CHAPTER – 3

MATERIALS AND METHODS
In the present research problem, the livestock of Kashmir Valley was checked for *Paramphistomum* species seasonally for a period of one year. The infection of parasitism was also checked by using different immunodiagnostic techniques. The collected parasites were processed and identified using routine methods/techniques. Further, the infected organs of the host were analyzed for histological alterations.

### 3.1. Incidence

#### 3.1.1. Collection of Paramphistomes

The entire stomach of sheep were collected from various slaughter houses at various districts of Kashmir province. The material thus collected were placed in covered buckets containing physiological saline solution (sodium chloride 0.85gm in 100ml distilled water) (Weisner, 1968) and taken to the Parasitology laboratory of Post Graduate Department of Zoology, University of Kashmir. The organs were dissected out in the laboratory and subjected to a thorough examination for different amphistome infections. As a part of the investigatory process, the host stomach infested with amphistome were cut roughly into two inch square pieces for histopathological study and the amphistomes were detached and picked up from the stomach. These were placed in separate petridishes, containing physiological saline solution. The above procedure was followed in order to facilitate the separation of different
amphistomes. The parasites were counted to maintain their monthwise incidence.

3.1.2. Fixation of Parasites

The whole-mounts of the amphistomes were pressed between two glass plates and fixed in Carnoy's fixative (Weisner, 1968). The time of fixation varied depending upon the size of the specimens. After fixation parasites were picked up with a fine brush and preserved in 70% ethanol.

Carnoy's Composition

| Absolute alcohol | - | 60ml |
| Chloroform       | - | 30ml |
| Glacial acetic acid | - | 10ml |

3.1.3. Preparation of whole mounts

After fixing, the parasites were washed in (3-4 times) distilled water to remove out all the traces of fixative. As the parasites were preserved in the 70% alcohol, they were hydrated by washing through descending grades of alcohol (50%, 30%) followed by distilled water before staining. The specimens were generally kept 30 minutes in the acetoalum carmine (aqueous) stain and the extent of stain was checked by transferring the specimens in distilled water and observing the gross appearance. In case of over staining, parasites were destained by using acid water (Weisner, 1968).

Acid Water Composition

| Distilled water | - | 100ml |
| HCl             | - | 1ml  |
Aceto-Alum-Carmine

Carmine Powder - 5gm
Glacial Acetic Acid - 5ml
Potash Alum - 5gm
Distilled Water - 20ml

After proper staining, specimens were washed in distilled water and dehydrated through various grades of alcohol.

3.1.4. Dehydration schedule

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% alcohol</td>
<td>5 min.</td>
</tr>
<tr>
<td>50% alcohol</td>
<td>5 min.</td>
</tr>
<tr>
<td>70% alcohol</td>
<td>10 min.</td>
</tr>
<tr>
<td>90% alcohol</td>
<td>10 min.</td>
</tr>
<tr>
<td>100% I-alcohol</td>
<td>15 min.</td>
</tr>
<tr>
<td>100% II-alcohol</td>
<td>15 min.</td>
</tr>
<tr>
<td>Xylene – I</td>
<td>10 min.</td>
</tr>
<tr>
<td>Xylene – II</td>
<td>10 min.</td>
</tr>
</tbody>
</table>

In lower grades they were kept generally for 3-5 minutes but in higher grades for 15-20 minutes. In 100% alcohol, two washings were given each of 10 minutes duration to ensure complete dehydration. After that the parasites were transferred to xylene to clear them. From the xylene, the parasites were placed on the slides, mounted in the desired amount of DPX mountant and covered by cover slips.

The processed parasites were observed with the help of Miopia Binocular microscope with the maximum lens combination of 100x objectives.
and 20x eye piece and Olympus Binocular Microscope with a maximum lens combination of 100x and 15x.

Drawings were made to scale with the help of prism type (Erma Japan) camera Lucida. Measurements were done by means of ocular micrometer which were first standardized at different magnifications with the help of an objective micrometer having 1mm divided into 100 divisions.

