gm of DEAE Sephadex A-50 resin, anion exchanger was preswelled in double distilled H₂O overnight at 4°C. Resin was cleaned prior to use and washed with 0.1NaOH and double distilled H₂O and 0.1N HCl and distilled H₂O. This step was repeated two times and the resin was washed in initial buffer till it reached the initial buffer pH.

DEAE Sephadex A-50 fractionation of the whole worm antigen

DEAE Sephadex A-50 fractionation of whole worm antigen was performed according to the method described by Dissanayake and Ismail (1980) in phosphate buffer pH 8.0. A 20 x 1.5 cm column of Sephadex A50 was packed and washed with 200 ml of same buffer until the pH of column
was stabilised. Aliquots of 5 ml of whole worm antigen (containing 2.8 mg/ml of protein) were loaded to the column and the unadsorbed materials were washed using 0.005 mol/lit pH 8.0. The proteins in crude antigen were either eluted in one fraction, using 0.005 mol/liter phosphate buffer containing 0.35 mol Sodium chloride/lit or with increasing concentration of sodium chloride. The antigen adsorbed to the column was eluted with increasing concentration of sodium chloride, with the constant flow rate of 30 ml/hr. Fractions of 2 ml were collected and optical density was measured at 280 nm in UV spectrophotometer. The fractions of protein peaks were pooled, dialysed against PB PH 8.0. The antigen solution was concentrated to 5 ml (4 mg/ml) by lyophilization (Refrigeration for science Inc., Island park, New York) and termed as *S.digitata* fraction I & II following the procedure described by (Dissanayake and Ismail 1980). The protein concentration of the crude antigen and purified fractions were estimated by method of Lowry *et al* (1951).

### III-li.c *S.digitata* antigen SD$_2$-4

Antigenic sub fraction of *S.digitata* viz. SD$_2$-4 was prepared as follows (Dissanayake and Ismail, 1980).

Adult worms of *S.digitata* were collected from the peritoneal cavity of cattle, washed repeatedly in PBS and homogenized in PBS pH 7.2. The homogenate was fractionated on DEAE - Sephadx A50 using NaCl gradient in 0.01 mol/l phosphate buffer, pH 8.0. The fraction eluting at 0.3 mol/l NaCl
was collected, concentrated, dialysed and stored at -20°C. This material contained antigenic subfraction of SD₂-4.

III-III.a Protein profiles of *W.bancrofti* mf antigen using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

*W.bancrofti* mf antigen was prepared as described in section II-iv. The number of peptide present in the antigen was characterized by SDS-PAGE as described by Lammeli (1970) using 10% polyacrylamide resolving and 4% polyacrylamide stacking gels.

**Preparation of reagents (Lammell 1970)**

**Materials**

1. Slab gel electrophoretic apparatus - Complete system.

**Acrylamide - 30% w/v**

- Acrylamide - 14.6 gms
- Bis - 0.4 gms

The above ingredients were dissolved in 30 ml double distilled water and the volume adjusted to 50 ml. It was filtered over Whatman No. 2 filter paper and stored at 4°C in a brown bottle.
SDS 10%

SDS - 2 gm
Distilled water - 20 ml

The ingredients were dissolved and the volume adjusted to 20 ml in double distilled H₂O.

Upper tris (pH 6.8)

Tris - 3.03 gms
10% SDS - 2 ml

Tris was dissolved in 30 ml double distilled water the pH was adjusted to 6.8 with conc HCl. and the final volume made to 50 ml.

Lower tris (pH 8.8)

Tris base - 18.15 gms
10% SDS - 4.0 ml

The ingredients were dissolved in 80 ml double distilled water, pH adjusted to 8.8 with conc. HCl and final volume made to 100 ml.

SDS sample buffer

Upper tris - 1.25 ml
10% SDS - 3.0 ml
Glycerol - 1.0 ml
D.DH$_2$O - 4.75 ml
Bromphenol blue - 10 mgs
(for reduction 0.5 ml of mercaptoethanol was added).

