CHAPTER 3
MATERIAL AND METHODS
The present study was undertaken to evaluate the seroprevalence of toxocariasis in human population of all the six districts of Kashmir valley and to observe the prevalence of *Toxocara canis* in dogs. This is the first comprehensive study of its type in the Kashmir valley and before this only a small hospital based study has been carried out. Blood samples were collected randomly from individuals including both male and female members of different age groups so as to know the prevalence of toxocariasis in human population. Faecal samples were collected from the stray dogs to find out the prevalence of *Toxocara canis* infection in dogs.

3.1. Prevalence of Toxocariasis in humans

The studied population was divided into three different age groups 0 - 16 (group I), 17-40 (group II) and 41 years onwards (group III) which represent children, young and old population respectively. Various demographic and social characteristics like source of drinking water, condition of drinking water, presence of pet (dog) at home, house fenced or not, playing and eating habits etc were noticed. The particular points in the questionnaire specially asked were as follows.
Department of Zoology  
The University of Kashmir  

Questionnaire  

<table>
<thead>
<tr>
<th>Name</th>
<th>Age (in years)</th>
<th>Gender: M/F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Residence</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paternal Education</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal Education</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Habits:  
Source of drinking water: Tap water/Well water/ River water/ other sources  
Condition of water: Boiled/ Unboiled  
Feeding habits: consuming raw vegetables or not.  
Geophagia  
House fenced/ unfenced  

Blood Examination  

<table>
<thead>
<tr>
<th>Haemoglobin (gm/dl)</th>
<th>Blood urea</th>
<th>Blood Glucose</th>
<th>Serum Bilirubin</th>
<th>Serum Creatinine</th>
<th>Alkaline Phosphatase</th>
<th>Differential Leukocyte count</th>
<th>Total Leukocyte count</th>
<th>Total Erythrocyte count</th>
<th>ELISA for Toxocariasis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 3.1.
Map of J&K, showing study sites.
1. Srinagar
2. Baramula
3. Budgam
4. Pulwama
5. Anantnag
6. Kupwara
3.1.1. Examination of blood

For the examination of blood in the laboratory following steps were followed.

3.1.1.1. Blood collection

Blood sample was taken from each individual to be studied. Before taking a sample each individual was made mentally prepared for it and only after his/her consent blood was taken. Blood was collected by commercially available 5 ml disposable syringes.

3.1.1.2. Isolation of serum and plasma

The blood collected was stored in two separate bottles one with anticoagulant EDTA (ethylene diamine tetra acetic acid) and another without anticoagulant from which later serum was separated.

3.1.1.3. Transportation

The bottles with blood samples were simultaneously labeled to prevent intermixing. Then the samples were transported to the laboratory for further investigation. The samples were stored at -20°C until tested.

3.1.1.4. Detection of serum IgG antibodies to *Toxocara*

ELISA was used for the qualitative screening of serum IgG antibodies to *Toxocara*. 
**Principle**

The micro test wells were coated with an excretory secretory antigen from the *Toxocara* larvae. During the first incubation with diluted patient's sera any antibodies which were reactive with the antigen bound to the coated wells. After washing to remove the rest of the sample, the enzyme conjugate was added. A complex would be formed with enzyme conjugate if antibodies were present. After another series of washes, a chromagen (Tetra methyl benzidine or TMB) was added. The enzyme peroxidase added would catalyse a reaction and consumed the peroxide and turned the chromogen from clear to blue. Addition of the stop solution ended the reaction and turned the blue colour to a bright yellow colour.

**Reagents**

- Test strip: micro wells containing *Toxocara* antigens – 96 or 48 test wells in a test strip holder.
- Enzyme conjugate one (1) bottle containing 11 or 6 ml of protein conjugated to peroxidase.
- Positive control serum ; one(1) vial containing 1 ml of diluted positive rabbit serum
- Negative control serum: one (1) vial containing 1 ml of diluted negative human serum.
- Chromogen: one (1) bottle 11 ml of the chromogen TMB.
- Wash concentrate solution: one (1) bottle containing 25 ml of concentrated buffer and surfactant.
- Dilution buffer: two (2) or one (1) bottle(s) containing 30 ml of buffered protein solution.
Material and Methods

Stop solution: one (1) bottle containing 11 ml of 1 M phosphoric acid.