Microphotography was done with an Olympus PM-6 camera.

3.2. Histopathology

Histology is the microscopic study of the normal biological material of the tissue whereas histopathology is the microscopic study of biological material affected by disease. The procedure adopted for the preparation of material for such studies are known as histological techniques. The characteristics of light microscope and the principle of image formed demand that the material must be relatively thin. If they are to be studied at high magnification, they should be coloured if maximal resolution is to be obtained and must be transparent if they are to be observed with transmitted light (Weisner, 1968).

For studying histopathological details of tissues, thin paraffin section were cut with help of microtome. By this method tissue was made fit for microscopic examination.

3.2.1. Fixation and Preservation

For histopathological preparations fixative used was either Bouin's fixative or 10% formalin.

Bouin's Composition

- Saturated aqueous picric acid - 75ml
- Formaldehyde - 25ml
- Glacial Acetic acid - 5ml
10% formalin Composition

Distilled water - 90ml
Formaldehyde - 10ml

3.2.2. Pre-embedding treatment

For the preparation of histological slides of infected and uninfected organs like stomach and small intestine of sheep, the organs were fixed in aqueous Bouin's fixative for 2 hrs. After the fixation the material was washed several times in 70% ethanol. Removal of picric acid was accelerated by warming the alcohol to about 40°C. The material was dehydrated by passing through 90% and two changes of absolute alcohol for 20-30 minutes in each. Dealcoholization was achieved with xylene. This was usually accomplished by transferring the material from the second change of absolute alcohol into a 1:1 mixture of absolute alcohol and xylene and then processed again for a period of 20-30 minutes.

3.2.3. Infiltration

The material for histological purpose after clearing in xylene was infiltrated with paraffin wax. The material was kept in a mixture of xylene and paraffin wax in the ratio of 1:1 for half an hour at 40°C - 42°C in an oven and finally two changes in pure wax (M.P. 58-62°C) maintained in a molten state in an oven for 2 to 4 hours.

3.2.4. Embedding

Embedding of infiltrated material was done in rectangular blocks, prepared by using L-shaped metal pieces. Fresh embedding paraffin from the oven was used to fill the blocks to the brim. The bottom layer of paraffin was allowed to solidify by keeping the blocks in cold water while the surface layer was kept melted by touching it occasionally with heated Spatula or forceps. The material was properly oriented in the wax with heated forceps. The
blocks were kept in water for 15-80 minutes and then stored in cold place for further processing.

Blocks were carefully trimmed with a clean sharp single edged razor blade. After proper trimming various sections of desired thickness (5μm to 7μm) were cut with the help of a rotatory microtome (Weswo Model). Continuous ribbons of the material were cut and passed over a ribbon carrier. The ribbons were placed in a section tray inorder in which they were cut.

3.2.5. Paraffin wax Embedding Schedule

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Materials fixed in Bouin's solution</td>
<td>Overnight</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>30% Alcohol</td>
</tr>
<tr>
<td>30% Alcohol</td>
<td>20min.</td>
</tr>
<tr>
<td>50% Alcohol</td>
<td>20min.</td>
</tr>
<tr>
<td>70% Alcohol</td>
<td>2 hr. (overnight)</td>
</tr>
<tr>
<td>90% Alcohol</td>
<td>20-30 min.</td>
</tr>
<tr>
<td>Absolute Alcohol I change</td>
<td>20-30 min.</td>
</tr>
<tr>
<td>Absolute Alcohol II change</td>
<td>20-30 min.</td>
</tr>
<tr>
<td>Absolute Alcohol + xylene (1:1)</td>
<td>20-30 min.</td>
</tr>
<tr>
<td>Pure xylene 1st change</td>
<td>20-30 min.</td>
</tr>
<tr>
<td>Pure xylene 2nd change</td>
<td>20-30 min.</td>
</tr>
<tr>
<td>Xylene saturated with paraffin wax.</td>
<td>20-30 min.</td>
</tr>
<tr>
<td>Molten paraffin wax. 58°C 1st change</td>
<td>30 min. - 2 hrs.</td>
</tr>
<tr>
<td>Embedded in fresh 58°C in paraffin wax.</td>
<td></td>
</tr>
</tbody>
</table>