**Running buffer**

Tris - 3.03 gms
Glycine - 14.40 gms
SDS - 1 gms
Volume was made to 1 liter.

**Ammonium persulphate salts**

0.1 gm ammonium persulphate was dissolved in 0.9 ml double distilled water.

**Method**

1. The slab gel was prepared between two glass plates. The glass plates were first cleaned with a detergent and rinsed thoroughly with distilled water.

2. These two glass plates were placed together, with the 3 spacers between them (along the side edges and along the bottom edge and the whole assembly was fixed tightly with clamps).

3. The two side edges and bottom edges were sealed with 2% agar and the assembly kept vertically on a flat surface.

4. The running gel was prepared according to the protocol given below,
Lower tris - 6.25 ml
DDH₂O - 10.4 ml
Acrylamide - 8.4 ml
Ammonium persulphate - 100 μl
TEMED - 15 μl

5. The solutions were mixed without TEMED in 100 ml conical flask and deareated.

6. TEMED, was added mixed and poured immediately between the glass plates, upto about 2.5 cm below the notch.

7. The gel was overloaded with H₂O in order to achieve an even surface. The polymerization was completed in 30-60 minutes at room temperature.

8. The unpolymerized gel was poured out and rinsed with water twice to ensure the removal of unpolymerized gel.

9. The stacking gel was prepared in a small beaker according to the protocol given below.

Upper tris - 2.0 ml
D.D.H₂O - 4.86 ml
Acrylamide - 1.0 ml
Ammonium persulphate - 300 μl
TEMED - 75 μl

The solution was deareated before adding TEMED.

10. Staking gel was poured and comb was inserted into stacking gel.

11. The stacking gel was allowed to polymerize for 15 to 20 minutes.

12. The comb was carefully removed by sliding vertically upwards.
13. The slots were rinsed with \( \text{DH}_2\text{O} \) 3 to 4 times to washout the unpolymerized gel.

14. The clamps were removed, the bottom spacer and the bottom edge were cleaned with a tissue paper. The gel assembly was fixed to the electrophoresis chamber with the help of clamps (with notched plate inside).

15. The slab gel plates were in air tight contact with the electrophoretic chamber because of the rubber gasket. The rubber gasket was sealed with 2% agar to prevent any possible leakage of buffer from the upper chamber.

16. The upper and lower chambers were filled with running buffer. The air bubbles trapped were removed at the bottom edge of the gel between the glass plates, using a syringe with a bent needle. The electrodes were connected to the power supply and pre run was made at a constant current of 20 mA for 15 minutes.

17. The power supply was switched off and the samples applied (5 to 50 \( \mu l \)) and molecular weights standards (10 \( \mu l \)) to the slots with the help of a micro syringe or micro pipette.

18. The power supply was switched on immediately and the electrophoresis was carried out at a constant current of 15 mA till the samples reach separating gel and then the current was increased to 25 mA.

19. Electrophoresis was carried out till the tracking dye reached the end of the separating gel.

20. After completion of the run, the power supply was disconnected, the gel assembly was removed from the electrophoresis chamber. The side
spacers and the notched glass plates were removed with the help of a spatula.

21. Later the gel was fixed and stained.

Fixation solution

<table>
<thead>
<tr>
<th>Substance</th>
<th>Quantity</th>
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<tbody>
<tr>
<td>Methanol</td>
<td>400 ml</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>70 ml</td>
</tr>
<tr>
<td>DH$_2$O</td>
<td>530 ml</td>
</tr>
</tbody>
</table>

Coomassie blue staining

Coomassie blue stain

Coomassie brilliant blue R-250 1.25gm was dissolved in 227 ml of methanol, 46 ml of glacial acetic acid was added and the volume made up to 500 ml with DH$_2$O. The Solution was filtered through Whatman No. 1 filter paper and stored in a brown bottle.

