MATERIAL AND METHODS

1. **Survey field:** A survey on filariasis was carried out in an urban area, Pedana Municipality (Krishna District) of Andhra Pradesh, which is located at the coastal belt of Bay of Bengal.

2. **Topography of the study area:** Pedana is located at 16.2667° N and 81.1667° E longitudes. Pedana is surrounded by paddy fields, aqua ponds and 8 freshwater ponds (Paidammatalli, Ballavari, Kotha, Tamara, Malli, Stambam, Muruguneetigunta, Kolukulavari ponds). The temperature fluctuations are in between 28° C to 38° C and some times it may go up to 40° C in the last week of May. Rainfall is moderate with relative humidity (60-66%). Pedana is known for its handlooms and attracts foreigners to visit several handloom firms like Kalamkari and Cheneta (weaving). Most of the natives in Pedana are cosmopolitan people. The streets with open drains have become the favourable sites particularly in weaver’s community; this area is usually loaded with soaked cloth materials, waste chemicals and dye solutions (particularly during rainy season, the drains have become more favourable sites for mosquito breeding). Freshwater ponds (8) surround the three sides of Pedana; excess of water during the rainy season over flows into the drains. Large pools and puddles in Pedana have become the favourable site particularly for *C. quinquefasciatus* breeding.

3. **Population in the study area:** Pedana municipality has a population of 30,835 as per 2011 census (23 wards in the urban area). The study was conducted in weaver’s colony of 23 wards at weekly intervals from July 2010 to June 2012.
4. Field work conducted in the study area: The size of the sample to be examined in a filarial survey varies with the type of survey; it may be a routine survey or survey for evaluation. The NICD (National Institute of Communicable Disease, Delhi) standard is to examine 5-7% of the population for routine surveys, and the samples must be random, representative and to cover all ages and both sexes. The present survey on filariasis was conducted following the statistical standardized schedule for conducting filarial surveys (WHO Expert Committee on filariasis, 1974). For convenience, the present investigations were divided into 2 phases. First phase consists of identification of filarial patients (July 2010 to June 2011) and second phase consists of treatment with DEC and observations on clinical, haematological and immunological indices (July 2011 to June 2012). In the first phase of routine survey i.e., from July 2010 to June 2011, seasonal survey was conducted in 23 wards of Pedana Municipality for the identification of filarial positive patients (WHO Technical Report Series, 1992). In this survey, random samples were collected from fever cases of both sexes.

5. Clinical aspects of filariasis

5.1 Clinical manifestations: Bancroftian and Brugian filariasis are characterised by a wide range of clinical manifestations; the signs and symptoms often differ from one endemic area to another.

5.1.1 Asymptomatic amicrofilaraemia: In all endemic areas a proportion of the population shows no microfilaraemia or clinical manifestation of filarial infection. Some of the population has probably not been exposed sufficiently to become infected at all. Other persons may have been sufficiently exposed but do not have infection (as detectable by current diagnostic techniques); they may be immune or partially immune to
infection. Still others in this group may have sub-clinical infections (without microfilaraemia), as indicated by the presence of filarial antigens in the blood.

5.1.2 Asymptomatic microfilaraemia: Certain individuals in the population of an endemic area may develop microfilaraemia but with no recognizable clinical manifestation of filariasis. Some remain microfilaraemic but asymptomatic for years (sometimes even for life); others develop clinical disease either after they have spontaneously become amicrofilaraemic or while remaining microfilaraemic.

5.1.3 Acute manifestations: The acute clinical manifestations of lymphatic filariasis are characterized by episodic attacks of adenolymphangitis associated with fever and malaise. In males with bancroftian filariasis this adenolymphangitis may be localized in the genitals and present as acute epididymo-orchitis. It is not certain whether some of these are episodes of adenolymphangitis triggered or accentuated by bacterial infection. Inflammatory nodules in the breast, scrotum or subcutaneous tissues (presumably reflecting inflammatory reactions around adult or developing adult worms) have also been reported as acute manifestations or infection.

5.1.4 Chronic manifestations: Hydrocele, lymphoedema, elephantiasis, and chyluria are the main clinical pathological consequences of chronic bancroftian filariasis. The incidence and severity of these chronic clinical manifestations tend to increase with age.

Genital manifestations: Hydrocele is extremely common in bancroftian filariasis and manifests clinically as a swelling of the peritoneal lining that surrounds each of the testicles. Usually clear, straw-coloured hydrocele fluid accumulates in this closed sac as
a result of blockage in the lymphatic draining in the retroperitoneal and subdiaphragmatic areas. Rarely, the fluid has a milky appearance caused by the presence of lymph, a condition known as a chylocele. Hydrocele is a common chronic disease manifestation of *W. bancrofti* infection but has only very rarely been recorded in *Brugia* infections.