3.2.6 Affixative of Paraffin Sections

The cleaned slides were coated with a thin film of affixative. The affixative used was Mayer's albumin.
Mayers albumin Composition

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg albumin</td>
<td>50ml</td>
</tr>
<tr>
<td>Glycerine</td>
<td>50ml</td>
</tr>
<tr>
<td>Sodium Salicylate</td>
<td>1gm</td>
</tr>
</tbody>
</table>

To stretch the sections, floating medium usually pre-warmed distilled water was used. The slides were dried by keeping on the hot plate to facilitate adhesion of sections.

**Preparation:** - The three ingredients were thoroughly mixed together and filtered. Preboiled distilled water was used as floating medium for the section, with this affixative. The ribbon of sections were divided into strips of the correct lengths, which were then placed with shiny side down in rows on the smeared slides.

Enough floating medium (water) was added from one end of the slide to facilitate the smooth adhesion. The slides were carefully transferred to a hot plate, which helps to flatten the sections and straighten the slides. The slides were removed from the hot plate and the excess fluid drained away. The slides were kept overnight for complete drying.

### 3.2.7 Processing of Paraffin sections

The removal of paraffin wax was accomplished by submerging the slides in pure xylene. Two changes were given each for 5 minutes.

**Removal of xylene:** - The xylene was removed from the sections by processing the slides through two changes of absolute alcohol for about 5 minutes in each change.

**Hydration:** - The slides were processed through a series of alcohol concentration in descending series (90%, 70%, 50% and 30%) and into distilled water for 2-3 minutes in each solution.
Haematoxylin stain Composition

- Haematoxylin Powder: 4gms
- Absolute alcohol: 25ml
- Glycerine: 100ml
- Methyl alcohol: 100ml

For globule leucocytes, Giemsa and Toluidine stain was used.

Giemsa Stain Composition

- Giemsa Powder: 2gm
- Phosphate buffer saline (pH 7.2): 2.0ml
- Distilled Water: 95ml

Toluidine Stain (A Rapid Method, Hamson 1979)

Method

1) Dewax. run slides at 60% alcohol: remove HgCl₂
2) Stain in 0.2% toluidine blue in 60% ethyl alcohol 1-2 min.
3) Rinse quickly in tap water and dehydrate in two changes of acetone in each, clear and mount.

Result

- Mast cell granules: deep reddish purple; background: faint blue

Eosin Composition

Aniline Blue (Rhodocyan Method, Lellie. 1994)

Fix in 10% neutral buffered formalin

- Eosin solution: 0.8ml
- Aniline blue: 0.2ml
- Citric acid: 0.2ml
- Disodium Hydrogen Phosphate: 0.9ml
- Distilled water: 37.0ml
The slides were dehydrated rapidly to 90% alcohol, skipping of 30% and 50% was found very useful and reducing the treatment from 2 minutes to about 20 seconds also gave good results. The slides were kept in 90% alcohol for 2 minutes and dehydrated completely by processing the slides through changes of absolute alcohol for 3 minutes in each change.