Destaining solution

<table>
<thead>
<tr>
<th>Substance</th>
<th>Quantity</th>
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<tr>
<td>Methanol</td>
<td>50 ml</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>70 ml</td>
</tr>
<tr>
<td>D.D$_2$O</td>
<td>880 ml</td>
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</tbody>
</table>

Preserving solution

<table>
<thead>
<tr>
<th>Substance</th>
<th>Quantity</th>
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</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>300 ml</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>100 ml</td>
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</tbody>
</table>
Glycerol - 100 ml
D.H₂O - 500 ml

1. The gel was placed in Coomassie blue stain for 1 to 2 hrs and then in the destaining solution for about 6 hrs. The destaining solution was changed frequently.

2. After the destaining was completed the gel was transferred to the preserving solution.

Relative front value (Rf)

The migration distance of the tracking dye and of the blue protein zones were recorded from the top of the separating gel.

To determine the relative front (Rf) of a protein, the distance from the top of the separating gel to the center of the protein band was divided by the migration distance of the bromophenol blue tracking dye from the top of the separating gel.

\[ R_f = \frac{\text{Distance of protein migration}}{\text{Distance of tracking dye migration}} \]

The Rf values were plotted against the known molecular weight standards (Sigma Dalton Mark VII) on Semi logrthmic paper. The molecular weight of the unknown protein was thus estimated.
Standard molecular weight markers (Sigma Dalton Marker)

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Approximate mol.wt.</th>
<th>Rf values</th>
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</thead>
<tbody>
<tr>
<td>Bovine albumin</td>
<td>66,000</td>
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<tr>
<td>Egg albumin</td>
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<tr>
<td>Carbonic anhydrase</td>
<td>29,000</td>
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<tr>
<td>Trypsinogen</td>
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<td>0.75</td>
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<tr>
<td>Trypsin inhibitor</td>
<td>20,100</td>
<td>0.81</td>
</tr>
<tr>
<td>Lactalbumin</td>
<td>14,200</td>
<td>0.98</td>
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</tbody>
</table>

The Folin Lowry method of protein Assay (Lowry et al 1951)

The protein concentration of the known antigen was calculated following the method of (Lowry, et al 1951).

III-iii.b Antigen characterisation

Characterization of *S. digitata* whole worm antigen and SD₂-4 Antigen by SDS-PAGE

The number of peptides present in the crude *S. digitata* ww antigen and affinity purified fractions were analyzed by SDS-PAGE. The gels were run as described by Lammeli (1970) earlier, using 10% polyacrylamide for resolving and 4% polyacrylamide for stacking gels.

Separated protein bands were stained with Coomassiae blue R250 (BDH), Rf values were calculated and compared with standard molecular weight markers (Dalton mark VII, Sigma Chemical Company).
IV IMMUNE RESPONSE TO W.BANCROFTI MF ANTIGEN IN RABBITS

IV-i Antibody response to W.bancrofti (Kallraj, et al 1981)

W.bancrofti microfilarial antigen was prepared as described in (II-iii). One ml of microfilarial whole cell lysate (2mg/ml) was emulsified in equal volume of Complete Freund's Adjuvant and injected intramuscularly at different sites as primary dose. After 30 days three booster doses were given subcutaneously without any adjuvant 9mg/ml. The kinetics of the development of antibodies were studied at various time intervals. Blood samples from the immunized rabbits were collected ten days after the booster dose. Sera were separated and stored at -20°C.

Antibody responses to primary dose and booster doses were determined by indirect hemaggultination test (Weir 1978) against the mf WCL antigen.

IV-ii Rabbit antisera to S.digitata ww antigen (Chatterjee et al (1978)

Rabbits were immunized with S.digitata ww antigen extract in complete Freund's adjuvant (CFA). 0.5 ml of antigen was emulsified in equal amount of CFA and injected intramuscularly in different sites. This was followed by three booster injections of antigen in incomplete Freund's adjuvant at 10 days interval, according to the method described by Chatterjee et al (1978). Blood samples from immunized rabbits were collected 10 days after the last booster dose. Sera were separated and stored at -20°C.
Antibody responses to primary dose and booster doses were determined by indirect hemaggultination test (IHA) (Weir 1978) against crude antigen.