**Lymphoedema and elephantiasis of the extremities:** Recurrent episodes of limb lymphoedema, first pitting oedema and then chronic non-pitting oedema with loss of skin elasticity and fibrosis are the result of anatomical and/or functional blockage of the lymphatics. The legs are more commonly affected than the arms. In *W. bancrofti* endemic areas, swelling of the leg often involves the thing as well as the lower leg. Secondary infections of the skin (bacterial and fungal) are common, particularly in subjects who do not use footwear.

**Occult filariasis:** Only a very small proportion of individuals in a community (where filariasis is endemic) develop occult forms of the disease, conditions in which the classical clinical manifestations are not present and where microfilariae are not found in the blood but may be found in the tissues. Tropical pulmonary eosinophilia is the classical example of occult filariasis. Males are affected about twice as often as females, and the disease is rarely seen in children. Extra pulmonary manifestations occur in about 15% of the patients, including mild to moderate splenomegaly, lymphadenopathy, and hepatomegaly. The syndrome is characterized by nocturnal paroxysmal cough, hypereosinophilia, elevated erythrocyte sedimentation rate, radiological evidence of diffuse military lesions or increased bronchovascular markings, extremely high titres of filarial antibody and a good therapeutic response to diethylcarbamazine (DEC). Low-
grade fever and weight loss may be present. In most cases lung function is impaired, with a reduction in the vital capacity, total lung capacity, and residual volume. Hypereosinophilia is the most constant feature of this syndrome. Absolute eosinophil counts generally range from 3,000 to 50,000 cells per mm$^3$ of blood, but the level of eosinophilia is not related to the severity of the symptoms. If untreated, tropical pulmonary eosinophilia progresses to a condition of chronic pulmonary fibrosis. Other conditions possibly associated with lymphatic filariasis a form of monoarthritis, usually involving the knee joint, is quite commonly seen in areas of endemic filariasis. The relationship between filariasis and endomyocardial fibrosis is not clear. Other conditions for which an association with lymphatic filariasis has been suggested include thrombophlebitis, tenosynovitis, nerve palsies and dermatoses.

5.1.5 Clinical features in previously uninfected individuals entering an endemic area: Persons from non-endemic areas (e.g.; expatriate visitors, immigrants, military personnel) have clinical presentations marked by prominent inflammatory reactions to the developing larvae and adult worms. These include lymphangitis, lymphadenitis, and the clinical pictures of epididymitis or funiculitis, as well as more generalized allergy-like symptoms. These individuals rarely become microfilaraemic unless exposure is prolonged and continuous. Elephantiasis trends to develop more often and sooner in immigrants than it does among the indigenous population; lymphoedema may develop within 6 months and elephantiasis 1-2 years after arrival.

5.2 Problems in differential diagnosis: Differences in the anatomical distribution of the clinical manifestations of brugian and bancroftian filariasis may be due in part to
different tropisms of the parasites for particular anatomical locations, which might govern the location of the adult worms in the human lymphatics. However, at present there is insufficient pathological evidence to support such hypothesis.

5.2.1 Adenolymphangitis: Although fever sometime precedes adenolymphangitis, fever alone, in the absence of this condition, should not be ascribed to filariasis, even when microfilaraemia is present. A characteristic feature of filarial adenolymphangitis is the retrograde extension of the lymphangitis from the affected node; this pattern is distinct from that usually found when bacterial infections cause adenolymphangitis.

5.2.2 Lymphoedema and elephantiasis: Persistent lymphoedema and elephantiasis are often difficult to distinguish clinically. Both are caused by the same pathological process and should be regarded as different stages of one clinical entity. Not all elephantiasis is caused by lymphatic filarial infection; even following acute or chronic infections, tumours, surgery, or irradiation, obstructive lesions involving a major lymphatic vessel may cause lymphostasis and subsequent elephantiasis. Another locally important form of elephantiasis in the tropics can be endemic non-filarial elephantiasis, a condition that is particularly common in highland areas in Africa, where alkaline red clay soils of volcanic origin are present and where people go barefoot. This condition is believed to be caused by the irritant effect of mineral particles (aluminium silicates and ferromagnesium compounds) that penetrate the skin and damage the vascular endothelium of the lymph nodes draining the lower limbs. The disease progresses slowly and centripetally but may be associated with episodes of acute local inflammation. Elephantiasis supervenes when there are persistent fibrotic changes in the skin and subcutaneous tissue.
5.2.3 Tropical pulmonary eosinophilia: In areas of endemic filariasis other helminthic infections, especially ascariasis and strongyloidiasis, may induce pulmonary syndromes with eosinophilia that must be differentiated from tropical pulmonary eosinophilia of filarial origin. Serological tests and clinical responsiveness help to distinguish these clinical syndromes and to treatment (WHO, 1992).

6. Diagnosis of filariasis

Filarial Test: Standard method adopted by the Andhra Pradesh State Medical and Health Department Laboratories i.e., Jeswanth Singh Bhattacharyaji (JSB-I). Staining method was adopted for identification and confirmation of both *W. bancrofti* and *B. malayi*.