Procedure

To detect the presence of IgG antibodies against *Toxocara canis* antigen in blood samples collected ELISA was performed by breaking off number of wells needed (two for controls plus number of samples) and placing them in strip holder. Added 100 μl (or two drops) of the negative control to well first, 100 μl of the positive control to well 2 and 100μl of the diluted (1:64) test samples to the remaining wells. Incubated at room temperature (15-25° C) for 10 minutes. Shook out contents and washed 3 times with diluted wash buffer. Added 2 drops of enzyme conjugate to each well. Incubated at room temperature for 5 minutes. Shook out contents and washed 3 times with wash buffer. Slapped plates against paper toweling to remove excess moisture. Added 2 drops of the chromogen to every well. Incubated at room temperature for 5 minutes. Added 2 drops of the stop solution and mixed by tapping strip holder.

Analysis

Visually observed each well against a white background and recorded as clear or +, ++, +++ reaction.

ELISA Reader

Set for bichromatic readings at 450/650-620 nm after zero reading on air and read the optical density of the samples and interpreted results.
Phg.3: Performing ELISA to detect anti-Toxocara Antibodies

Phg.4: Yellow coloured wells showing Toxocara positive samples
against the standard (reference) value given on the kit.

3.1.1.5. Examination of blood for haematological investigation

1. **Estimation of blood haemoglobin**

   Estimation of blood haemoglobin was done by Sahli’s acid hematin method.

   **Procedure**

   It was done by following procedure. The blood sample was mixed in the collected bottle by rotation. Then N/10 HCl was placed in the diluting tube up to mark 10 on the percentage side. The EDTA mixed blood up was drawn up to the mark in the pipette supplied. This indicated a volume of 20 cmm. Then the tip was wiped if the blood had over shot the mark, and the blood level was reduced to the mark by tapping on the finger or cotton. The pipette was introduced into the tube in which N/10 HCl was already taken. Allowed the blood to flow out. After the blood had flown out, the pipette was raised slightly and the acid was sucked and then blown in the tube gently without causing any acid bubbles to disturb the acid and blood. This was repeated twice. Then the distilled water was drawn into the pipette and added to the fluid in the tube. The tube was shook gently to mix the blood and acid. The tube was kept in a dark place for 30 minutes. At the end of this period, the tube was placed in the comparator and distilled water was added drop by drop with a continuous stirring with the glass stirrer. This procedure was repeated till the colour in the tube matched with the colour of the standard. When colour matched, then the tube was
removed from the comparator and reading of level of the fluid was recorded in grams per 100 ml of blood.

II. Total leucocyte count

Counting of the total leucocyte count is clinically significant, when accompanied by a differential leucocyte count. Improved Neubauer chamber was used to do total count of leucocytes.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glacial acetic acid</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>1% aqueous solution of Gentian violet</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>98 ml</td>
</tr>
</tbody>
</table>

Procedure

> Using a WBC pipette of a haemocytometre we drew well mixed venous blood or capillary blood and fill till the 0.5 mark. Cleaned the tip of the tube. Now drew WBC diluting fluid till the 11 mark (or to 38 ml of diluting fluid add 0.02 ml of blood with an Hb pipette).

> Mixed the fluid and blood mixture gently, avoiding bubbling.

> Placed the cover slip of the counting chamber at the right place.

Shook the fluid - blood mixture and transferred the mixture using a fine bore Pasteur pipette on to the counting chamber (called charging the chamber), taking care that the mixture did not overflow, washed and dried the chamber to be re-charged again. The cells were then allowed to settle at bottom of the chamber for 2 minutes. (Placed a wet filter paper at the bottom of the slide in a Petri dish to avoid drying up of fluid)

Using 10X objective counted the WBC’s uniformly in the four larger corner squares. Cells present on the outer most lines were counted on
one side and those present on the line opposite were not counted.

Calculation

\[
\text{WBC count in undiluted blood} = (W \times 10) \times 20 \\
= W \times 50 \\
20 \rightarrow \text{dilution factor} \\
W \rightarrow \text{cells counted} \\
0.4 \text{ cumm} = 4 / 10 \text{ mm}
\]

III. Differential leucocyte count (DLC)

The differential leucocyte count calculates the relative proportion of 5 types of leucocytes. It is expressed as a percentage of each type of 100 leucocytes counted in a suitable area of the smear.

