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EBINAT WOREDA LIVESTOCK RESOURCE DEVELOPMENT AND PROMOTION OFFICE

LABORATORY MANUAL OF VETERINARY PARASITOLOGY AND MICROBIOLOGY FOR ALL ANIMAL HEALTH PHYSICIANS

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Abstract: In addition to best clinical diagnosis of patient animals for reaching tentative diagnosed disease of animal, it is better aided by best laboratory working procedure to confirm whether the tentative diagnosis of patient animals are real or not. So performing the whole clinical diagnosis procedures within laboratory working for best drug selection and recommendation for patient animal is very important. Therefore, doing laboratory work helps directly or indirectly reduces development of drug resistance in veterinary science. In this manual mostly focused in most important parasitology and microbiology laboratory tests like: direct fecal smear, sedimentation technique, flotation technique, baerman technique and gram stain, California mastitis test rose Bengal test etc.
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SUMMARY

In addition to best clinical diagnosis of patient animals for reaching tentative diagnosed disease of animal, it is better aided by best laboratory working procedure to **confirm** whether the tentative diagnosis of patient animals are real or not. So performing the whole clinical diagnosis procedures within laboratory working for best drug selection and recommendation for patient animal is very important. Therefore, doing laboratory work helps directly or indirectly reduces development of drug resistance in veterinary science. In this manual mostly focused in most important parasitology and microbiology laboratory tests like: direct fecal smear, sedimentation technique, flotation technique, baerman technique and gram stain, California mastitis test rose Bengal test etc.

Key words:- *laboratory, manual, microbiology, parasitology and veterinary*

1. INTRODUCTION

The study of the development and transmission of parasitic nematodes started in the middle of the 19th century with early investigations on the transmission of *Trichinella spiralis* by Herbst, Virchow, Leuckart, Zenker and others. This was followed a few years later (1865–1866) by the discovery of alternation of generations in *Rhabdias bufonis* in amphibians by Metchnikoff and Leuckart. During the same period Leuckart and Marchi discovered that *Mastophorus muris* of the stomach of mice developed in flour beetles (*Tenebrio*) and Metchnikoff and Leuckart showed that *Camallanus lacustris* of freshwater fish developed in copepods. The latter finding apparently led directly to Fedchenko's important discovery in 1871 of the transmission of the human guinea worm by means of copepod intermediate hosts.

Principles of classification**:-**All animal organisms are related to one another, closely or remotely, and the study of the complex systems of inter-relationship is called systematics. It is essentially a study of the evolutionary process. When organisms are examined it is seen that they form natural groups with features, usually morphological, in common. A group of this sort is called a taxon, and the study of this aspect of biology is called taxonomy.

The taxa in which organisms may be placed are recognized by international agreement, and the chief once are: kingdom, phylum, class, order, family, genus and species. The intervals between these are large, and some organisms cannot be allocated to them precisely, so that intermediate taxa, prefixed appropriately, have been formed; examples of these are the suborder and the snperfamily. As an instance, the taxonomic status of one of the common abomasal parasites of ruminants may he expressed as shown below. Kingdom (Animalia), Phylum (Nemathelminthes), Class(Nematoda),Order(Strongylida),Suborder(Strongylina), Superfamily (Trichostrongyloidea), Family (Trichostrongylidae), Subfamily (Haemonchinae), Genus (*haemonchus)* and Species (*contortus).*

The names of taxa must be adhered to according to the international rules, but it is permissible to Anglicize the endings, so that members of the superfamily Trichostrongyloidea in the example above may also be termed trichostroneyloids.

The names of the genus and species are expressed in Latin form the generic name having a capital letter, and they must be in grammatical agreement. It is customary to print foreign words in italics, so that the name of an organism is usually underlined or italicized. Accents are not permitted, so that, if an organism is named after a person, amendment may be necessary; the name of Muller, for example, has been altered in the genus Muellerius.

Generally the parasites divide in to three categories such as **helminthes** (nematode, trematodes and cestodes), **protozoa** and **arthropods.**

1.1. Materials and equipment

Beaker, universal bottle, slide, cover slip, lens powder, distilled water, vaccutainer tube, needle with holder, cryo-vial, measuring cylinder, strip cup, stick, paddle, milk sample, conical test tube, mortar and pistil, mesh sieve, personnel protective equipment (PPE), blade with handle, scissor and forceps, lancet, guaze, cotton, syringe, disposable/surgical glove, eye goggle, mask, test tube rack, filter/bloating paper, scalpel, compound microscope, refrigerator, chair and table, separate room, water supply, centrifuge (manual/automatic), weighing balance, deep freezer (-20^{o}c).

1.2. Reagents

The serum, positives control serum, negative control serum, brucella/salmonellosis antigen, California mastitis reagents (CMT reagents).

1.3. Chemicals

10% buffered formalin, oil immersion, xylene, saturated salt/sugar solution, grams iodine, safranine, alcohol, giemsa powder, methylene blue and crystal violet

1.4. General safety and rules in laboratory

- ✓ Use all personnel protective equipment
- ✓ Pipetting should be done by mouth
- ✓ Used and contaminated materials should be stored safely and accordingly
- ✓ Long hair should be secured behind your head
- ✓ Labelling all materials
- ✓ Avoid faulty apparatus and careless behaviour
- ✓ Avoid direct contact with culture of pathogenic organisms
- ✓ Wash skin and contaminated clothing immediately
- ✓ Personnel hygiene of her/his own safety by following rules
- ✓ Never eat, drink, smoke in laboratory, food and drink must not be taken into or stored in laboratory.
- ✓ Hand washing and removal of protective clothing before leaving the laboratory.
- ✓ Don't put the microscope at edge of table

1.5. Method of cleaning and sterilization cleaning method

- ✓ Clean by tap water with soap or other detergent

Disinfection method

- ✓ Clean by using different disinfectant

Sterilization method

- ✓ Sterilize, by heat (moist/autoclave) or dry/dry oven/ or filtration.

1.6. Blood collection sites in animals for laboratory examinations

1.6.1. Jugular vein:

- ✓ The most commonly used sites in the horse, cattle, sheep, goat and camel
- ✓ Placement of the thumb of the left hand in the jugular furrow to occlude and anchor the jugular vein while manipulating the syringe and needle with the right hand
- ✓ Clipped and rubbed the site with alcohol

1.6.2. Cephalic vein:

To collect the small amount of blood in the dog. by the constrict the area on the dorsal aspect of the forelimbs at level of elbow, above the carpal vein.

1.6.3. Wing vein (used for birds)

After the feathers in the auxiliary region are plucked, the alar vein is seen running from beneath the pectoral muscle then along the ventral surface of humerus.

1.6.4. Tail vein:

Can be used in pig, cattle, and sheep.

Vein puncture- of the coccygeal vein on the ventral side

1.6.5. Mammary vein: used for dairy cattle

The vein appears at anterior border of the mammary gland lateral from the linea alba and run forward passing through a foramen in the abdominal wall posterior to ribs.