6.1 Blood examination: Peripheral blood is examined for the identification of microfilariae. Two to three drops (20 cu. mm.) of peripheral blood was collected by finger prick. The blood was collected during the hours when a large number of microfilariae are found in the peripheral blood circulation. Blood was collected as follows: Nocturnal periodic *W. bancrofti*: between 10 P.M. and 4 A.M. in the night. Subperiodic nocturnal *W. bancrofti*: between 8 P.M. and 10 P.M. during the night. Subperiodic diurnal *W. bancrofti*: between 2 P.M. and 6 P.M. in the afternoon. Microfilariae can be demonstrated in the blood by microscopy by the following methods:

Direct wet mount: In this method, 2 to 3 drops of blood was collected on a clean glass slide and examined microscopically after placing a cover slip on it. Live microfilariae were identified by their characteristic serpentine movement in the blood plasma.
Stained thick blood film smear: Thick blood smear stained with JSB-I (Jeswanth Singh Bhattacharyaji) is the most commonly used method for the demonstration of microfilariae. The presence of sheath but the absence of nuclei in the tail end of the microfilaria is the diagnostic feature of *W. bancrofti* microfilaria.

DEC provocation test: This is a test in which diethyl carbamazine (DEC) was given orally to stimulate nocturnal periodic microfilariae to circulate in the peripheral blood during the daytime. It is recommended for diagnosis of nocturnal periodic *W. bancrofti* filariasis, in which night blood collection is difficult or unacceptable to the population. In this test, DEC was given orally at a dose of 2-8 mg/kg body weight. After 30 minutes, the capillary blood was collected by finger prick for demonstration of microfilariae by direct wet-mount or staining the blood smear (Parija, 2010).

Haematological, serological and immunological studies: Blood samples were collected from fever patients based on the classification of filarial manifestation stages; they were classified as occult, acute and chronic stages of infection (WHO, 1992; Parija, 2010). Blood was analyzed for Hb. and for RBC, WBC and platelet counts following the method of Fulwyler (1965) by BC-2300 hematology Analyzer (Shenzhen Mindray, Bio-Medical Electronics Company Limited). Estimation of Aspartate transaminase (AST) was done by UV Kinetic method using RANDOX IFCC kit following the method of Bergmeyer and Bowers (1977). Estimation of Alanine transaminase was (ALT) done following the UV Kinetic standard method (Wroblewski and La Due, 1956) by using ERBA LIQUIXX kit. Alkaline phosphatase (ALP) was estimated following the method of Bowers and McComb (1975) using Ensure biotech diagnostic kit. Estimation of protein and albumin
was done using Biuret method (Gornall et al., 1949) and BCG colorimetric test (Spencer and Price, 1977). Serum IgG, IgA and IgM were estimated by turbidimetry method (Haven et al., 1994) using SPINREACT kits. CD3, CD4 and CD8 cells were counted by Flow Cytometry using CD45 GATING- BDIS Multi TEST IMK kit (Ormerod, 1999).

**Haematological tests:** 1. Collected the whole blood sample from venous blood with a K₂EDTA (1.5 to 2.2 mg/mL) anticoagulant collection tube. Rapidly and thoroughly mixed the blood with the anticoagulant. 2. Pressed [MODE] to select “Whole Blood” mode. 3. Wiped the surface of the diluent dispenser with lint-free tissue. 4. Presented the mixed sample under the diluent dispenser so that the tip was well inserted into the tube. 5. Pressed the diluent key and removed the sample when the beep was heard. A message box was popped up. 6. Wiped the surface of the diluent dispenser with lint-free tissue from the top to the bottom. Precaution was taken not to bring out the blood sample in the dispenser. 7. Presented a cleaned sample cup under the diluent dispenser and made sure that the cup was tilted towards the dispenser, to avoid spills and bubbles. Pressed the diluent key to dispense the sample (the dispensing volume is controlled by the analyzer) into the cup. Kept the dispenser tip away from the sample. 8. After the dispensation had finished, shook the cup and mixed the sample.

**Running whole blood samples:** Pressed MENU and SELECTED “The count” screen, pressed [MODE] to select “Whole Blood” mode

**Entering sample information:** Entered the sample information mode of Patient number at “count” screen, used the bar-code scanner to scan the sample into the analyzer; or at the count “Count” screen, pressed [F1] to enter the “ID” window and ENTERED the
sample ID. Finished the entering of the sample ID, pressed MENU and the dialog box was popped up. Clicked “Yes”

**All information modes (external key board needed):** Entered the Edit menu at the “Count” screen, pressed [F1] and an edit window was popped up.