**IV-iii Rabbit antisera to S.digitata antigen SD₂₄ fraction**

(Dissanayake et al 1982)

A group of three rabbits were immunized with antigen of SD₂₄ in CFA. Approximately 1 mg of antigen in 0.5 ml of complete Freund's Adjuvant was injected intramuscularly. This was followed by four booster injections of antigen in incomplete Freund's adjuvant at 10-days interval and after another 10 days, the animals were test bled.

Antibody responses to primary and booster doses were determined by IHA against SD₂₄ fraction of S.digitata. The animals were bled, the sera were pooled and stored at -20°C.

**IV-iv IHA Proceedure**

Humoral immune response was studied by measuring the antibodies by indirect haemagglutination assay (IHA), using pyruvic aldehyde fixed sheep RBC sensitized with the antigens prepared above, by the following procedure.

**Pyruvic aldehyde fixation of SRBC**

Sheep blood collected in Alsever's solution was washed three times in physiological saline and finally made up to a 50% packed cell suspension in saline. 4 ml of pyruvic aldehyde was added to 12 ml of 1.7% sodium chloride
and the mixture was adjusted to pH 7.0 by the addition of about 35 ml of 1% sodium carbonate solution. To this 7 ml of Sorenson phosphate buffer pH 8.0 was added. 10 ml of red cell suspension was then added to the pyruvic aldehyde preparation and the mixture was left 4°C for 24 hrs with occasional agitation. At the end of this period the cells were washed three times in saline and resuspended to approximately 10% packed cell volume in phosphate buffer pH 7.2 containing 0.1% sodium azide. They were subsequently stored at 4°C.

Preparation of antigen coated RBC

Preserved cells were washed three times with physiological saline or PBS and then packed at 750 x g for 15 minutes. 0.6 ml of the packed cells were pipetted into each of two bottles and suspended in 10 ml of PBS. 10 ml of tannic acid (1:20,000) was added to the cell suspension in each bottle and the contents of each bottle was mixed well. The bottles were incubated for 15 minutes at 37°C in a water bath. Both bottles were centrifuged for 5 minutes at 750 x g to bring down the cells, the supernatant was removed and the cells were resuspended and washed once with about 20 ml of PBS. Finally they were resuspended in 10 ml of PBS in each bottle. One bottle was set aside and washed for controls and absorption. To the other 10 ml of PBS containing RBC, the antigen 2 mg/ml was added. After gentle mixing, this bottle was incubated at 37°C for 30 minutes in a water bath. The cells in both bottles were spun down, the volume in the bottle of control cells having been made up to 20 ml with PBS in order to balance the centrifuge. After removal of the supernatant, the contents of each bottle was suspended in 20
ml of PBS containing 1% of inactivated and absorbed normal rabbit serum. The cells were washed three times with this serum saline and resuspended to 50 ml with the same serum saline. This 1% suspension is used for the tests.

Test sera absorption and the test

This was conveniently done by making an initial 1:10 dropwise dilution of the serum with 1% suspension of uncoated tanned cells. The mixture was incubated at room temperature for 30 minutes. It was centrifuged and the supernatant was collected and dilutions were made from the supernatant, and 0.05 ml of sensitized cells were added to each serum dilution. The test was carried out in the wells of microtitre agglutination plates. A control consisting of serum at the initial dilution and tanned but uncoated cells were set up for each titration. Controls with coated and uncoated cells in saline alone were also prepared for each group of tests (Weir 1978). The plates were incubated at 37°C and the readings were taken after 2-4 hrs.