### 3.2.8. Haematoxylin Eosin Staining Schedule

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylene I</td>
<td>5 min.</td>
</tr>
<tr>
<td>Xylene II</td>
<td>5 min.</td>
</tr>
<tr>
<td>Absolute Alcohol 1st</td>
<td>5 min.</td>
</tr>
<tr>
<td>Absolute Alcohol 2(^{nd})</td>
<td>5 min.</td>
</tr>
<tr>
<td>90% alcohol</td>
<td>5 min.</td>
</tr>
<tr>
<td>50% alcohol</td>
<td>2-3 min.</td>
</tr>
<tr>
<td>30% alcohol</td>
<td>2-3 min.</td>
</tr>
<tr>
<td>Dist. H(_2)O</td>
<td>2 min.</td>
</tr>
<tr>
<td>Haematoxylin staining solution</td>
<td>15-20 min.</td>
</tr>
<tr>
<td>Dist. H(_2)O</td>
<td>3 min.</td>
</tr>
<tr>
<td>Dehydrated upto 90% very rapidly</td>
<td></td>
</tr>
<tr>
<td>(occasionally skipp 30% or even</td>
<td></td>
</tr>
<tr>
<td>30% 50% and 70% alcohol)</td>
<td>20 sec.- 2 min.</td>
</tr>
<tr>
<td>Eosin stain</td>
<td>15 min</td>
</tr>
<tr>
<td>Absolute Alcohol 1st</td>
<td>3 min.</td>
</tr>
<tr>
<td>Absolute Alcohol 2(^{nd})</td>
<td>3 min.</td>
</tr>
<tr>
<td>90% alcohol</td>
<td>5 min.</td>
</tr>
<tr>
<td>Xylene 1st</td>
<td>5 min.</td>
</tr>
<tr>
<td>Xylene II</td>
<td>5 min.</td>
</tr>
</tbody>
</table>
Dealcoholization and clearing: - Xylene was used for dealcoholization process. The slides were placed for 5 minutes in each of the two changes of the de-alcoholizing agent for complete clearing.

3.2.9. Mounting

The slides were removed from the second change of xylene. A drop of mountant was placed at the left end of each slide. A large cover glass was then placed in contact with the drops of mountant and lowered slowly until it covered the material.

Clearing the Prepared slides: - The prepared slides were finally cleared by removing the excess mountant with the help of xylene.

3.3. Immunology

Several immunodiagnostic methods have been used recently for the diagnosis of trematode infections in ruminants and also to assess the immune response elicited by these parasites in the experimental animals. Immunodiagnostic methods for the detection of these parasitic infections, however, usually suffer from problems of low sensitivity.

3.3.1. Collection of Gut content from the slaughtered sheep at the local abattoir

The gut of sheep slaughtered at local abattoir was collected and brought to the laboratory and they were minutely observed for trematode parasites. The main sites observed for parasite infection were rumen, abomassum, reticulum and small intestine, and the parasites especially trematodes were kept in separate tubes. The parasites recovered from rumen and small intestine were Paramphistomum cervi, Gastrothylax crummifer and Cotylophoron cotylophorum.
The infected parts of gut were carried to the laboratory for further investigations.

3.3.2. Preparation of soluble PBS antigen (Phosphate buffer saline)

Adult worms of *Paramphistomum* ranging from 9.25 to 10.35mm in length were collected from the rumen of sheep. The worms were washed with Physiological saline and stored at -20°C until use. Somatic antigen of adult fluke was prepared by following the technique of Yadav and Gupta (1996). Paramphistomes were cut into small pieces and then blotted over sterile filter paper and were freeze dried. 1gm of fluke was immersed in 5ml of PBS (pH 7.2) and homogenized for 30 minutes in a tissue glass grinder. The emulsion was transferred into a refrigerator for 24 hrs for extraction with occasional stirrings. This mixture was then centrifuged at 10,000 rpm at 4°C for 15 min.

The supernatant was stored as a clear PBS antigen in clean glass vials. The Protein content of the antigenic homogenate was estimated by the method described by (Doumas, 1981).

3.3.3. Total Protein Estimation

It is based on the principle that proteins and peptides containing at least two adjacent peptide bonds react with cupric ions in alkaline solution forming violet coloured complex having absorption maximum at 540nm. The concentration of low molecular weight peptides is too less to interfere.

Procedure (for 3 ml Cuvette).