**Entering sample ID:** ENTERED the patient number in the “ID” box.

**Selecting patient gender:** SELECTED the desired item from the “gender” pull-down list.

**Entering the patient name:** ENTERED the patient name into “NAME” box.

**Entering the patient age:** This analyzer provided three ways to enter the patient age in years, in month and in days. To enter the patient age in years: ENTERED the desired number, an integer from 0 to 200, into the “Years” box. To enter the patient age in months: ENTERED the desired number, an integer from 0 to 12, into the “months” box. To enter the patient age in days: ENTERED the desired number, an integer from 0 to 31, into the “Days” box.

**Exit edit:** After finished entering the all the desired sample information, Clicked the “Yes” button to save the changes and returned to the “COUNT” screen.

**Running the samples:** 1. Pressed down the cup stand and presented the sample cup under the sample suction nozzle so that tip was well inserted into the tube, replaced the cup stand lightly to hold the cup. 2. Pressed the [COUNT] key and the analyzer started aspirating sample; and the analysis progress was displayed on the screen. 3. When the analysis was finished, the result was displayed on the screen and the sample ID
automatically increased by 1. And if the auto print function was enabled, the analysis result would be automatically printed out. 4. Repeated the above steps on other samples.

**Differential Leucocyte Count (DLC):** Enumeration of WBC was done by microscopic rapid slide method. For differential leucocyte counts an area had chosen where the morphology of the cells was clearly visible. Ensured that there was no tailing of the WBC’s. Differential count had been done by moving the slide as zigzag movement in order to include central and peripheral areas of the smear. While doing DLC, looked for vacuolation, toxic granulation, size and maturity of the white blood cells. Counted 100 cells and percentage of the cells had drawn. Counting had become easier since 100 squares were made on a paper and the letters P for neutrophil, L for lymphocyte, M for monocyte, E for eosinophil and B for basophil were entered in each square.

**Collection of blood and separation of serum:** Blood samples were collected by standard vein puncture techniques to plastic tubes with barriers. Ensured complete clot formation has taken place prior to centrifugation. Serum was separated from red blood cells immediately after the collection.

**Serological tests:** Samples were analysed for the quantitative estimation of Aspertate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), protein, albumin, IgA, IgG and IgM.

**Estimation of AST:** For the quantitative *in vitro* determination of AST, RANDOX (Modified IFCC method) As 1204 R1a. Buffer/Substrate 1 x 105ml, 10 x 10 ml R1b
Enzyme/Coenzyme/α-oxoglutarate kit was used; serum collected from filarial positive patient was employed.

**Principle:** α-oxoglutarate reacts with L-aspartate in the presence of AST to form L-glutamate plus oxaloacetate. The indicator reaction utilizes the oxaloacetate for a kinetic determination of NADH consumption.

\[
\text{AST} \\
\begin{align*}
\alpha\text{-oxoglutarate} + \text{L-aspartate} & \rightarrow \text{L-glutamate} + \text{oxaloacetate} \\
\text{oxaloacetate} + \text{NADH} + \text{H} & \rightarrow \text{L-malate} + \text{NAD}^+
\end{align*}
\]

**Reagent composition:**

**Reagent-I: Buffer/Substrate** Tris buffer - 80 mmol/L, pH 7.5; L-aspartate - 240 mmol/L

**Reagent-II: Enzyme/coenzyme/ α-oxoglutarate** 12mmol/L; MDH - ≥ 420 U/L; LD - ≥ 600 U/L; NADH – 0.18 mmol/L

**Stability and preparation of reagents:**

**R1a. Buffer/Substrate:** Contents were ready to use. Stabled up to the expiry date when stored at +2 to +8 °C.

**R1b. enzyme/Coenzyme/ α-oxoglutarate**

Reconstituted one vial of Enzyme/ Coenzyme/ α-oxoglutarate R1b with the appropriate volume of Buffer/Substrate R1a:10 ml for the 10 x 10 ml kit (AS 1204) stable for 14 days at +2 to +8 °C.

**Procedure:**

Mixed and read initial absorbance after 1 minute and read again after 1, 2 and 3 minutes.
If the absorbance changed per minute is between 0.11 and 0.16 at 34/Hg 334 nm; 0.06 and 0.08 at Hg 365 nm values were used for the first 2 minutes of the calculation.

**Calculation:**

Calculated the AST activity using the following formula: $U/L = 1746 \times \Delta A_{340\text{ nm/min}}$; $U/L = 1780 \times \Delta A_{334\text{ Hg nm/min}}$; $U/L = 3235 \times \Delta A_{365\text{ Hg nm/min}}$.

**Estimation of ALT:** For the estimation of ALT, LIQUIXX SGPT-R (Modified IFCC Method) kit was used; serum collected from filarial positive patient was employed.