IV-v Cell mediated immune response

CMI responses in experimental rabbits were evaluated at various time intervals by means of leucocyte migration inhibition test using W. bancrofti microfilarial antigen prepared as described earlier (II-iii). CMI responses in experimental rabbits were also evaluated by means of Leucocyte migration inhibition test using the S.digitata ww antigen as discribed earlier (II-iv).
V-1.a UTILITY OF HOMOLOGOUS AND HETEROLOGOUS ANTIGENS IN THE DIAGNOSIS OF BANCRFTIAN FILARIASIS

Counter current immunoelectrophoresis (CIEP) was carried out to detect antibody from Patients’ sera (Desowitz and Una, 1976). In CIEP, two parallel wells in agar gel (pH 8.6) are filled with antigen and antibody. Voltage is applied across the gel so that antigen and antibody move towards each other at a faster rate, as a result of electroendosmosis and precipitin lines are formed.

Material

1. Noble agar
2. 0.1M veronol buffer, pH 8.6
3. Normal saline
4. Amido black (1%)
5. 2% Acetic acid
6. Glass slides
7. Humid chamber
8. Electrophoretic tank with power supply.
9. Whatman number 3 filter papers.
10. Template and gel punch.

*W. bancrofti* microfilarial antigen was placed in agarose wells at the cathodic end and human serum sample was placed at the anodic end. The wells were spaced 5mm apart between antigen and antibody. Electrophoretic run (at 5 mA per/25 x 75 mm slide) was carried out for 1 hr.
Following completion of electrophoresis, the precipitation bands were visualized and the results were read and recorded. The plates were then kept in humid chamber for 24 hrs, and the results were recorded. Finally the slides were washed in physiological saline for 24 hrs and reexamined. In a number of instances precipitin bands developed only after this procedure. Permanent preparations were made by washing the plates in several changes of distilled water for 24 hrs and stained with Amidoblack and dried. Final results were read after staining and the slides were preserved.

V-1.b CIEP for antibody detection using *S. digitata* whole worm antigen

CIEP was performed as described above with minor modifications. Instead of *W. bancrofti* mf antigen, *S. digitata* whole worm antigen was used at the cathodic end. The patient's serum was placed in the anodic wells. The rest of the procedure was same as described earlier.

V-1.c Utility of fractionated antigen in the diagnosis of filariasis

One of the antigenic fractions namely SD₂-4 is antigenically similar to the one present in *W. bancrofti* mf antigens (Dissanayake *et al* 1982). Using this fraction, antibodies from the patients' sera were detected by CIEP.
COUNTER IMMUNO ELECTROPHORESIS (CIE)

A. Chequer board Titration of antigen and antibody

Ag: S. digitata ww antigen
Ab: Rabbit anti S. digitata antibody

Demonstration of antigen in patient's sera
V-ll  Enzyme linked immunosorbent Assay (ELISA): for antillarial IgG Antibody

ELISA test was used to detect antibody in various bacterial, viral and parasitic diseases. The test is simple sensitive and does not involve radio isotopes as in radio-immunoassay (Voller et al 1976).

Procedure

Each well of 96 well microtiter plates (NUNC Intermed Denmark) or individual strip was coated with 0.1 μg of antigen in 100 μl of 0.06m carbonate buffer pH 9.6. The plates were incubated at 4°C overnight. The sensitized plates were sealed and stored at 4°C till used in the test.

At the time of the test, the plates or individual strips were washed three times with PBS pH 7.2, containing 0.05% Tween 20 v/v, (PBS-T20) and the following steps were performed.

Determination of optimal dilution of conjugate

The wells were further incubated with 200 μl of 2% BSA in carbonate buffer at 37°C/ for 2 hrs. The plates were washed with PBS-T20 (2x). 1:100 dilution of positive and negative control sera in PBS-T20 was made and 100μl added to wells and incubated at 37°C for one hour. Then the strips were washed 5 times with PBS-T20 (x5). Serial two fold dilutions of antihuman IgG HRPO conjugate in PBS-T20, starting from 1:250 to 1:3200 were prepared and 100μl of each dilution added into the positive and negative serum wells
and the strips were incubated at 37°C for 1 hr. After washing the strips 5x with PBS-T_{20}. 0.1 ml of freshly prepared OPD substrate was added into each micro wells and incubated at 37°C for 30 min in the dark. The reaction was stopped with 2.5 m HCl and the results were read quantitatively by ELISA reader (Bio tech) at 492 nm.

