Techniques

Direct Wet Mount Fecal Exam

1. Place a small amount of feces on a microscope slide.

2. Add a drop of liquid to the feces and mix thoroughly. The type of liquid added depends on what you hope to accomplish with the technique. If you are examining a liquid fecal sample for the presence of protozoan trophozoites (live active protozoa) then use saline (if any extra liquid is needed). If you are looking for helminth eggs and protozoan cysts in a small sample (bird droppings, rectal smear, etc) then either water or iodine may be used.

3. Cover with a cover slip. Move the cover slip around until it lays flat. You should be able to read through the smear (light from the microscope must be able to pass through the sample in order for you to examine it).

4. Examine the slide using the 10X objective, and then go over it with the 40X objective.

Because this technique examines only a very small amount of feces, it should only be used in the following circumstances:

a. Liquid feces where protozoan trophozoites may be present.

b. Fecal samples where the amount of feces obtained is too small to handle with any other technique.

c. As an adjunct to a flotation technique where you are looking for eggs that do not float. (In this case you probably would be better off running an ethyl acetate sedimentation and then examining the resultant pellet using the direct wet mount method.)

Note: Circumstances "b" and "c" occur frequently when dealing with small fish, birds, amphibians and reptiles and thus the direct wet mount has some utility in dealing with fecal samples from these animals.
The OVASSAY PLUS Kits used in today’s lab were a gift from Synbiotics Corp., San Diego, CA.
The Fecalyzer units used in this lab were donated by EVSCO Pharmaceuticals, Buena, NJ (a division of Vétoquinol).
ZINC SULFATE CENTRIFUGAL FLOTATION METHOD

1. Fill a 15 ml centrifuge tube with ZnSO4 solution (1.18 specific gravity)\(^1\) and pour into a glass dish or plastic specimen cup.

2. Using a tongue depressor, push the feces (2 to 5 grams, a piece the size of a large grape) through the strainer into the ZnSO4 solution in the dish. **TIPS:** 1. **The sieve must be in the liquid in order for the feces to be passed through.** 2. **The more feces you use, the more likely you will be able to find eggs which are present in low numbers.**

3. Using a funnel, pour the ZnSO4-fecal mixture back into the centrifuge tube.

4. Centrifuge for 2 min at high speed (1500 - 2000 rpm).

5. Using a headed-rod or loop (see pg 6 for instructions), remove a sample from the surface of the solution and place on a microscope slide. (Make 2 or 3 dips with the rod or loop to get enough material to examine, you want the equivalent of a large drop on the slide.) Add a drop of iodine\(^2\) (to stain the cysts and ova) and a coverslip. Examine at 10X and then part of slide at 40X.

**TIP:** To increase the sensitivity of this technique either use more feces or do the following:

After removing the tube from the centrifuge, fill the tube with ZnSO4 to just over the top of the tube, place a coverslip over the top of the tube and wait 10 min. Place a drop of iodine on a slide and place the coverslip onto the drop of iodine and examine at 10X. This modification also allows you to skip using the loop or headed rod to obtain your sample, and thus may be easier to do at a veterinary practice. OR place a cover slip on the tube before centrifuging (see hints on the next page).

**TIP:** If the sample contains a large amount of fat or other material that floats in water, you may want to wash the sample before doing the flotation. To do this, start at step 1 but use water instead of ZnSO4. When you centrifuge the water-fecal mixture, the eggs, being heavier than water, will sink but the fat and other material will float. After centrifugation pour off the supernatant, add the ZnSO4 solution and mix well. Centrifuge as in step 4 and examine as in step 5. OR use the ethyl acetate sedimentation technique (pg. 8) to get rid of the fat.
Solutions for ZnSO4 Centrifuge Flotation Technique

1. **ZnSO4 solution** (1.18 sp. Gr.) is made by adding 386 grams of ZnSO4 to 1 liter of water. The mixture should be checked with a hydrometer and adjusted to 1.18. The ZnSO4 solution should be stored tightly capped to prevent evaporation (and the resulting change in the specific gravity of the solution).