**Principle:** ALT present in the sample catalyzes the transfer of the amino group from L-alanine to $\alpha$-ketoglutarate forming pyruvate and L-glutamate. Pyruvate in the presence of NADH and lactate dehydrogenase (LDH) is reduced to L-lactate. In this reaction NADH is oxidized to NAD. The reaction is monitored by measuring the rate of decrease in absorbance at 340 nm due to the oxidation of NADH to NAD. The reagent incorporates a reaction mechanism of regenerating the NADH for the extended stability of the working reagent.

**Reagent Composition (concentration and activity in the test):**

**Reagent–I:** Tris Buffer – 100 mmol/L; L-alanine – 440 mmol/L; LDH >4 U/ml; $\alpha$-ketoglutarate – 13.20 mmol/L and also contains non-reactive fillers and stabilizers.

**Reagent – II:** $\beta$-NADH – 1.52 mmol/L

**Reagent Preparation:** Prepared the working reagent by mixing 4 part of R-I with 1 part of R-II per assay tube.
Reagent Storage: The unopened reagents were stable until the expiration date was stored at 2-8°C. Reagent stability after mixing of R-I & R-II was 90 days at 2-8°C.

Assay Parameters

<table>
<thead>
<tr>
<th>Mode</th>
<th>Kinetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength 1 (nm)</td>
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</tr>
<tr>
<td>Wavelength 2 (nm)</td>
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</tr>
<tr>
<td>Sample volume (µl)</td>
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</tr>
<tr>
<td>Working reagent volume (µl)</td>
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</tr>
<tr>
<td>Lag time (sec.)</td>
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</tr>
<tr>
<td>Kinetic interval (sec.)</td>
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<tr>
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<tr>
<td>Reaction direction</td>
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<td>Normal low</td>
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<tr>
<td>Normal high</td>
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<tr>
<td>Linearity low</td>
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</tr>
<tr>
<td>Linearity high</td>
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<tr>
<td>Absorbance limit (min.)</td>
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</tr>
<tr>
<td>Blank with</td>
<td>water</td>
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</tbody>
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Assay procedure

<table>
<thead>
<tr>
<th>Pipette</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td>500 µl</td>
</tr>
<tr>
<td>Sample</td>
<td>25 µl</td>
</tr>
</tbody>
</table>

Mixed well and aspirate.

Calculation: Calculated the ALT activity using the following formula.

\[
[IU/L] = \Delta A/\text{min.} \times \text{factor (3376)} \times A.
\]

Estimation of ALP: For the quantitative analysis of ALP (p-NPP-DEA Method) Ensure biotech diagnostic Pvt., Ltd., kit was used; filarial positive patient’s serum was used for analysis.

Principle: Alkaline Phosphatase at an alkaline Ph hydrolysis p NPP formed yellow coloured p NPP. The rate of PNP formed is directly proportional to the ALP activity.
p-Nitro phenyl phosphate + H₂O ⇄ ALP ⇄ Phosphate + p-Nitro phenol

**Reagent Composition:**

Reagent 1: p NPP substrate reagent 5x10 ml, 2-8°C

**System Parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
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<td>Wavelength</td>
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<tr>
<td>Reagent volume</td>
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<td>Delay time</td>
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<tr>
<td>Kinetic interval</td>
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<td>Factor</td>
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</tr>
<tr>
<td>Reaction Incubation</td>
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</tr>
<tr>
<td>Abs. Maxim</td>
<td>≤ 1.2</td>
</tr>
<tr>
<td>Units</td>
<td>IU/L</td>
</tr>
<tr>
<td>High normal</td>
<td>280 (Adults)</td>
</tr>
</tbody>
</table>

**Procedure:**

<table>
<thead>
<tr>
<th>Working reagent</th>
<th>1.0 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>25 µl</td>
</tr>
</tbody>
</table>

Mixed thoroughly and read absorbance against distilled water at 405 nm as follows

\[ A_o – \text{Exactly after 30 seconds} \]
A1, A2 and A3 – Exactly after every one minute for 3 minutes

Calculation:

Concentration of ALP in serum (IU/L) = $\Delta A/\text{Min} \times \text{Factor 2757}$. 

Normal Ranges:

Children up to 15 years old       < 644 IU/L 
Children 15-17 years              < 483 IU/L 
Adults                              up to 280 IU/L 

Estimation of total protein: Iris health care test kit was used for the estimation of proteins.

Principle: Cupric ions, in an alkaline medium, interact with protein peptide bonds results in the formation of a coloured complex.

Reagent composition:

Reagent – I : Biuret reagent 
Protein standard: 6g/dl (store at 2-8°C)

Sample collection and preservation: Serum collected by standard procedures. 
Stable for 8 days at 2-8°C.