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein reagent</td>
<td>3.0 ml</td>
<td>3.0 ml</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>50 μl</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Total protein standard 6 gm/dl</td>
<td></td>
<td>50 μl</td>
<td>-</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td>50 μl</td>
</tr>
</tbody>
</table>
The tubes labelled as standard, blank and test were mixed well and incubated at 30°C for 10 minutes and the optical density of all the tubes were measured at 540nm (Spectrophotometer) against blank adjusted to zero using yellow filter.

Calculation

\[
\text{Total Protein} = \frac{\text{O.D. test}}{\text{O.D. Stand.}} \times 6.0 \text{ mg/dl.}
\]

Composition of 0.2M PBS

- NaCl - 87.7gms
- Na₂HPO₄ - 10.91gms
- NaH₂PO₄ - 2.77gms

This was made to one litter distilled water and was diluted 1/10 with distilled water before use.

3.3.4. Immunization of Rabbits with PBS antigen for the Production of antibodies

Newzealand white rabbits of 6-8 months age and weighing about 1-2kg were immunized with 0.5ml of the antigen (2.5mg/dl protein concentration) subcutaneously along with the same amount of Freund's Complete Adjuvant (Geni). Six injections of the antigen doses were given at 5 days intervals of gap. Rabbits were bled after 7 days of last injection. The blood sample collected was allowed to clot for few hours, later on the clot was removed and the serum was separated by centrifugation at 2500 r.p.m for 15 minutes and the clear supernatant was stored in aliquotes at -20°C for further use.

3.3.5. Preparation of Emulsion

0.5ml of Antigen in PBS was mixed with 0.5ml of Freund's Complete Adjuvant (FCA) in a clean, dry sterile narrow bottle. The dispersion of the
water phase was increased by repeatedly taking up the emulsion into the syringe and then ejecting it with force through a 18G needle. As the dispersion increased the emulsion became more viscous.

Before use, water in oil emulsion was tested to determine its integrity by dispersing few drops onto surface of water in a petridish which would not disperse easily.

3.3.6. Route of Immunization

Emulsified antigen was injected subcutaneously at various sites on the back of the rabbits after proper preparation of sites.

3.3.7. Collection of Blood (Berlin and McKinney 1968)

To obtain clear serum or plasma, animal was bled before feeding. Blood was collected in a tube without anticoagulant. Rabbits were bled from the marginal vein of the ear. About 5ml of blood was collected in a single bleed from a immunized rabbits. Each rabbit was held by its head protruding over the edge of the table. Downward pressure was applied at the back with one hand and the neck region was held with other. Lateral margin of the ear was dry shaved and cleaned with 70% alcohol. Some times if the vein was not prominent, it dilated by rubbing the ear with finger or cotton swab moistened with xylene. To prevent premature clotting a thin coating of petroleum jelly was applied on the lateral margin of the ear. With the help of a sharp scalpel blade a diagonal incision was made across the vein. Immediately after making incision, blood was collected by pressing the venous return, proximal to the cut. After collecting the required amount of blood, pressure at the base of ear was released. Bleeding was stopped by pressing the vein at the site of cut with dry cotton, and held by applying a clip. Blood stains were cleaned off by applying 70% alcohol and the rabbits were observed for at least 1 hour before the animal was returned to cage.
3.3.8. Ouchterlony Gel Diffusion Test

Immunodiffusion test was carried out as per method described by Ouchterlony (1958). The antigen-antibody reaction demonstrated in gel media was indicated by precipitin bands. 1% agarose (SRL) solution was prepared by adding 1gm of agarose in 100 ml barbitone buffer having pH 8.6 and contained 1% thiomersol as preservative. The mixture was then boiled at 60°C, so that agarose got dissolved completely in buffer. The agarose solution was poured onto slides and then kept at room temperature so that gel solidifies. With the help of gel punch, wells were punched in the agarose gel at a distance of 3mm between the wells. The test serum samples (both normal and infected/immunized) were added to the peripheral wells and central well was filled with trematode antigen. The slides were kept in a moist chamber at room temperature (18-22°C) for 72 hrs for immunodiffusion. To rule out the possibility of cross reactions with other flukes, the AGDT was also performed with the Fasciola antigen and the positive reference sera against Paramphistome antigen. The gel slides were then fixed in methanol for 15 minutes and then washed in quick changes of distilled water. The slides were dried in an incubator and then stained by comassie brilliant blue R -250 and amido black (0.5%) stain dissolved in 90ml methanol and 10ml glacial acetic acid for 15 minutes. The slides were finally destained in a destaining solution to remove out excess stain and air dried.