2. **Iodine solution**: 10 gms Potassium Iodide (KI) added to 1 liter of distilled H₂O. Shake to dissolve. Add 10 gms of Iodine (I₂) to the above solution. Allow to stand over-night with stirring, at this time you may still have Iodine crystals at the bottom, this is OK, just leave them there. This solution will stain (and kill) most parasite eggs and cysts (coccidial oocysts are an exception, they do not take in the iodine). Iodine will also turn starch blue, so a quick test for amylase insufficiency (pancreatitis) is to add a drop of iodine to a small amount of feces on a slide, mix and observe for a color change over a white piece of paper (keep in mind that some kitty litter is made of corn cobs (starch)).
Use of a loop to recover parasites from a ZnSO4 centrifugation float.

Correct technique:

1. Place loop into ZnSO4.
2. Gently break the float.
3. Loop forms inside loop.
4. Loops release loop.
5. Transfer under the microscope.
6. Repeat steps 2 to 5, until you have enough.
7. Apply a coverslip and seal.

Common mistakes:

1. Loop forms outside loop.
2. Loops do not release loop.
3. Loops break before releasing loop.
4. Loops break because of ZnSO4.

Proper placement of loop:

- Place loop into ZnSO4.
- Gently break the float.
- Loop forms inside loop.
- Loops release loop.
- Transfer under the microscope.
- Repeat steps 2 to 5, until you have enough.
Many veterinary customers are using the Straight 8 series for fecal floatation. The ova recovery rate is much higher using the centrifuge method where a coverslip is placed directly onto the top of a tube while spinning. The ova, being lighter than the floatation solution, are forced upward onto the glass of the coverslip. After spinning, simply remove the coverslip and place directly onto your microscope slide.

Using fecal floatation solution in both the sample tube and the balance tube, fill the tube to the top with a slight bulging meniscus over the top. Then place a coverslip onto the top of the tube, and tap it down with your finger to seat it on the rim.

It is fine to have a small bubble beneath the coverslip, but make sure that mostly liquid is contacting the coverslip.

Place the tube into the centrifuge.

**Bulging Meniscus**

**Note:** Fecal Floatation solution is much heavier than water, and must be used for balancing. Using a water tube will cause the centrifuge to spin off balance, causing coverslips to break or damage to the centrifuge.

Make sure that the coverslip is turned so that the edge is parallel to the edge of the rotor.

If the corner of the coverslip is pointing towards the center nut, then it will contact the flat metal portion of the rotor when spinning horizontal and it will fall off.

**Incorrect**

**Correct**

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Be careful using this technique - if one coverslip comes off or if it breaks (use the heavier coverslips for this method) it may throw the centrifuge out of balance and cause the coverslips on the other tubes to come off.
ETHYL ACETATE SEDIMENTATION
(For Fat Extraction Prior to ZnSO₄ Centrifugal Flotation)

1. Pass a grape-sized piece of feces through a sieve into about 9 ml of water and pour into a 15 ml centrifuge tube.

2. Add about 3 ml of ethyl acetate, plug the tube with a rubber stopper and shake the tube vigorously. **CAUTION**: Test materials before placing Ethyl Acetate into them. This solvent will dissolve many types of plastic!! The white plastic centrifuge tubes used in the lab are OK, but clear hard plastic tubes and the disposable polystyrene cups will dissolve.

3. Remove the rubber stopper and centrifuge the tube (1500-2500 rpm) for 1 to 2 minutes.

4. Using a stick, "ring" the plug of fat at the water - ethyl acetate interface (the plug adheres to the side of the tube and must be detached before the liquid contents of the tube can be poured off).

5. Pour off the supernatant, being careful to leave the pellet at the bottom of the tube intact. (Flush the ethyl acetate down the sink with plenty of water.)

6. Resuspend the pellet in distilled water, centrifuge, then pour off the water (this removes any leftover fat and ethyl acetate). Resuspend the pellet in flotation solution, centrifuge again, and remove the material from the top of the float to examine for eggs (see ZnSO₄ technique on Lab 1 Pg. 9).

When removed from centrifuge, your tube will have clearly defined layers:

A. An ethyl acetate layer on top.
B. A plug of dissolved fat in the middle.
C. A layer of water.
D. A pellet of sediment at the bottom.
THE BAERMANN TECHNIQUE

In 1917, while working in Java, the Dutch physician Dr. Baermann developed a simple method for isolating nematodes from soil. Today veterinarians use his method for the extraction of live larval stages of nematode parasites from feces.