Automated parameters:

Wavelength : 540 nm 
Cuvette : 1 cm light path 
Reaction temperature : 20-25°C
Measurement : Against Reagent Blank

Reaction type : End point

Reaction Direction : Increasing

Sample/Reagent Ratio : 1:50

Incubation : 10 minutes

Low normal : 6.6 g/dl

High normal : 8.3 g/dl

Linearity : 10 g/dl

**Assay Procedure:**

Pipetted into test tubes

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>STD</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>20 µl</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>20 µl</td>
<td>-</td>
</tr>
<tr>
<td>Reagent</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
</tbody>
</table>

Mixed well, and waited for 10 minutes at 20-25°C temperature. Measured the absorbance of the sample (Ac) and Standard (As) against the reagent.

**Calculation:**  \( \frac{Ac}{As} \times \text{Concentration standard} = \text{g/dl protein} \)

**Estimation of albumin (BCG colorimetric test):** Iris health care test kit was used for the analyze quantitative determination of albumin in serum filarial positive sample.

**Principle:** The measurement of serum albumin is based on its quantitative binding to the indicator 3, 3’, 5, 5’– tetrabromo-m-cresol sulphophthalein (bromocresol green, BCG). The albumin – BCG complex absorbs maximally at 578 nm.
**Reagent composition:**

Reagent I: BCG reagent

Albumin standard: 4g/dl (store at 2-8°C)

**Sample collection and preservation:** Used non-haemolysed serum collected without prolonged venous stasis. Specimens were stable for at least 20 days when stored at 4°C.

**Reagent stability:** Up to expiry date when stored at 2-8°C.

**Linearity:** The method was linear to a concentration of 8.0 g/dl.

**Automated parameters:**

- Wavelength: 620 nm
- Cuvette: 1 cm light path
- Reaction temperature: Room temperature
- Measurement: Against Reagent Blank
- Reaction: End point
- Reaction Direction: Increasing
- Sample/Reagent Ratio: 1:200
- Incubation: 5 minutes
- Low normal: 1.8 g/dl
- High normal: 5.1 g/dl
- Linearity: 8.0 g/dl

**Assay Procedure:** Pipetted into test tubes
Mixed well, and waited for 5 minutes at room temperature, measured the absorbance of the sample (Ac) and Standard (As) against the reagent.

\[
\text{Absorbance of sample} \quad \text{Albumin} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Standard value}
\]

**Quantitative determination of human immunoglobulin A (IgA) IVD**

**Intended used:** The IgA was a quantitative turbidimetry tested for the measurement of IgA in human serum or plasma.

**Principle of the method:** Anti-human IgA antibodies mix with samples containing IgA, from insoluble complexes. These complexes cause an absorbance change, depends upon the IgA concentration of the patient sample, which quantifies by comparison from a calibrator of known IgA concentration.

**Reagents**

- Diluent (R₁): Tris buffer 20 mmol/L PEG 8000, pH 8.3 sodium azide 0.95g/L
- Antibody (R₂): Goat serum, Anti-human IgA, pH 7.5. sodium azide 0.95g/L
- Optional: Cod: 1102003 PROT CAL

**Calibration:** The assay was calibrated to the referenced material CRM 470/RPPHS (Institute of reference of materials and measurements, IRMM). It used the PROT CAL
Calibrator to calibrated the reagent. The reagent (both mono-reagent and bi-reagent) were recalibrated every month, when the control was out of specifications and when changing the reagent lot or the instrument settings.

**Preparation:**

Reagents: Ready to use

**Additional equipments required:**

1. Thermostatic bath at 37 °C
2. Spectrophotometer or photometer thermostatable at 37 °C with a 600 nm filter (580-620 nm).

**Samples:** Fresh serum or plasma. EDTA or heparin was used as anticoagulant. Stabled for 7 days at 2-8 °C or 3 months at-20 °C.

The samples with presence of fibrin were centrifuged.

Did not used highly hemolized or lipemic samples

**Procedure:**

1. The reagents and the photometer (cuvette holder) were brought to 37 °C
2. Assay conditions: Wavelength: 600 nm
   
   Temperature: 37 °C

   Cuvetee light path: 1 cm

3. Adjusted the instrument was zero with distilled water
4. Pipetted into a cuvette: Reagent R1 800 µl; sample or calibrator 10 µl
5. Mixed and read the absorbance (A1) after the sample addition.
6. Immediately, pipetted into the cuvette reagent 2 (R2) 200 µl
7. Mixed and read the absorbance (A2) of calibrators and sample exactly 2 minutes after the R2 addition.

Calculations: Calculated the absorbance difference (A2-A1) of each point of the calibrated curve and plotted the values obtained against the IgA concentration of each calibrated dilution. IgA concentration in the sample was calculated by interpolation of its (A2-A1) in the calibrated curve.


Content: R1 Diluent: 1x 40 mL; R2 Antibody: 1x 10mL. ITIS39-I

Quantitative determination of human immunoglobulin G (IgG) IVD

Intended used: The IgG was a quantitative turbidimetry tested for the measurement of IgG in human serum.