1.7. Collection of faecal samples

Fecal samples for parasitological examinations should be collected from the rectum of animals. If the fecal sample can't be obtained, fresh fecal sample may be collected from the pasture. Samples should be dispatched as soon as possible to a laboratory in suitable containers.

1.7.1. Procedure for fecal sample collection:

1) Wear gloves and lubricate the index and middle finger

2) Collect feces directly from the rectum of animal

3) Collect fresh uncontaminated feces from the ground only if collection from the rectum is not possible.

4) Preserve the feces by 10% formalin, +4^{o}c for 72 hours, 72% alcohol for adult parasites and -20^{o}c for 15 months.

5) Shake the feces in preservatives.

6) Notice feces for ova culture is not kept in preservatives.

2. QUALITATIVE TECHNIQUES FOR SEPARATING AND CONCENTRATING EGGS/LARVAE

A number of different methods are available for separating, concentrating and demonstrating eggs, oocytes and larvae in fecal samples. These include:

2.1. Gross examination of feces:

In the gross examination of feces we can appreciate **gross parasites** like: tape worm and nematodes by direct macroscopic examination of feces, **consistency of feces**: the condition of feces that is whether the feces are soft, watery (diarrheic), very hard (constipation) should be noted. This description should be varying with animal of species for example cattle feces are normally softer than those of horse and sheep, **color** e.g. light gray feces indicate excusive fat in the feces, clay color feces indicate bile obstruction, bloody feces are evidence of hemorrhage stomach or intestine, **mucus feces** indicate intestinal parasitism.

2.2. Direct fecal smear preparation (Direct microscopy examination)

2.2.1. Procedure:

1. Place a small drop of water on clean slide.
2. Add small fecal sample of about 3mm in diameter to the water and mix with a loop/tooth pick.
3. Cover with cover slip.
4. Examine first using the low power (10x) and then high dry power (40x) to confirm.

2.2.1.1. Advantage of direct smear

1. Require short time and minimal equipment.
2. Does not distort egg or larvae (nematode) unlike the other methods due to their effect by the concentrating media.

2.2.1.2. Disadvantage of direct smear

The amount of fecal sample is so small that is not represent a good sample size, therefore negative finding are inconclusive but positive results are just as valid as those obtained in more efficient concentration techniques.

This procedure leaves a lot of fecal debris on the slide which may confuse with nematode eggs.

2.2.2. Interpretation (this works actually for all qualitative techniques)

No eggs in the whole sample negative (-)

Few eggs in the whole sample moderate infection (+)

Few eggs in each microscopic field medium infection (++)

Many eggs in each microscopic field heavy infection (+++)

2.3. Simple floatation technique

This is done based on the separating of eggs from fecal material and concentrating them by means

of a flotation fluid within an appropriate specific gravity.

2.3.1. Principle of floatation technique

The basis of any flotation method is that when worm eggs are suspended in a liquid with a specific gravity higher than that of eggs, the latter will float up to surface. Specific gravity refers to the weight of an object for example the parasite eggs compared with the weight of an equal volume of pure water. Most parasitic eggs have a specific gravity between 1.1 and 1.2 g/ml whereas tap water is slightly higher than 1. Therefore the parasite eggs are too heavy to float on tap water. To make the eggs float a liquid with a higher specific gravity than that of eggs must be used. Such fluids are called flotation fluids and consist of concentrated sugar or various solutions which usually have specific gravity between 1.2 and 1.25. In this range fecal materials much of which have a specific gravity of 1.3 or greater, does not float. If the specific gravity below the desired range (1.2 to 1.25), add more reagents until the hydrometer indicates this range.

If the specific gravity is above 1.25 add water until the proper reading is obtained. But the problem of increasing the specific gravity to a higher level will distort the egg or cyst. In general this techniques which is based on the flotation principle work well for nematode and cestodes eggs and protozoan cyst but, fail to float some trematodes eggs. Trematodes eggs are much heavier and require a specific gravity of 1.30 to 1.35, whereas nematode and cestodes eggs float in a liquid with a specific gravity of between 1.10 and 1.20.

2.3.2. Procedure floatation technique

1. Put approximately 3g of faces in to baker or plastic container
2. Put 50ml of floatation fluid in the baker or plastic container containing 3g of faces.
3. Mix (stir) faces and flotation fluid thoroughly with a stirring device (tongue, blade and fork).
4. Pour the resulting fecal suspension through a tea strainer or double layer of cheese cloth into another beaker or plastic container.
5. Pour the fecal suspension in to test tube from the second container.
6. Place the test tube in the test tube rack.
7. Gently top up the test tube with the suspension, leaving a convex meniscus at the top of the tube and carefully place a cover slip on the top of the test tube.
8. Let the tube to stand for 20 minutes.
9. Carefully lift off the coverslip from the tube vertically together with drop of fluid adhering to it and immediately place the cover slip on the microscope slide and examine under microscope.

2.3.3. Preparation of common flotation fluid

1. Saturated salt solution:

Dissolve 350-400 grams of salt in 1000 ml of tap water /specific gravity is 1.18-1.2/

2. Saturated sugar solution:

The 454 grams of granulated sugar in 355 ml of hot water. Use low heat and stir continually. Finally add 2ml of formalin or phenol crystal to avoid the growth of mold. The specific gravity is 1.27.

3. Zinc sulphate solution:

Dissolve 331-385 grams in 1000ml of tap water /specific gravity is 1.18-1.2/.

Dissolve 703 grams in 1000ml of tape water /specific gravity 1.3/.

4. Zinc chloride solutions

Dissolve the 436 grams in 1000 ml of tap water /specific gravity 1.3/.

5. Magnesium sulphate solution:

Dissolve 400 milligrams in 1000 ml of tape water /specific gravity is 1.2/.

6. Sodium nitrate solution:

Dissolve 850 mg in 1000 ml of water /specific gravity is 1.4/

Dissolve 400 mg in 1000 ml of tap water/ specific gravity is 1.18/

NB most flotation fluids distort the shape of eggs and oocyst. Examine processed samples as quickly as possible.

2.4. Sedimentation techniques

2.4.1. Principle of Sedimentation techniques

This technique concentrates eggs in the sediment and primarily used to detect eggs or cysts that have too high specific gravity to float or these would be severely distorted by floatation fluid.