Preparation of the smear

- Placed a small drop of blood in the central line of the glass slide about 1 or 2 cms from one end.
- Put the spreading edge of the spreader at an angle of 45 degrees to the slide just in front of the blood drop.
- Moved the spreader back a little until it touched the drop and let the blood run along the edge of the spreader.
- Prepared the smear by a rapid, smooth and forward movement of the spreader.
- Allowed the smear to air-dry.
- Labeled the smear with the patient’s name and date.
Staining of the smear

Modification of Ramanowsky’s stain (Marshalk et al., 1975) namely Leishman’s stain/Wright’s stain and Giemsa stain were employed for staining of air dried blood smear. For Giemsa staining the air dried smears were first fixed in the acetone free methanol for 5 minutes. For Leishman’s staining methanol was already mixed with the stain. The stain was allowed to act for 1-2 minutes. Added water/buffer to the stain. Mixed thoroughly and carefully water with the stain and allowed it to react for 10-15 minutes. Washed off the stain with distilled or tap water. Now allowed the slide to air dry and examined by microscopy.

IV. Total erythrocyte count

Diluting fluid: This should be isotonic so that RBC’s are not haemolysed. Normal saline can be used but it may cause crenation of the R.B.C.’s

A) Formal- citrate solution
   Sodium citrate : 3 gm
   Formalin : 1 ml
   Distilled water : 100 ml

B) Hayem’s fluid
   Mercuric chloride : 0.5 gm
   Sodium chloride : 1 gm
   Sodium sulphate : 5 gm
   Distilled water : 200 ml

Method

Blood was drawn to 0.5 mark in the R.B.C. Pipette. Wiped tip clean and drew diluting fluid to the 101 mark. Shook for 3 minutes, charged the
chamber and counted the RBC's using 40x objective in the 80 smallest squares.

**Calculation**

\[
\text{Number of cells counted} \times \text{dilution factor} \times \text{depth factor} \]

\[
\text{Area counted} = \frac{(\text{Number counted} \times 200 \times 10)}{1/5} \\
\text{Area counted} = \frac{80}{400} \\
\text{Area counted} = \text{Number counted} \times 10,000.
\]

3.1.1.5. Examination of blood for various biochemical parameters

**I. Estimation of blood urea:** END POINT DAM method is usually employed for the quantitative determination of urea in serum/plasma.

**Principle**

Urea reacts with diacetyl monoxime in acidic medium at 95°C to 100°C to give pink coloured complex. Ferric ions were used to oxidize hydroxylamine formed in the reaction. Absorbance of pink colored end product was measured at 520 ± 15 nm. The intensity of colour developed was proportional to the concentration of urea present in the specimen.

**Kit contents**

<table>
<thead>
<tr>
<th>Kit contents</th>
<th>Description</th>
<th>Qty.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent I</td>
<td>Urea color Reagent A</td>
<td>190ml</td>
</tr>
<tr>
<td>Reagent II</td>
<td>Urea color Reagent B</td>
<td>190ml</td>
</tr>
<tr>
<td>Reagent III</td>
<td>Urea color Reagent C</td>
<td>250 ml</td>
</tr>
<tr>
<td>Reagent IV</td>
<td>Urea standard (40 mg/dl)</td>
<td>30 ml</td>
</tr>
</tbody>
</table>

**Procedure:** Pipetted into three test tubes labeled as B-Blank, Standard-S, and T-Test as follows.
Material and Methods

<table>
<thead>
<tr>
<th>Reagent</th>
<th>B</th>
<th>S</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Urea colour reagent A</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>2. Urea colour reagent B</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>3. Urea colour reagent C</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>4. Distilled Water</td>
<td>0.50 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5. Urea standard (40 mg/dl)</td>
<td>-</td>
<td>50 µl</td>
<td>-</td>
</tr>
<tr>
<td>6. Specimen</td>
<td>-</td>
<td>-</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

Mixed by lateral shaking and incubated all tubes in a vigorously boiled water bath for exactly 10 minutes. Cooled all tubes in tap water for 3 minutes. Measured absorbance (O.D.) of all tubes at 520 ± 15 nm against blank adjusted to zero.

Calculation

The concentration of urea in unknown sample

\[
\text{RT/RS} \times 40\text{mg/dl}
\]

(\(\text{RT}=\text{optical density of unknown sample}\))

(\(\text{RS}=\text{optical density of standard}\))

Normal Values

Serum-

- Adults = 10-46 mg/ dl
- Children = 10-32 mg/ dl

II. Blood sugar estimation

End point O-TOLUIDINE method was employed for the quantitative determination of glucose in blood.