Principle of the method: The IgG is a quantitative turbidimetry test for the measurement of IgG in human serum. Anti-human IgG antibodies when mix with samples containing IgG forms insoluble complexes. These complexes cause an absorbance change depends upon the IgG concentration of the patient sample, that quantifies by comparison from a calibrated of known IgG concentration.

Reagents: Diluent (R1): Tris buffer 20 mmol/L, PEG 8000, pH 8.2 sodium azide 0.95g/L.; Antibody (R2): Goat serum, anti-human IgG, pH7.5 sodium azide 0.95g/L

Optional: Cod: 1102003 PROT CAL
**Calibration:** The assay was calibrated to the reference material CRM 470/RPPHS (Institute for reference materials and measurements). It was recommended the use of PROT CAL calibrator.

**Preparation:** Ready to use.

**Additional equipment:**

1. Thermostatic bath at 37 °C
2. Spectrophotometer or photometer thermostatable at 37°C with a 600 nm filter (580-620 nm).

**Samples:** Fresh serum or plasma. EDTA or heparin were used as anticoagulant. Stabled 7 days at 2-8°C or 3 months at-20°C.

The samples with presence of fibrin were centrifuged.

**Procedure:**

1. Brought the reagents and the photometer (cuvette holder) to 37 °C
2. Assay conditions: Wavelength: 600 nm  
   Temperature: 37 °C  
   Cuvette light path: 1 cm
3. Adjusted the instrument to zero with distilled water
4. Pipetted into a cuvette: Reagent R1 950 µl; sample or calibrator 7 µl
5. Mixed and read the absorbance (A₁) after the sample addition.
6. Immediately, pipetted into the cuvette reagent 2 (R₂) 50 µl
7. Mixed and read the absorbance (A₂) of calibrators and sample exactly 2 minutes after the R₂ addition.
**Calculations:** Calculated the absorbance difference \((A_2-A_1)\) of each point of the calibrated curve and plotted the values obtained against the IgG concentration of each calibrated dilution. IgG concentration in the sample was calculated by interpolation of its \((A_2-A_1)\) in the calibrated curve.


**Content:** R\(_1\) Diluent: 1 x 50 mL; R\(_2\) Antibody: 1x 2mL. ITDTT14.

**Quantitative determination of human immunoglobulin M (IgM) IVD**

**Intended used:** The IgM was a quantitative turbidimetry tested for the measurement of IgM in human serum.

**Principle of the method:** Anti-human IgM antibodies when mix with samples containing IgM, form insoluble complexes. These complexes cause an absorbance change, depends upon the IgM concentration of the patient sample that can be quantified by comparison from a calibrator of known IgM concentration.

**Reagens:** Diluent (R\(_1\)): Tris buffer 20 mmol/L, PEG 8000, pH 8.3 sodium azide 0.95g/L.; Antibody (R\(_2\)): Goat serum, anti-human IgM, pH 7.5 sodium azide 0.95g/L

Optional: Cod: 1102003 PROT CAL

**Calibration:** The assay was calibrated to the reference material CRM 470/RPPHS (Institute for reference materials and measurements). It used the PROT CAL calibrator to calibrated the reagent.

**Preparation:** Ready to use.
**Additional equipment:**

1. Thermostatic bath at 37 °C
2. Spectrophotometer or photometer thermostatable at 37 °C with a 340 nm filter (320-360 nm).

**Samples:** Fresh serum or plasma. EDTA or heparin was used as anticoagulant. Stabled for 7 days at 2-8 °C or 3 months at -20 °C.

The samples with presence of fibrin were centrifuged.

Do not use highly hemolized or lipemic samples

**Procedure:**

1. The reagents and the photometer (cuvette holder) were brought to 37 °C
   
   Temperature: 37 °C

   Cuvette light path: 1 cm
3. Adjusted the instrument to zero with distilled water
4. Pipetted into a cuvette: Reagent R₁ 800 µl; sample or calibrator 10 µl
5. Mixed and read the absorbance (A₁) after the sample addition.
6. Immediately, pipetted into the cuvette reagent 2 (R₂) 200 µl
7. Mixed and read the absorbance (A₂) of calibrators and sample exactly 2 minutes after the R₂ addition.

**Calculations:** Calculated the absorbance difference (A₂-A₁) of each point of the calibrated curve and plotted the values obtained against the IgM concentration of each
calibrated dilution. IgM concentration in the sample was calculated by interpolation of its \((A_2-A_1)\) in the calibrated curve.


**Content:** R₁ Diluent: 1 x 40 mL; R₂ Antibody: 1x 10mL. IT IS 41-I.

**Immunological tests:**

**Standard operating procedure for CD3, CD4 and CD8 on FACS Comp., BDIS multi test IMK kit with true count tubes:**

**Purpose:** Identification of CD3, CD4 and CD8 cells in randomly selected 45 filarial positive patients in Pedana

**Specimen collection, transport and handling for flow**

**Specimen collection:** Peripheral blood is collected in K₃ EDTA AcCuvt-PLUS (2ml is sufficient for this assay) vacutainers should be filled properly to account for the amount of anticoagulant. All specimens were regarded as potentially infectious and universal precautions for blood collection and handling were properly followed.