2.4.2. Procedure of Sedimentation techniques

1. Weigh or measure approximately 3g of feces in to bakers or plastic container.
2. Pour 40-50 ml of tap water.
3. Mix (stir) with stirring device (fork, tongue blade).
4. Filter the fecal suspension through a tea strainer or double layer of cheesecloth into another baker or plastic container.
5. Pour the filter material in to centrifuge tube ($3/4^{th}$ of test tube only).
6. Balance the centrifuge tube and centrifuge the sample at about 1500 rpm for 2 minutes. If the centrifuge is unavailable allow the mixture to stand undisturbed for 20 to 30 minutes.
7. Discard (pipette, descant) the supernatant carefully.
8. In case of trematodes- stain the sediment by adding one or two drop of 5% aqueous methylene blue. This contrast staining facility recognition of the yellow or colorless fluke eggs against the blue backgrounds of stained fecal particles under the microscopes.
9. Using a pipette and bulb transfer a small amount of the top of the layer of sediment to the microscope

slide. Cover with cover slip. If the drop is too thick, dilute it with a drop of water.
10. Then examine under 4x, 10x or 40x objectives. NB to obtain a clear smear resuspend or centrifugal repeatedly (once or twice). This can be repeated until the fecal debris is cleared.
2.4.3. Interpretation**:**

Fasciola hepatica and f. gigantica eggs are large golden yellow and contain zygote while paramphistomum is dark grayish in content. Paramphistomum eggs are large but colorless and contain zygote. Dicrocoelium eggs are small dark brown and contain miracidium these eggs sediment slowly thus diagnosing of low level of infection by this technique is uncertain.

2.4.3.1. Advantage

Sedimentation is more sensitive than direct smear in terms of the number of the number of organisms demonstrated and the slide is easier to read because much of fecal debris has been removed. Sedimentation is particularly appropriate for trematodes and acanthocephalan eggs, amoebas and ciliates. Sedimentation has greatest advantage in suspected trematodes (flukes) infections. Some laboratories increase the specific gravity of their flotation fluid to 1.3 to ensure the recovery of fluke eggs by the flotation technique. The problem with the use of flotation methods for recovery of fluke's eggs is that the eggs may be damaged by high concentration of the solution and become hard to identify.

2.4.3.2. Limitations

Sedimentation is less sensitive than flotation in concentrated sucrose or most nematode eggs and coccidian oocytes including cryptosporidium.

3. QUANTITATIVE FECAL EXAMINATIONS.

This used to determine the degree of parasitism based on the number of fecal eggs per gram of feces or larvae. All the procedures previously have been qualitative which means that they reveal whether the parasites are present or not. Quantitative procedures indicates the number of eggs or cyst, larvae present in each gram of feces.

3.1. McMaster method

This is used to determine the number of egg/eggs present per gram of feces (egg per gram).

3.1.1. Principle of McMaster method

The McMaster slide consist of two glasses or plastic slides joined together (by aquarium cement) between the marked areas of the upper and bottom slide, two chamber of 0.15ml volume (10*10*1.5mm) each are formed. The chambers are filled with a suspension of feces in the floatation solution. Nematode and cestodes eggs float to lie immediately below the upper glass of the chamber where they can be readily counted under the microscope while most fecal debris sinks to the bottom.

NB For qualification of trematodes eggs and nematode larvae a modified McMaster chamber has been developed.

3.1.2. Procedure of McMaster method

1. Weigh 3g of feces or if the feces are diarrheic, 3 teaspoonful
2. Break up thoroughly in 42 ml of water in a plastic container
3. Pour through a fine mesh sieve (aperture 0.15-0.25mm)
4. Collect, filtrate, agitate and fill a 15ml test tube.
5. Centrifuge at 2000 rpm for 2 minutes
6. pour off supernatant agitate sediment and fill tube to previous level with flotation fluid.
7. Invert the tube six times and remove fluid with pipette to fill both chambers of McMaster slide. Leave no fluid in the pipette or else pipette rapidly, since the eggs will rise quickly in the flotation fluid.
8. Allow the counting chamber to stand for 5 minutes (important!!)
9. Examine at 10x objective
10. Count all eggs, cyst or larvae with in the engraved area of both chambers.
11. To find the number eggs per gram of feces (epg) or larvae per gram of feces multiply the number under one etched area by 100 or two chambers by 50. If 3g of feces are dissolved in 42ml the total volume is 45ml, therefore, 1g is equivalent to 15ml. the volume under etched (each) area is 0.15ml. Therefore the number of eggs, cyst or larvae counted is multiply by 100 or if two chambers are counted it is multiplied by 50 to get the number of eggs per gram of feces.

3.1.3. Interpretation of McMaster method

Eggs are less than valuable in making judgment about the clinical condition of individuals animals, since many factors affect the accuracy of fecal egg count.

- ✓ The consistency of the fecal material may affect the epg markedly. The more watery the feces are the more the eggs are diluted.
- ✓ Species of the parasite their eggs production varies depending on the type of parasite.
- ✓ Immunity-this can suspended ovulation on the part of the worms.
- ✓ New infection (recent) – immature worms are unable to produce an eggs through in a number of species they are highly pathogenic. Therefore, the epg result should be interpreted carefully.

However it is generally considered that:

>1000 ……is heavy infection

>500……..……is moderate infection

Table: 1: Interpretation of egg in sheep and cattle.

Helminths	Epg levels indicative of intermediate and degree of infection.	
Mixed infection with unspecified GIT nematodes	Sheep	Cattle
	1000-2000	200-700
Oesophagostomum	1000-2000	200-700
Bunostomum	?	50-100
Haemonchus	2500-8000	200-700
Ostargia, Trichostrongyle	250-2000	100-500
Nematodirus	100-600	?
Fasciola	200-500	10-25

Source T. Kassai (1999): Veterinary Helminthology

For young animals (cattle)

	Light	moderate	heavy infections
Mixed infection	50-200	200-800	800+
Pure haemonchus	200	200-600	600+
Trichostrongylus	50-100	100-400	400+
Pure cooperia	200-300	300-2500	2500+

3.2. Modified Stoll's egg counting method

The stoll egg counting technique is a method for determining the numer of nematode eggs per gram of feces in order to estimate the worm burden in an animal. The advantage of this technique is that it requires no specialized equipment. The disadvantage is that the counting takes a long time because of the amount of extra (non-egg) materials on the slides.

3.2.1. Procedure Modified Stoll's egg counting method

1. Weigh out 3g of feces
2. Measure out 42ml of water and place it into a dish. This using a tongue depressor pushes 3g of feces through the sieve in to water. Lift the sieve and hold over the dish. Push out any remaining water from the feces.
3. While stirring the water-feces mixture take 0.15ml of suspension and spread over two slides. Cover each slide with a long cover slip (or 2 regular cover slips).
4. Examine both slides for worm eggs. The total amount or eggs counted times 100 represents the number of eggs per gram of feces.

3.2.2. Interpretation

The recent obtained by this method is as valid as that of McMaster egg counting technique.

3.3. Larval recovery, larval identification and larval count

Fecal culture: fecal culture is a method of incubating the eggs to hatch so that the larvae hatched can be identified according to the criteria given in the keys.

3.3.1. Interpretation

Used to differentiate parasites whose eggs and cysts cannot be distinguished by examination of fresh fecal sample and could be isolated from fecal sample, fecal culture and pasture.

3.3.2. Procedure

1. Take a certain amount of feces in a tray (the amount of feces required can be determined by the number of eggs found per gram of feces by running quantitative test first).
2. Moisten sample with water if too dry or add sterilized dry sheep feces or animal charcoal if two wet (N.B sheep feces may be sterilized using autoclave at 120 ^{o}c for one hour).
3. Incubate at ambient temperature for one week
4. Collect the larvae using Baerman's technique or other techniques mentioned below.