Principle

The quantitative determination of glucose in body fluids by use of O-Toluidene as a colour reagent was first reported by Hilltman in 1959. This
method has been used extensively and has proved to be simple reliable and rapid. O-Toluidene reacts with the aldehyde group of glucose, in hot acetic acid solution to form an equilibrium mixture of glycosylamine and corresponding Schiff's base. The blue green coloured end product has maximum absorption at 630 nm. Intensity of colour developed was proportional to the original concentration of glucose present in the specimen.

KIT Contents

<table>
<thead>
<tr>
<th>Description</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose Reagent</td>
<td>500ml</td>
</tr>
<tr>
<td>Glucose standard (100 mg/ dl)</td>
<td>2 x 10 ml</td>
</tr>
<tr>
<td>TCA Reagent</td>
<td>Supply on request</td>
</tr>
</tbody>
</table>

Procedure

The three test tubes were labeled as B-Blank, S-Standard, and T-Test (unknown).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>B</th>
<th>S</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Glucose Reagent</td>
<td>3.0 ml</td>
<td>3.0 ml</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>2. Distilled water</td>
<td>50 μl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3. Glucose standard</td>
<td>-</td>
<td>50 μl</td>
<td>-</td>
</tr>
<tr>
<td>4. Specimen</td>
<td>-</td>
<td>-</td>
<td>50 μl</td>
</tr>
</tbody>
</table>

Mixed by lateral shaking. Put all tubes into vigorously boiling water bath (100°C) for exactly 9 minutes. Quickly removed the tubes and cooled to room temperature by placing in cold water for 3 minutes.

Transferred the contents of tubes to cuvette and measured the absorbance (O.D) of all tubes against blank adjusted to zero at 630 ± 20 nm.
Calculation

Concentration of glucose in the unknown sample

\[ \frac{RT}{RS} \times 100 \text{ mg/dl} \]

Normal values: Serum or plasma → 70 - 110 mg/dl

- RT - Optical density of test
- RS - Optical density of standard.

II. Serum bilirubin estimation

The total serum bilirubin was estimated by the method described by Jendrassik and Graf's (1938).

Principle

In this test, the bilirubin reacts with diazotized sulphanilic acid to form an azo dye, which is red in neutral and blue in alkaline solution whereas the water soluble bilirubin glucoronides react directly. The direct bilirubin was measured as the red azo dye at 546nm using the method of Schellong and Wende (1960).

The reagents used were:

A) Sulfanilic acid 9 29 mmol/l C₉H₇NO₃S 170mmol/l

B) Sodium nitrate (29 mmol/NaNO₂)

C) Accelerator (130mmol/lit. caffeine)

D) Fehling’s Solution II (930mmol/1 potassium sodium tartarate, 1.9mmol/1 sodium hydroxide solution).

Procedure

Neat and clean dry test tubes were selected and the contents were
added as per the following method.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Sample</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium nitrate</td>
<td>1 drop</td>
<td>-</td>
</tr>
<tr>
<td>Sulfanilic acid</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>Accelerator</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Serum</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
</tr>
</tbody>
</table>

The contents were mixed well and incubated for 10 to 60 minutes at room temperature (20°C).

<table>
<thead>
<tr>
<th>Fehling's solution</th>
<th>1 ml</th>
<th>1 ml</th>
</tr>
</thead>
</table>

The test tube contents were mixed well and after 5 to 30 minutes the absorbance of sample was measured against blank (578 Spectrophotometer).

**Calculation**

Total bilirubin = \( A \times 10.5 \, \text{mg/dl} \) (\( A = \text{Absorbance} \))

**III. Estimation of serum creatinine**

A manual method was developed by Folin-Wu. Brod and Sirota (1948) applied the method of Bronsnes and Taussky (estimation of urine creatinine) to determine the serum creatinine. Later on, Owen et al (1954) and Ralston (1955) developed a technique for determining (true creatinine).

In all these methods, the principle is the same (Jaffe reaction)

**Alkaline picrate method**

It is a simple and economical method.

**Principle**

Creatinine in a protein free solution reacts with alkaline picrate with
development of a red coloured complex. The intensity of the developed
colour is measured.

Sample: Serum / plasma, 1 ml
       Urine (1:25 dilution)

Reagents

Reagent 1: Saturated picric acid solution.

Took 13 gm picric acid in a measuring cylinder and added water to 1
litre. Excess of the picric acid was allowed to remain in contact with water,
shaking occasionally. The Filtered substance was stored in a polyethylene
bottle.