**Specimen Transport:** Specimens were placed in well stoppered tubes. Tubes were placed in adequate absorbent material, in side a zip blocked bag and placed in a closed leak proof container for delivery and care was taken to main temperature in between 18°C to 25°C during transportation and storage. The samples were tested within 24 hours from the time of collection.

**Equipment, materials and reagents** (Plate 3, Fig. 4)

1. Micropipette with sterile disposable tips, 10 µl to 100 µl
2. Single pipette 450 µl for dispensing 1 X multi test lysing solution
3. BD FACS Flow sheath fluid (BD Biosciences)
4. FACS Calibur Cytometer (BD Biosciences)
5. Cell Quest Software (BD Biosciences)
6. Printer

Reagents:
1. Multi test IMK kit (BD Biosciences) kept at 2-8°C in the dark until use; did not freeze
2. Multi test CD3 FITC, CD4 APC and CD8 PE
3. True count tubes (BD Biosciences)
4. 1 X multi test IMK kit lysing solution diluted.

Staining procedure:
1. Diluted the 10 X Multi TEST IMK kit Lyse concentrate
2. For each patient sample, labeled two TruCount tubes with the sample identification number. Used letters A and B to differentiate the two tubes.
3. Pipetted 20 µl of Multi TEST CD3/CD4/CD8 reagent into the bottom of each tube labeled A & B separately just above the metal retainer. Avoided touching the fluorescent bead pellet.
4. Pipetted 100 µl of well mixed EDTA anticoagulated whole blood onto the side of each tube just above the metal retainer
5. Cap the tubes and vertex gently to mix and incubated for 15 minutes in dark at room temperature.
6. Added 450 µl 1 X Multi TEST Lysing solution to each tube
7. Cap the tubes and vertex gently to mix and incubated for 15 minutes in dark at room temperature. The samples were now ready to be analyzed on the flow cytometer.

8. Filled designated flow assay work sheet to include sample PID and MoAb kit lot #, tech initial and date for staining, acquisition and analyzing.

**Sample acquisition and analysis:**

1. Performed FACSComp on “Lyse/Wash” and Lyse/No Wash” settings

2. Choosen worklist manager from the toolbar on the bottom of the screen

3. The application window opened. Signed in as the operator. Clicked ‘Accept” to continue to the Set Up view.

At the Set Up View, verified the following:

Data files, laboratory report, physician report, export document, and used date generated file name

Clicked “Accept” upon satisfactory.

The FACS Comp view appeared. Either launched into FACS Comp or Clicked “Skip FACS Comp” if already done.

Proceeded to the test preference view.

**Verification:**

All subset results are chosen (CD3+ CD4+ %lymph)

Report reference ranges was selected

QC Message for “Out of Normal Range.”

**Laboratory report choices:** Report percent.

File ID and sample ID were corrected to save for list mode data files.
Continued to sample view. Entered sample PID.

Clicked on “Assign Rack” and put in the number of the rack in which the samples were loaded.

Loaded the samples onto the rack. Samples were loaded starting at position 1, with each sample A tube run before sample B tube. Made sure that the FACS Clean tube was loaded in position 39 and FACS Rinse was loaded in position 40.

Clicked “OK.”

Loaded the rack into the flow cytometer. Made sure the flow cytometer was set to “Run” and “Med.”

Clicked on “Run Tests.”

When the acquisition and automatic analysis were done, the reports were automatically printed.

Viewed the analysis. Checked the gating visually to make sure it is accurate. Verified that QC values were acceptable. Values were: Lymphosum = 95 to 105

CD3% Lymph Difference = 0% to 2%.

Filarimetric parasitic indices

1. Microfilaria Rate (mf Rate): It is the proportion of microfilaria carriers to the total no. of persons examined.

\[
\text{mf rate} = \frac{\text{No. of mf carriers}}{\text{Total no. of blood samples examined}} \times 100
\]

2. Disease Rate: It is the proportion of persons with disease to the total no. of persons examined.
Disease rate  =  \frac{\text{No. of persons with disease}}{\text{No. of persons examined for filarial disease}} \times 100

3. Endemicity Rate: It is the proportion of sum total no. of mf carriers and total no. of persons with disease to the total no. of persons examined.

\text{Endemicity rate}  =  \frac{\text{No. of persons with mf} + \text{no. of persons with disease}}{\text{Total no. of persons examined in the survey}} \times 100

4. Average mf Density: It is the proportion total no. of mf among mf carriers to the no. of microfilaria carriers,

\text{Average mf Density}  =  \frac{\text{Total no. of microfilariae among mf carriers}}{\text{Total no. of blood samples \text{\textsuperscript{+}ve for mf}}}