3.4. Baerman's technique

This is used to recover the larvae of nematodes from feces, soil and animal tissue.

3.4.1. Procedure of Baerman's technique

1. Take a funnel fitted to a stand.
2. Attach a rubber tube to the funnel with clamp on the lower ends
3. Fill the funnel with Luke worm water
4. Wrap the feces to be examined for larvae in double layer gauze.
5. Keep the wrapped feces on the tea strainer and lower it into the water in the funnel (the wrapped feces should be completely covered with water).
6. Keep a beaker under the funnel in case the rubber tube leaks.
7. Twenty four hours latter open the clamp and collect the aliquot in test tube.
8. Allow the larvae to settle at the bottom
9. Discard the supernatant and examine the sediment for larvae.

3.5. Isolating infective larvae from herbage

3.5.1. Principle

This is a qualitative and semi quantitative procedure for isolating, counting and identifying L_3 from representative samples of herbage taken from defined grazing areas. This procedure allows the estimation of larval availability on the pasture and can be used to define larval seasonality and distribution.

3.5.2. Equipment

1. Scissor and collecting bags
2. Small bucket (e.g. 7liter), calibrated at one-half liter graduations.
3. Guaze bag made from Terylene netting or cheese-cloth, long enough to fit over the rim of the bucket.
4. large plastic or glass filter funnel (20cm diameter), fitted with a length of flexible, transparent tubing carrying 2 screw clamps arranged so that about 15 ml can be trapped between the 2 clamps.
5. Domestic detergent and balance.

3.5.3. Procedure

1. Collect a small sample of grass from the large number of sites randomly scattered throughout the area to be sampled using a W" and N" Collecting route. Avoid heavy fecal contamination and avoid collecting soil.
2. Grass can be collected by hand. Where grasses are coarse, scissor, may be used. The grass collections of approximately 300-600 grams should be placed in a plastic bag.
3. Place the collected grass sample inside gauze bag and immerse the bag in water in a bucket but, keep the bag clear of the bottom of the bucket.
4. In the first 3-4 hours, remove drain and replace the bag in the bag in the water several times to agitate the sample. Leave the bag in the water at room temperature overnight.
5. The next morning, remove the bag and run fresh tap water over it and into bucket, leave the contents of the bucket to sediment for about 1 hour.
6. The bag of grass should be dried (sun/oven/incubator) and weighted when completely dry.
7. Carefully decant or syphon off the supernatant, leaving about 1 liter containing all the sediment.
8. Suspend the sediment and pour it into the large funnel but discard any heavy debris that sediments rapidly.
9. Leave the funnel to stand for another hour.
10. Close the top clamp and collect the trapped sediment with about 15 ml of fluid in test tube. Procedures (h) and (i) may need to be repeated when large volume of sediment have to be processed, or when only a small funnel is available. Leave to cool at 4^{o}c for at least 1 hour.
11. Siphon off the supernatant to leave 3-5 ml of which is stained with 3-5 drop of iodine for at least l hour. Counter stain with 3 drop of sodium thiosulphate.
12. Count and identify the parasite larvae seen. There will usually be many more non parasitic nematodes and larvae present than parasitic larvae and the count may therefore be rather laborious.
13. Parasitic larvae all have sheaths discernible at the tail end and tend to retain the brown colorations of iodine for 30-40 minutes.
14. Number of larvae per kg of dry herbage =count times 100/weight of dry herbage/in grams.

4. EXAMINATION TECHNIQUES FOR PROTOZOAN PARASITES.

Smears can be made from blood, brain crush, organ impression etc.

4.1. Blood smears

The materials needed to take blood from the ear vein of animal include: grease free slide, lancet, alcohol, cotton, and wool, a pair of scissor or sharp blade.

4.1.1. Procedure

- ✓ Cut the hair of the ear with a pair of scissor or sharp blade
- ✓ Clean the shaved area and disinfect with alcohol or other disinfectant
- ✓ Locate a small vein on the ear
- ✓ Prick the vein using a lancet or small sharp needle
- ✓ Take the first drop of blood and make thick thin smears as required
- ✓ Use the pencil to identify the smears when they are dry.

4.2. Thick blood smears:

The principle is to concentrate hemoparasites

1. Take a small drop of blood on clean grease free slide
2. Spread it to size of about 2cm in a diameter in such way that you can just read a script through
3. Air dries quickly so that it is protected from flies
4. Dehemoglobinize by gently running of distilled water on the smear by immersing the smear in distilled water for 5-10 minutes.
5. Fix with methylene alcohol for 2 minutes
6. Stain with giemsa diluted in buffered distilled water 1:10 for 30 minutes
7. Was with buffered distilled water till it assumes a blues purple color
8. Examine under the microscope (x4 and x100)

4.3. Thin blood smear:

Use to detect blood parasites, to study the morphology of RBC and WBCs etc.

4.3.1. Procedure

1. Take a small drop of blood on clean grease free slide

2. Spread the on the slide using cover slip or clean slide at angle of 45 degree
3. Dry it quickly and fix with methylene alcohol for 2 minutes
4. Stain with giemsa diluted 1:10 neutral phosphate buffer for 30 minutes

4.4. Wet film

Wet films are mostly made from blood to see motile organisms like trypanosomes. But wet films could be made from peritoneal fluids, aqueous and vitreous humour of the eye etc. But the morphology of the parasite cannot see, it is difficult identify the species.

- ✓ Take a drop of the blood or other body fluids on clean slide
- ✓ Apply a cover slip gently
- ✓ Look under the microscope (40x)

4.5. Buffy coat

4.5.1. Procedure:

1. Fill heparinized or citrated capillary tubes with blood from the animal to be examined.
2. Centrifuge the sample using hematocrit centrifuge.
3. Transfer the capillary tube on to a slide
4. Use a small adhesive tape to attach the tube on to a slide.
5. Examine the buffy coat in the capillary tube under the microscope.(The buffy coat is the grayish narrow space found between the plasma and the red blood cells in the capillary tube.) The motile organisms such as the trypanosomes are seen flickering at this junction.)
6. Cut the capillary tube at the junction between the buffy coat and the red blood cells. (More towards the red blood cells)
7. Blow the capillary tube containing the plasma, the Buffy coat and some red blood cells on a clean slide.
8. Make a smear of this content and stain with Giemsa to identify the organisms.

4.5.2. Interpretation**:**

Whitish foam is formed between PCV and plasma.

4.6. Pack Cell Volume

This technique is used for determine the blood volume; it is expressed as percentage or as a decimal fraction.

4.6.1. Procedure:

1) Mix the blood collected with anticoagulant by inversion of tube about 10 times
2) Draw blood 2/3 of the way up a micro-haematocrit capillary tube.
3) Wipe blood tip of capillary and seal the vacant end of the tube with special clay or by holding it in the flame of a burner.
4) Place the tubes on the head in slots with the open ends toward the center and sealed
5) Ends outwards.
6) Centrifuge for 4 to 5 minutes at 10,000 rpm.
7) Take out the capillary tube from the centrifuge and read on micro-haematocrit reader.