Reagent 2: Sodium hydroxide: 0.75 N
       Sodium hydroxide: 3 gm
       Distilled water : 100 ml

Reagent 3: Stock creatinine standard, 150 mg, dissolve 150 mg in 100 ml Hcl
(100 mmol/1)

Diluted 0.1 ml of stock creatinine standard to 10 ml. with distilled
water and mixed well to prepare working creatinine standard.

Procedure

Step A: Deproteinization of test sample

Serum/plasma 1.0ml
Distilled water, ml 1.0ml
Reagent 1: picric acid, 6.0

The mixture was mixed well and kept in a boiling water bath for
exactly one minute. The mixture was then cooled immediately under a
running tap water and centrifuged or filtered.

Step B: Colour development
Material and Methods

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank (B) (ml)</th>
<th>Standard(S) (ml)</th>
<th>Test(T) (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filtrate/supernatant (from step A)</td>
<td>---</td>
<td>----</td>
<td>4.0</td>
</tr>
<tr>
<td>Working standard</td>
<td>---</td>
<td>1.0</td>
<td>---</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.0</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Reagent 1: picric acid</td>
<td>3.0</td>
<td>3.0</td>
<td>---</td>
</tr>
<tr>
<td>Reagent 2: sodium hydroxide (0.75N)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Mixed well and allowed to stand at room temperature exactly for 20 minutes and measured immediately optical density of blank (B), standard (S) and test (T) against distilled water on calorimeter with a green filter.

For spectrophotometer: All the volumes mentioned under calorimetric procedure could be halved. The rest of the procedure remained unchanged.

Measured the optical density at 520 nm.

Calculation


IV. Serum alkaline phosphatase activity

Kind and King’s method

It is popular simple and economical method.

Principle

Alkaline phosphatase at a pH of 10 catalyse the following reaction

\[
\text{Phenyl phosphate + alkaline phosphatase} \xrightarrow{\text{pH 10}} \text{phenol + pi}
\]

Phenol so formed reacted with 4-aminoantipyrine (4-amino
phenazone) in the presence of an alkaline oxidizing agent - potassium ferricyanide with formation of an orange red colored complex. The intensity of colour was proportional to the enzyme activity and was measured spectrophotometrically

Specimen: Serum 0.4 ml

Procedure

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank(B)</th>
<th>Standard(S)</th>
<th>Control (C)</th>
<th>Test(T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent 1: buffered substrate</td>
<td>-</td>
<td>-</td>
<td>0.5ml</td>
<td>0.5ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1ml</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

Mixed well and incubate at 37°C for 3 minutes

<table>
<thead>
<tr>
<th></th>
<th>Working standard</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5ml</td>
<td>-</td>
</tr>
</tbody>
</table>

Mixed well and incubated at 37°C for 15 minutes.

<table>
<thead>
<tr>
<th>Reagent 2</th>
<th>Sodium hydroxide (0.5N)</th>
<th>0.4ml</th>
<th>0.4ml</th>
<th>0.4ml</th>
<th>0.4ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td></td>
<td>-</td>
<td>-</td>
<td>0.05ml</td>
<td>-</td>
</tr>
<tr>
<td>Reagent 3</td>
<td>Sodium bicarbonate</td>
<td>0.6ml</td>
<td>0.6ml</td>
<td>0.6ml</td>
<td>0.6ml</td>
</tr>
<tr>
<td>Reagent 4</td>
<td>4-aminoantipyrine</td>
<td>0.5ml</td>
<td>0.5ml</td>
<td>0.5ml</td>
<td>0.5ml</td>
</tr>
<tr>
<td>Reagent 5</td>
<td>Potassium ferricyanide</td>
<td>0.5ml</td>
<td>0.5ml</td>
<td>0.5ml</td>
<td>0.5ml</td>
</tr>
</tbody>
</table>

Mixed well after the addition of each reagent and measured the O.D. of blank (B), Standard(S) Control(C) and Test (T) at 510nm.

Calculation

Serum alkaline phosphatase activity, KA/ units/100ml

3.2. Prevalence of *Toxocara canis* infection in dogs

Besides the screening of blood samples for detection of *Toxocara* antibodies and various effects caused by the larva, prevalence of *Toxocara canis* in dog population of the Kashmir valley was also studied. For this faecal samples both dry as well as fresh, were collected from the different places i.e., streets, playgrounds, parks etc. of six districts of Kashmir valley. The collected faecal samples were immediately transferred in a bottle containing 10% formalin carefully. The collected samples were then transported to the parasitology laboratory 1 in the department of zoology, the University of Kashmir Srinagar for further examination.