**Barbitone buffer (pH 8.6) Composition**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barbituric acid</td>
<td>1.83gm</td>
</tr>
<tr>
<td>Sodium barbitone</td>
<td>10.6gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000ml</td>
</tr>
</tbody>
</table>
**Amido black stain (0.5%)**

- Amidoblock Powder: 0.5gm
- Methanol: 90ml
- Glacial acetic acid: 10ml

**Comassie brilliant blue R - 250 Stain**

- Glacial acetic acid: 50ml
- Methanol: 200ml
- Distilled water: 250ml
- Comassie brilliant R - 250 Powder: 1.0g

**Destaining Solution I**

- Methanol: 90ml
- Glacial acetic acid: 10ml

**Sol. II**

- Methanol: 90ml
- Glacial acetic acid: 10ml
- Distilled water: 10ml

**Sol. III**

- Methanol: 90ml
- Acetic acid: 10ml
- HCl: 1ml

After washing, the slides were put upside down on a plain surface on water soaked filter paper for the absorption of the gel.
3.3.9. Electrophoresis and Immunoelectrophoresis

Immunoelectrophoresis technique was followed as given by Grabar and Burtin (1964). The barbitone buffer and 1% agar solution prepared as described in Ouchterlony test.

In simple electrophoresis a small piece of Whatman filter paper (Size 0.1 x 1 cm) was soaked in the test serum and applied on the slides towards the negative end at the junction of 1/3 and 2/3 or small troughs were made and then filled with test serum towards the negative end.

Where as in the immunoelectrophoresis a trough was cut in the gel slide and on either side of this trough a small hole was made which was filled by Paramphistome antigen.

After charging the slides with suitable reactants both in electrophoresis and immunoelectrophoresis, the slides were subjected to 200 volts for 3hrs in electrophoretic apparatus (Densek Denmark).

After running, the slides were taken from the apparatus and the serum slides were fixed in pure methanol for 15 minutes.

Further steps were the same as described earlier, whereas in case of immunoelectrophoresis, the antigen slides, which have been separated by electrophoresis in the electric field, were filled with suitable anti-serum (in the trough) derived from infected sheep and immunized rabbits and then the slides were kept in the moist chamber for 72 hours for diffusion and precipitation reaction. Further steps regarding washing, fixation staining and destaining of plates were same as described in the Ouchterlony test. After 72 hours the precipitin arcs appeared were photographed.

3.3.10. Indirect Haemagglutination Test

Passive haemagglutination is the agglutination of red cells by antibodies specifically directed to soluble antigens that have been previously
adsorbed or otherwise attached to the red cell surface. Red cells are very popular as inert particles. erythrocytes of sheep can be readily obtained in bulk amounts with little effort (Johnson et al., 1966). The most important feature of these cells is that they are red in colour and their agglutination is very easy to observe. Agglutination of red cells in a tube shows a typical pattern. The non-agglutinated cells settle to the bottom of the tube as a clear red "button" while as agglutinated cells settle as mat like structure.

**Fresh Sheep Red Blood Cells:** - Sheep blood was collected defibrinated aseptically into an equal volume of Alsever’s solution and then kept at 4°C.

**Alsever’s solution**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrose</td>
<td>2.05g</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>0.80g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.42g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100ml</td>
</tr>
</tbody>
</table>

Therefore pH of this solution was adjusted to 6.1 with 10% citric acid before autoclaving at 10lbs for 15 minutes. antibiotic (streptomycin - 5mg/100ml) was added.