4.6.2. Examination:

- The capillary tube is placed in the groove in the plastic slide. Adjust the slide and the capillary tube.
- The line in the center is moved by a knob on the left so, that it just touches the top of the packed cells and the haematocrit is read where the line crosses the scale on the right.

4.6.3. Interpretation**:**

Normal hematocrit value/PCV value is 24-36%.

5. DETECTION OF ECTOPARASITE

5.1. Preservation of ticks

Collect ticks from infected animals with forceps or using thumb

Keep them in 70% ethanol

5.2. Lice

Collect lice from an animal or bird by pulling the hair or feather

Place the lice on slide

Examine under 10x if the lice is blood sucking, having relatively narrow heads with piercing moth part. Example **linognathus**

If the lice are biting lice, have a broader head with mouth parts adapted from biting. Example **damalina (reddish brown)**

5.3. Skin scraping for mites examination

5.3.1. Procedure:

- ➢ Place a drop of mineral oil on a slide.
- ➢ Clean the scalpel blade by wiping it with paper.
- ➢ Dip the clean scalpel blade into the drop of oil on the microscope slide.
- ➢ Pick up a fold of the patients skin at the **edge** of the suspected area pinching it firmly between the thumb and forefinger, Scrape the crest of the fold several times in the same direction with oily scalpel. Scraping will adhere to the blade.
- ➢ Stop scraping when a small amount of blood appears.
- ➢ Transfer the scraping from the scalpel blade into the drop of oil on the slide, using a slight rotary motion.
- ➢ Apply a cover glass to the scraping on the slide by gently lowering it by means of a cover glass forceps. (Additional oil may be added at the cover glass edge in order to fill the pace beneath it. Do not press on the cover glass.)

- Examine the preparation under low power (X10) in a methodical manner so that all portions of the cover glass area are seen. (oily preparations of mites may be kept for days as demonstration specimens. The mites show motion for many hours.)
- Boil the skin scraping in 10% potassium hydroxide solution to facilitate identification.

6. MAJOR BACTERIOLOGICAL EXAMINATIONS TECHNIQUES

- ✓ Methylene blue stain
- ✓ Giemsa stain
- ✓ Gram stain
- ✓ Direct microscopic examinations
- ✓ California mastitis test (CMT)

6.1. Light Microscopy and common staining techniques

6.1.1. Procedure to use your microscope effectively:

1. Turn the light *all the way on*.
2. Insert the blue filter.
3. Place a slide on the stage, and focus it as best you can with the low power lens.
4. Close down the illuminator diaphragm about half-way, until you see a circle of light.
5. Move the condenser assembly up and down until the edges of the diaphragm are sharp and clear.
6. Open the condenser diaphragm *all the way* to fully illuminate the field; if there is a coarse graininess to the field background, move the condenser down slightly to eliminate it.

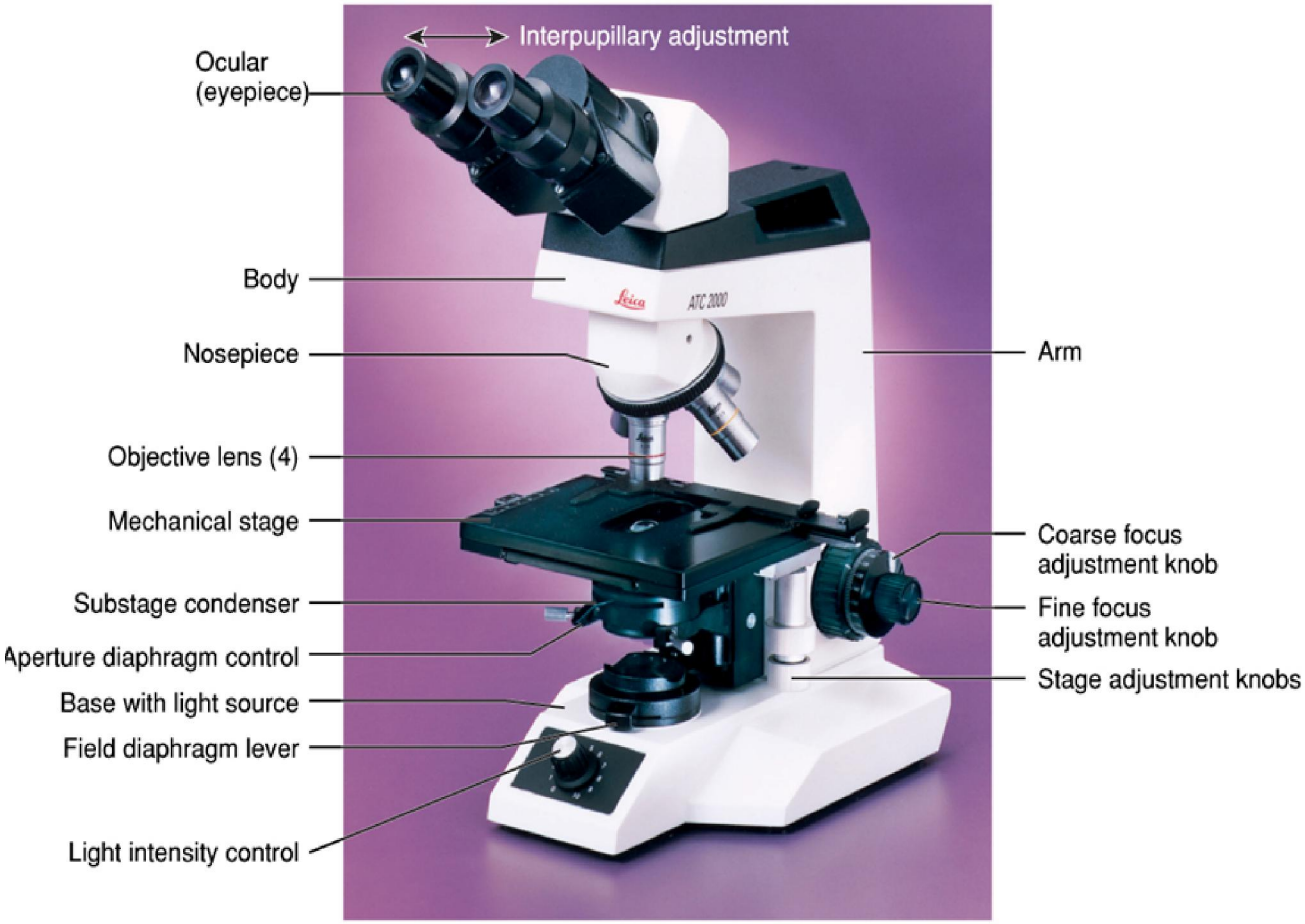
6.1.2. Principles of Light Microscopy

Two key characteristics of microscopes: **magnification** and **resolving power**

6.1.2.1. Magnification:

Results when visible light waves pass through a curved lens, The light experiences **refraction**. An image is formed by the refracted light when an object is placed a certain distance from the lens and is illuminated with light. The image is enlarged to a particular degree- the power of magnification. Magnification- occurs in two phases. 1) Objective lens- forms the **real image** and Ocular lens- forms the **virtual image**. Total power of magnification- the product of the power of the objective and the power of the ocular

Figure: 1. Compound microscope



6.1.2.2. Resolution

It is the ability to distinguish two adjacent objects or points from one another, also known as **resolving power**. Shorter wavelengths provide a better resolution. Oil immersion lenses increase the numerical aperture. **Numerical aperture**- describes the relative efficiency of a lens in bending light rays.