Optimal dilution of the conjugate was the highest dilution giving maximum reactivity with positive serum and minimum reactivity with negative serum at 30 minutes time.

V-ii.a Detection of filarial antibodies using \textit{S.digitata} whole worm antigen by ELISA

ELISA procedure employed was essentially of the microtitration test as described by Ruitenberg \textit{et al} (1975), Kaliraj \textit{et al} (1981a & b) and as described above. The plates were coated with optimum diluted (50 µl) crude antigen (20 µg/ml) of protein in carbonate buffer (0.06m, pH 9.6). Further procedure was essentially the same as described above.

V-ii.b Detection of filarial antibodies using \textit{S.dlg} whole worm SD2-4 fraction (Dissanayake and Ismail 1980) by ELISA

Above mentioned indirect ELISA procedure was applied for SD2-4 fraction with minor modification.
QUALITATIVE INDIRECT ELISA FOR THE DETECTION OF ANTIBODIES FROM PATIENTS SERUM

A. Using S. digitata ww antigen

Patient s' sera diluted to 1:50 and used : positive control row C, well 6.

B. Using S.D 2-4 antigen

Patient s’ sera diluted to 1:50 and used positive control :Row C and D, Wells 8.
An optimum antigen concentration of 5 μg/ml was found to be sufficient for coating the plates, rest of the procedure was same as was followed for *S. digitata* whole worm antigen.

V-iii Antigen detection

V-iii.a CIEP for antigen detection (Kaliraj *et al* 1979)

For antigen detection, the CIEP procedure described in section V-i.a was followed with slight modification. In place of microfilarial antigen, at the cathodic end patients' sera were placed. Rabbit antiserum to mf antigen was placed in the anodic wells. The rest of the procedure was same as that was followed for antibody detection by CIEP. Similarly for antigen detection, antibody to *S. digitata* ww antigen and antibody to SD2-4 were used.

V-iii.b Co-agglutination test

Preparation of Reagents

Co-agglutination test was done according to standard procedure (Rahman *et al* 1987). *Staphylococcus aureus* CowanI, bacterial strain was obtained from Christian Medical College, Vellore. *S. aureus* stain was grown over night in trypticase soy broth with intermitent shaking at 37°C. The broth was centrifuged at 2000 rpm for 10 min and supernatant was discarded. The cell suspension was washed thrice in PBS pH 7.2 and stabilised with 0.05% formaldehyde. The cell suspension was heated for 1 hr at 80°C and washed. Later 10% (V/V) suspension was made in PBS. Rabbit anti mf antiserum, (0.1 ml) was mixed with 1ml of 10% suspension and incubated for 3 hrs at room temperature with intermitent shaking. The
antibody coated sensitized *Staph aureus* (SSA) suspension was washed with PBS and bacterial suspension was made 2% V/V in PBS containing sodium azide (0.1%).

For negative control, formaldehyde stabilized, heat treated 2% V/V suspension of unsensitized *Staphylococcus aureus* (USSA) was used as a negative control. Reagents were stored at 4°C until used.

**Slide coagglutination test**

The reagents were brought to room temperature and shaken to obtain a uniform bacterial suspension. Two rectangles were made with a wax pencil on a glass slide. In the first rectangle a 20 μl of serum sample from which antigen was to be detected was added. One drop of negative control (USSA) reagent was added to the 1st rectangle, and one drop of SSA reagent was added to the second rectangle. Cell suspension and serum were mixed well with sticks. After rotation by hand for 1 min the slides were examined against a dark background with a viewing lamp. The Co-agglutination test was considered positive if agglutination was seen with SSA reagent but not with the control USSA reagent.
CO-AGGLUTINATION TEST FOR THE DETECTION OF
THE ANTIGEN

1. Positive Control
2. Test
3. Negative Control