**3.3.11. Tanning of SRBC (Sheep Red Blood Cells)**

Sheep RBC was washed three times with normal saline by centrifugation for 10min. Then 0.5ml pellet of RBC was added with 25ml PBS (7.2) suspended slowly and properly. 2.5mg of tanic acid was added with 50ml of PBS and mixed with 50ml of 4% sheep RBC suspension.

Incubation was done at 37°C for 20min, followed by centrifugation slowly at 1000 r.p.m and then supernatant was discarded.
3.3.12. Agglutination of Sheep Red Blood Cells (SRBC)

Pellets was re-suspended in 20ml PBS (4% SRBC), then 0.5ml of antigen suspension was added with 0.5ml of 4% SRBC, mixed slowly and properly and incubated at 37°C for 30min. Following incubation, the cells were centrifuged and washed thrice with PBS. Finally the cells were re-suspended in 50ml of the same buffer (1% suspension). Each well of the microtitration plate was filled with 20µl PBS. Well number 1 and 2 was used as negative control. 20µl antisera was added well-3 and a serial dilution was performed there after. 1% tanned antigen (Ag) coated SRBC were added in all wells except well number first and second, where tanned SRBC without antigen was added. Plate was incubated at 37°C for 30min. and agglutination in wells was observed.

3.3.13. Enzyme Linked Immunosorbent Assay (ELISA)

In order to perform ELISA assay the first requirement was to absorb the prepared parasite antigen onto the wells of the microtitre plate.

Microtitration plate (Nunc) was coated with antigen diluted 2µg/ml in carbonate buffer (pH 9.6) and incubated at 37°C before keeping overnight at 4°C. Coated plates were blocked with 3% skimmed milk in PBS for 2 hrs at 37°C. After washing the plate with phosphate buffer (3 times). Both positive and negative serum samples were diluted in appropriate dilution, from 1:100, 1:400 etc) to each well @ 100µl/well. Serum was diluted in PBS - 1% skimmed milk and then incubated at 37°C for 2hrs. The plate were again washed with PBS tween -20.

After washing, 100µl of antiovine and anti rabbit IgG - peroxidase conjugate (1:5000 dilution, Sigama Chemical company USA) was added. The plate was kept at 37°C for 2hr. After washing five times with OPI (Orthophenylene diamine, Sigama, Chemical company USA)) in 100ml of
phosphate citrate buffer (pH 9.6) and 40μl H₂O₂ was added. Therefore the plate was kept in dark for 7 minutes. The reaction was stopped by adding 50μl of 3N HCl per well and the optical density was measured at 492nm by ELISA reader (Multiscan ELISA Reader).

In the present study an attempt has been made to assess the anti Paramphistomum antibodies in rabbit and sheep sera collected from local abattoir using ELISA. Indirect ELISA was performed as per method described by Yadav and Gupta (2005) with some modification.

**Chemical composition of substrate solution and coating buffer as follows:**

1) **Chemical Composition of Substrate (OPD)**

   (i) Orthophenylene-damine = 40mg
   (ii) H₂O₂ = 21μl/ml
   (iii) Phosphate buffer = 100ml

2) **Citrate Phosphate Buffer (pH 9.6)**

   A. Citric acid = 0.479g
   B. Distilled Water = 100ml
   C. Disodium hydrogen phosphate = 0.730g

   Mixed equal volume of solution (1) and (2) to prepared complete substrate buffer.

**Coating Buffer**

   (i) Sodium carbonate (Na₂CO₃) = 15mg
   (ii) Sodium bicarbonate (NaHCO₃) = 293mg
   (iii) Distilled H₂O = 100ml
   (iv) Adjust pH (9.6) (at 4°C)
**PBS Tween - 20 (PBST)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween-20</td>
<td>0.50ml</td>
</tr>
<tr>
<td>PBS</td>
<td>1000ml</td>
</tr>
</tbody>
</table>

Mixed properly and used before the solution turns milky within (2-3 days).