Magnification and Resolution: Increased magnification decreases the resolution**.** Adjusting the amount of light entering the condenser using an adjustable iris diaphragm or using special dyes help increase resolution at higher magnifications

6.2. Fixed and Stained Smears

Smear technique developed by Robert Koch. Spread a thin film made from a liquid suspension of cells and air-drying it. Heat the dried smear by a process called heat fixation and some cells are fixed using chemicals.

Staining creates contrast and allows features of the cells to stand out. Applies colored chemicals to specimens, Dyes become affixed to the cells through a chemical reaction and Positive and Negative Staining.

1) Positive staining (The dye sticks to the specimen to give it color)

2) Negative staining (The dye does not stick to the specimen, instead settles around its boundaries, creating a shape).

6.2.1. Simple Stains:

Require only a single dye. Examples include malachite green, crystal violet, basic fuchsin, and safranin. All cells appear the same color but can reveal shape, size, and arrangement.

6.2.2. Differential Stains:

Use two differently colored dyes, the primary dye and the counterstain. Distinguishes between cell types or parts. Examples include Gram, acid-fast, and endospore stains.

6.2.3. Differential Stains: Gram Stain:

Gram stain procedure was developed by **H. Christian Gram**. The most universal diagnostic of staining technique for bacteria, differentiation of the microbes as gram positive **(purple)** or gram negative **(red).**

6.2.3.1. The difference between Gram positive and Gram negative bacteria:

The difference is in the permeability of the cell wall to "purple iodine-dye complexes" when treated with decolorizing solvent. Gram positive bacteria **retain** purple iodine-dye complexes after the treatment with decolorizing solvent. Gram negative bacteria do **not retain** complexes when decolorized, so we use a red counterstain with safranin to observe Gram negative bacteria.

*N.B: This is because; the method is used for identification of Gram negative and Gram positive bacteria. The Gram positive retain the crystal violet colour or **blue** in colour. The Gram negative lose the crystal violet colour when are treated with a decolourizer and are counter stained by safranin appeared **red** in colour. This method is mostly used in bacteriological stain.*

6.2.3.2. Procedure of gram stain

- Prepare thin smear, allow it to dry and fix by heating
- Apply crystal violet solution for 1 minute and wash
- Replace lugols iodine for 1 minute and wash with tap water
- Decolorize for 1-2 seconds with absolute alcohol or acetone
- Wash with tap water
- Counter stain with dilute carbol- fuchsin for 30 seconds
- Wash in water, bloat, dry and examine uder oil immersion.
- N.B Gram positive bacteria result blue/puple color and gram negative bacteria result **red** color

6.2.4. Differential Stains: Acid-Fast Staining (Zielh Neelsen):

- ✓ Important diagnostic stain.
- ✓ Differentiates acid-fast bacteria (pink) from non-acid-fast bacteria (blue).
- ✓ *Mycobacterium tubercolosis,* the etiologic agent of tuberculosis, is the most common pathogen of this group.
- ✓ Other microoganisms, particularly the *Nocardia,* can be identified by their acid-fast characteristic.
- ✓ The term acid-fast is derived from the resistance displayed by acid-fast bacteria to decolorization by acid once they have been stained by another dye.
- ✓ Cell wall contains fatty acids and phospholipids responsible for acid fast stain
- ✓ Important diagnostic stain
- ✓ Differentiates acid-fast bacteria **(pink)** from non-acid-fast bacteria **(blue**)
- ✓ Important in medical microbiology

6.2.5. Differential Stains: Endospore Stain

- ➢ Dye is forced by heat into resistant bodies called spores or endospores
- ➢ Distinguishes between the stores and the cells they come from (the vegetative cells)
- ➢ Significant in medical microbiology

6.2.6. Special Stains

- ✓ Used to emphasize certain cell parts that are not revealed by conventional staining methods
- ✓ Examples: capsule staining, flagellar staining

6.3. California mastitis test (CMT)

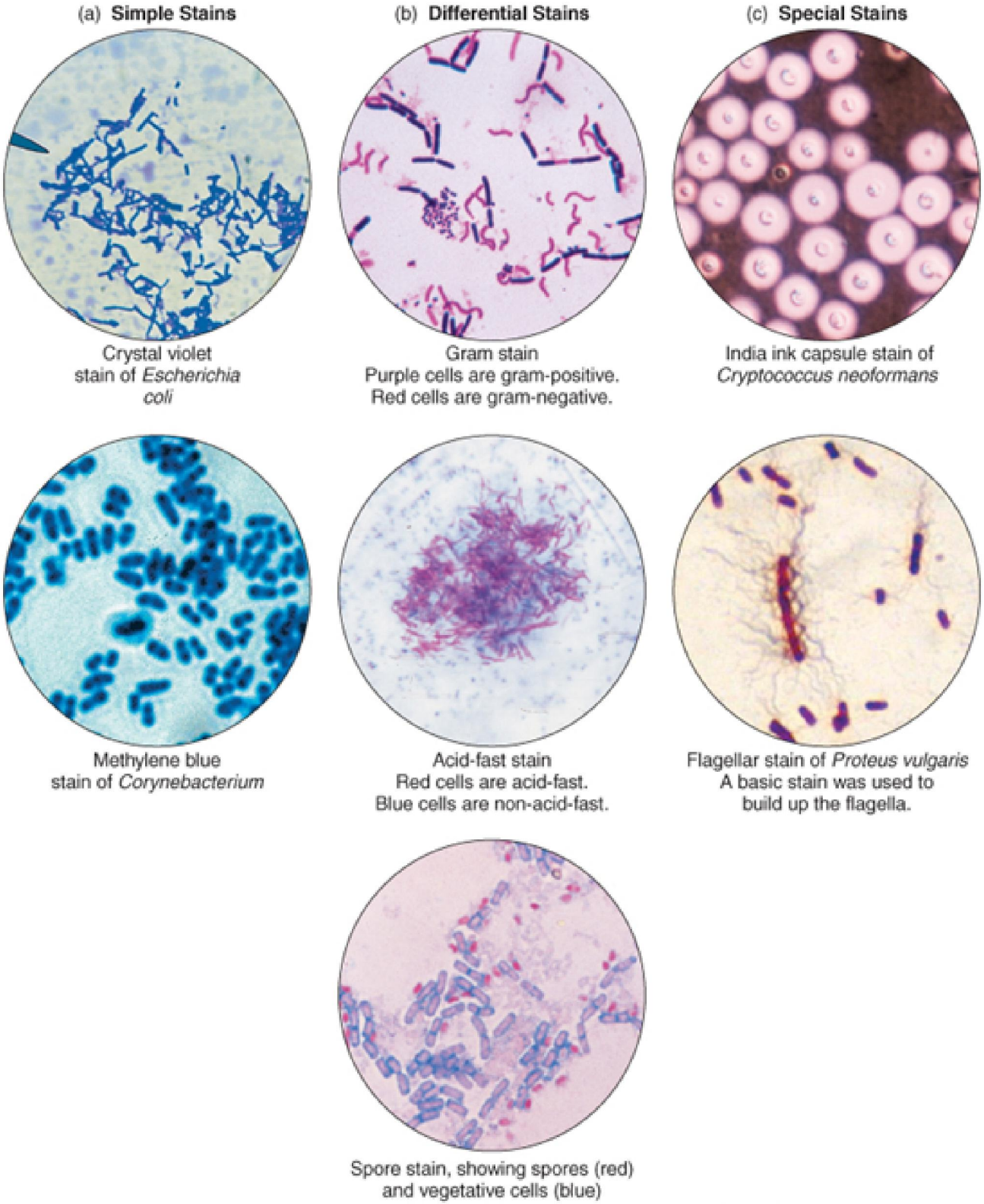
- ➢ Take a small amount of milk sample in the test tube
- ➢ Add a drops of CMT reagent on the paddle
- ➢ Add a milk sample and then mix thoroughly.