**Procedure**

Samples were quickly processed and examined by the following techniques.

3.2.2. Faecal examination for *Toxocara canis* eggs

Examination was carried out by:

3.2.1.1. Naked eye examination.

Naked eye examination was used for the detection of adult worms. The specimen for examination was diluted with water and poured on to a sieve (meshes 30 to an inch) the faecal matter washed away & the worms left were easily picked out by holding the sieve against a dark background. In positive cases detection of adult worms may be different at times but the eggs are generally present in the specimens. For the detection of nematode infection the specimen was submitted for microscopically examination.
3.1.2.2. Microscopic examination

I. Direct smear

A small quantity of the faeces, about the size of a pin head was placed on a slide along with 2 or 3 drops of water, thoroughly emulsified with a needle, evenly spread over the 3"X 1" glass slide and examined under the microscope. This method was useful when the number of eggs was larger or infection was heavy. In case where the results were negative or doubtful or eggs were less in number, the diagnostic yield was enhanced by subjecting the samples to any one of the concentration methods.

II. Concentration method

The rationale of the concentration is to separate parasite objects from the bulk of the material in the specimen. For this purpose two methods, the sedimentation and floatation or a combination of the two were employed.

A. Flotation technique: This method was mostly useful in the examination of the nematode infection. The principle was to utilize a liquid suspending medium that was heavier than the parasite objects, or that they raised to the surface and can be skimmed out of the surface film. Faecal matter was dissolved in a solution of higher density than that of eggs causing the eggs to float in superficial portion of fluid.

A.1. Simple floatation technique (Maplestone, 1940)

Material required

1. Glass or metal container of 5-20 ml capacity with a flat bottom, vertical edges and diameter of not more than 1.5 inch.
2. Glass slides 3x2 inch instead of 3x1 inch

3. Saturated salt solution of specific gravity 1.200. This was prepared by allowing an excess of common salt to boil in a basin until a scum formed on the surface. When cooled it was stored in a bottle leaving an excess of undissolved salt at bottom

4. A sheet of glass on which the container was placed

One ml of stool was taken in container and a few drops of salt solution were added. It was stirred with a glass rod to make an even emulsion. After this more of salt solution 15-20 ml was added till the container is nearly full with continued stirring. The container was then placed on a level surface and final filling was done by means of a dropper, until a convex meniscus was formed. A glass slide 3x2 inch was carefully laid on top of the container till it comes in contact with fluid preparation. It was allowed to stand for 10 -20 minutes after which the glass slide was quickly lifted and turned over smoothly. So as to avoid spilling of liquid and was examined under microscope. The surface of the film was focused with both low and high power objectives for detection of eggs.

A.2. Zinc sulphate centrifugal flotation (Faust et al., 1939): A fine faecal suspension was made by taking 1 gm of freshly passed stool and 10 ml of luke warm distilled water. The coarse particles were removed by straining through wire gauze (40 meshes to an inch). The filtrate was collected in a Wasserman’s tube and centrifuged for one minute at the rate of 2500 rpm. The supernatant fluid was poured off and distilled water was added to sediment. It was then shaken well, centrifuged and process was repeated 2
- 3 times till the supernatant fluid was clear which was then poured off. To the sediment 3-4 ml of a 33% ZnSO₄ solution having sp. gravity 1.800 was added. The sediment was stirred and further ZnSO₄ was added to fill the tube up to the top and centrifuged again for at least one minute at 2500 rpm. The surface film was then removed by a platinum wire loop 5mm in diameter. On a clean glass slide onto which a coverslip was put and the specimen was examined

A. Sedimentation method: This type of method is reliable for all types of parasite eggs

Procedure

A small quantity of faeces made up from different areas of the sample was thoroughly mixed with 10-15 ml of water. This emulsion was strained through a sieve to remove all coarser particles. This filtrate was poured into a centrifuge tube just up to 1 inch below the brim and centrifuged at 1000 rpm for 1-2 minutes. All the eggs got packed, at the bottom as sediment and a drop of sediment was placed on a slide, covered with a cover glass and examined under the microscope.

3.3. Statistical Analysis

A computer program (SPSS 10.0, Minitab for windows) and online SISA software were used for data analysis. The descriptive data was given as a mean ± standard deviation (SD). The Chi-square test and student's T-test were used for the analytic assessment. The differences were considered to be significant when the p-value obtained was less than 0.05.