N.B reddish colour of milk indicate positive

6.4. Rose Bengal plate test (RBPT)

- ✓ Drawn blood from jugular vein with sterile needle and vaccutainer tube
- ✓ Left the blood at room temperature to clot for maximum hours not exposed to sunlight or centrifuge at 2500 rpm for 5 minutes
- ✓ Decant 3-4 ml of serum into vial
- ✓ Label clearly and place on wet ice box (-4^{o}c)
- ✓ Leave the antigen and sera at room temperature, for 0.5-1 hour before test
- ✓ Place one drop of serum by holding the dispenser upright and one drop of antigen on white tile alongside (but not into serum) of serum sample
- ✓ Mix antigen and serum thoroughly with wooden applicator
- ✓ Rock backward and forward gently for up to 4 minutes
- ✓ Read the test by examining agglutination in good light (view box)

Figure: 2: stain of gram positive and gram negative



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ANNEX: 1: SHAPE OF MITES AND EGGS OF HELMINTH PARASITES

Figure female of the genus *Demodex,* drawn to show the diagnostic features.

Figure of female and male mites of the genus *Sarcoptes*.

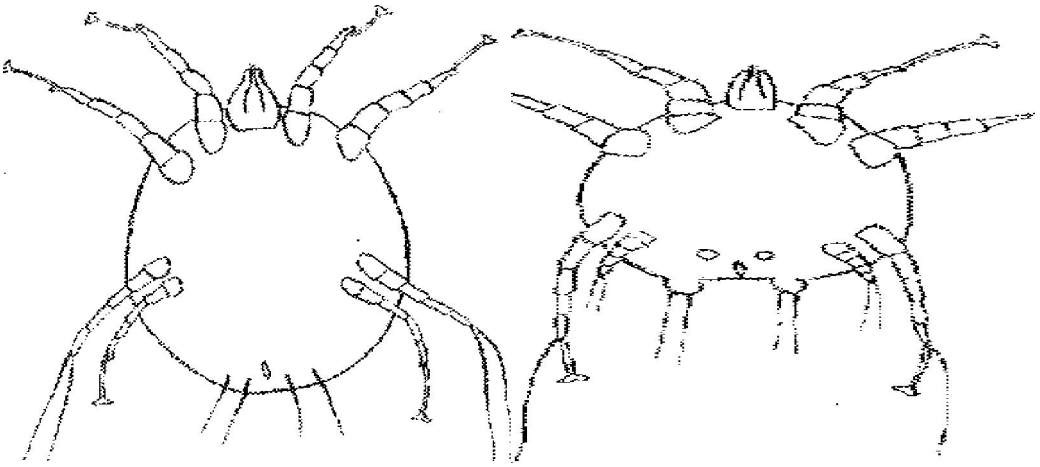
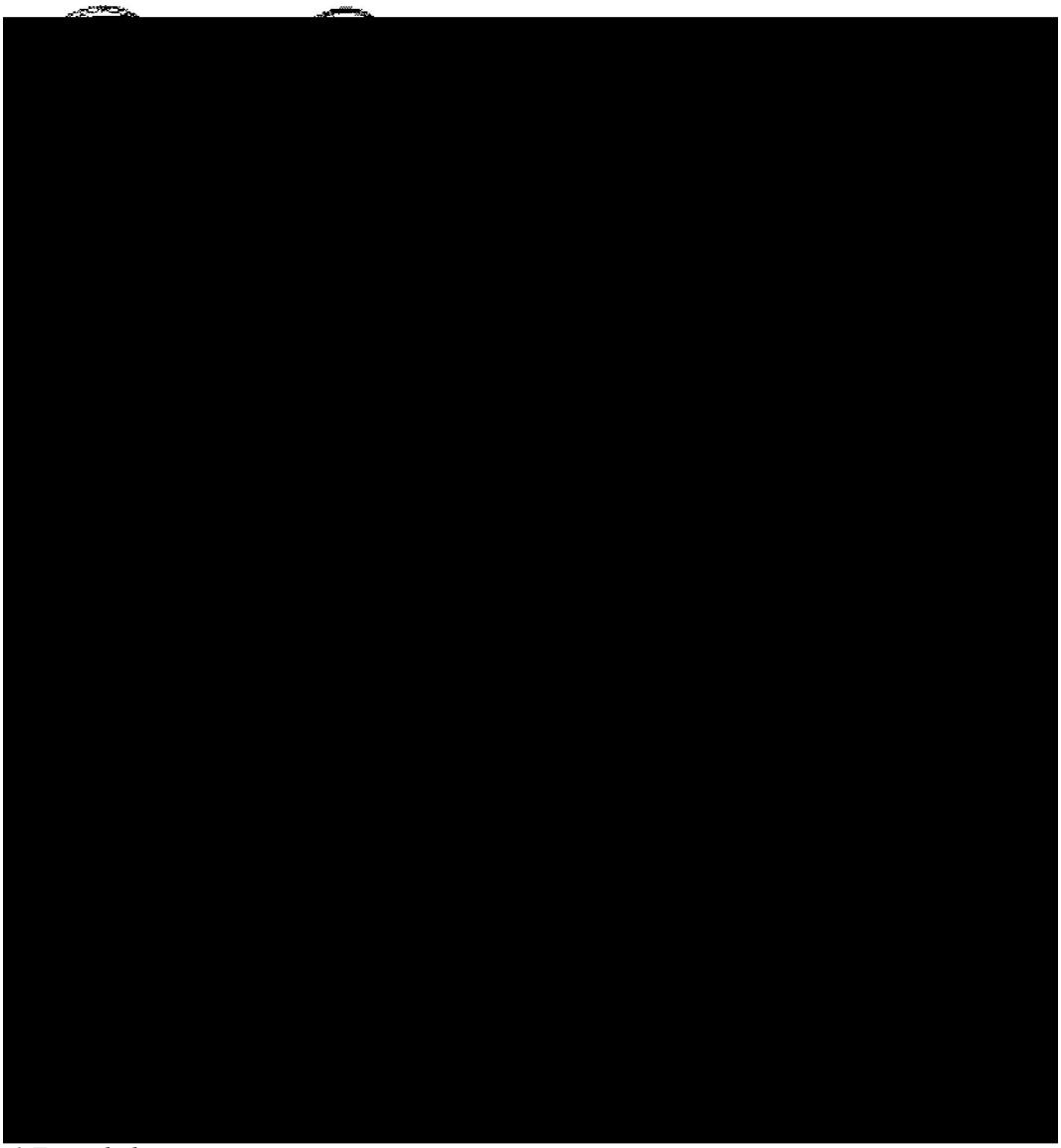


Figure of female and male mites of the genus *Psoropie*.



1.*Fasciola hepatica*
2.*Paramphistomum cervi*
3.*Thysaniezia giardi*
4.*Moniezia expans a*
5. *Moniezia benedeni*
6. *Deirocoelium dendi itic um*
7.*Strongyloides papilosuc*
8. *Go'i l o n e m a pulchrum*
9.*Trichuris globulos a*
10.*Fesciola gigant[i]ca*
11.*Nemato,hus spathigzr*
12.*Gaigeria pachyscelis*
13 *Trichostrongylus spp.*
14 *Skrjabinema ovis*
15 *Avitellina centripunctata*
16 *Chabertia ovina*
17 *Haemonchus contortus*
18 *Bunoistotnum trigonocephalum*
19 *Oesophagostomum colwnbianum*
20 *Cotylophoron colaphorum*
21 *Fascioloides magna*
22 *Ostertagia circumc Meta*
23 *Marshalagia mars halli*

Figure of Eggs of worm parasites *of sheep*

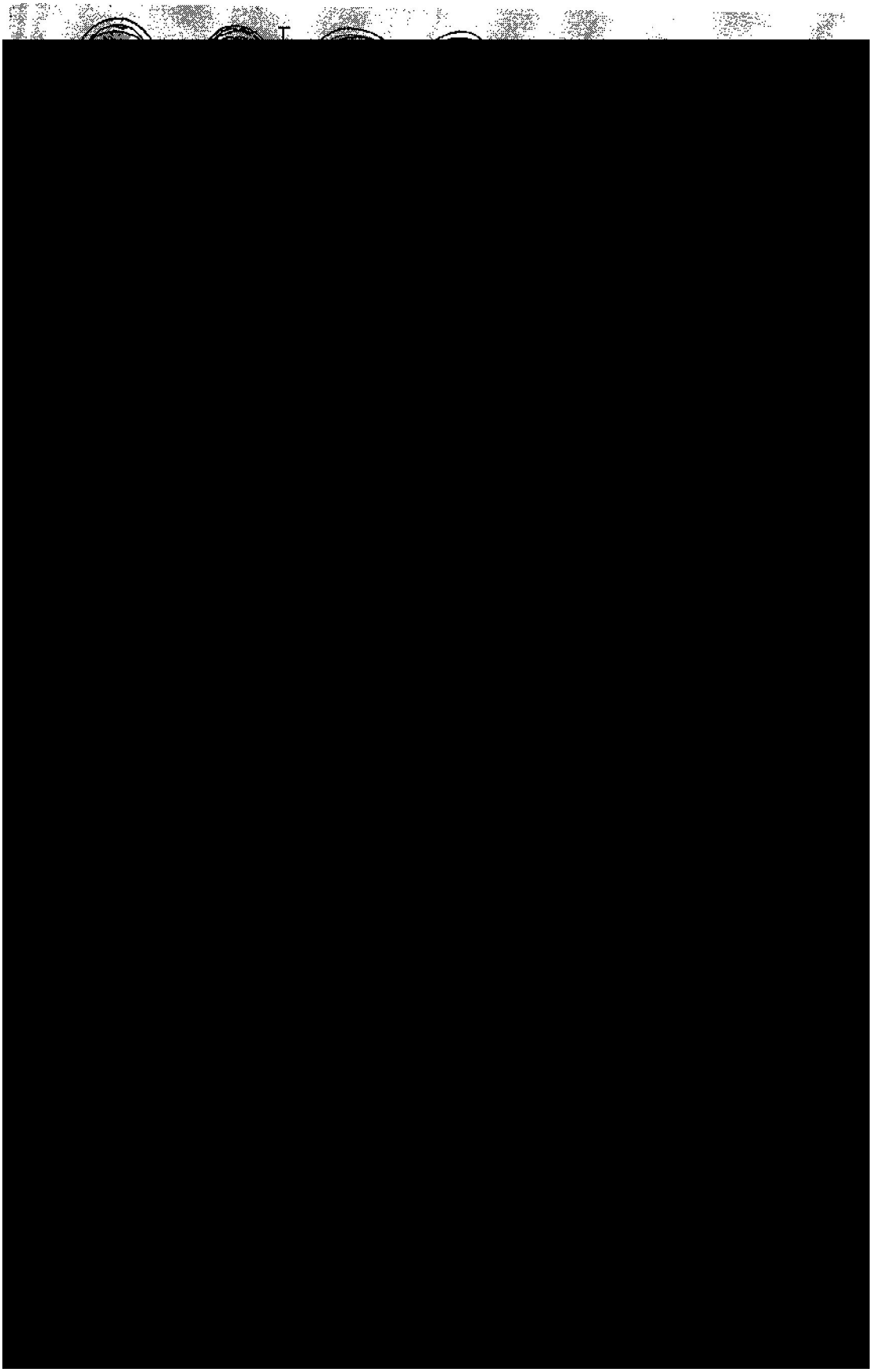


Figure of eggs of worm parasites of dog and fox

1. Toxocara canis
2.. Toxoascaris leonina
3. Dyplidium caninum
4. Linguatula serrata
5. Ancylostoma caninum
6. Ancylostoma brazilense
7. Spirocerca lupi
8. Dioctophyma renale
9. Mesocestoides lineatus
10. Diphylobothrium latum
11. Euryhelmis squamula
12. Echinococcus granulosus
13. Taenia hydatigena
14. Taenia ovis
15. Uncinaria stenocephala
16. Necator americanus
17. Oncicola canis
18. Troglotrema salmincola
19. Physaloptera canis
20. Trichuris vulpis
21. Capillaria plica
22. Capillaria aerophila
23. Filaroides osleri

8/19/2022