Technique (modified for use in the office lab):

1) Place a sieve in a custard dish or other similar container.
2) Spread about 10 grams of feces* on a piece of tissue paper and place it into the sieve.
3) Place warm* * water in the custard dish until it just covers the feces, taking care not to disrupt the feces.
4) Allow to sit for at least one hour.†
5) Lift off sieve.
6) Pour liquid into a 50 ml centrifuge tube.
7) Let sit for 20 minutes.
8) Using a Pasteur pipet, remove a drop of the sediment at the bottom of the tube and place it on a microscope slide for examination. (Be careful not to resuspend the sediment before you take a sample from it.)

* Use fresh feces - refrigeration may kill Strongyloides stercoralis larvae.
** This technique makes use of two characteristics of parasitic larval nematode behavior:
1) The warmer it is, the more active the larva (up to a point, you don’t want to cook them!; 37 to 40°C is as warm as you want to get), and, in addition, some larvae are thermotaxtic and will move towards the warmer water under the filter paper.
2) Most parasitic larval nematodes are poor swimmers.

Therefore, the following events take place when the sieve is placed in the water:
The larvae will be moving around in a random fashion and within any given time interval some of them will migrate through the tissue and fall into the water. Because they can’t swim they sink to the bottom and over time a number accumulate there. The more active the larvae are (i.e. the warmer the water) the greater the number of larvae that accumulate at the bottom in a given time interval.

† The longer you wait, the more larvae will fall to the bottom of the dish, but with time, the fecal sample breaks down and begins to pass through the tissue leading to an accumulation of sediment along with the larvae.
STOLL EGG COUNTING TECHNIQUE
A method for determining the number 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 the counting takes a long time because of the amount of extra (non-egg) material on the slides.

1. Weigh out 3 grams of feces.
2. Measure out 42 ml of water and place it into a dish. Using a tongue depressor, push the 3 grams of feces through a sieve into the 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 0.15 ml of the suspension and spread over 2 slides. Cover each slide with a long coverslip (or 2 regular size coverslips).
4. Examine both slides for worm eggs, the total number of eggs counted X 100 represents the number of eggs per gram of feces.

5. The mathematics: 0.15 ml is 1/300 of 45 ml (42 ml water and 3 gm feces) so the number of eggs in 0.15 ml X 100 is equal to 1/3 of the total number of eggs in the original 3 grams and thus equal to eggs per gram (EPG).

McMASTER EGG COUNTING TECHNIQUE
This is another method for determining the number of nematode eggs per gram of feces in order to estimate the worm burden in an animal. The advantage of this method is it is quick as the eggs are floated free of debris before counting, the disadvantages are you must use a special counting chamber and it has a detection limit of 100 EPG (unless multiple counts are done on the same sample or more feces is added to the same volume of flotation solution).

1. Weigh out 2 grams of feces.
2. Pass the feces through a sieve into a dish containing 60 ml of ZnSO4 or saturated salt solution. Lift the sieve and hold over the dish. Push out any remaining solution from the feces.
3. While mixing vigorously (you may want to put the solution into a flask to prevent spillage) take a sample of the mixture with a pipette and transfer it to one of the chambers of the McMaster slide. Repeat the procedure and fill the other chamber.
4. Wait 30 sec, then count the total number of eggs under both of the etched areas on the slide. Use your 10X objective (first check to see that this objective can be swung into place without hitting the slide, if it hits the slide, count with the 4X lens). Focus first on the etched lines of the grid, then go down a tiny bit, the eggs will be floating just below the top of the chamber. Multiply the total number of eggs in the 2 chambers by 100, this is the eggs per gram (EPG).

5. The mathematics: The volume under the etched area of each chamber is 0.15 ml (the etched area is 1 cm X 1 cm and the chamber is 0.15 cm deep) so the total volume examined is 0.3 ml. This is 1/200 of 60 ml. Since you started with 2 gms of feces and then multiplied by 100, the final result is eggs per gram of feces.
Modified Wisconsin Sugar Flotation Method

This method of determining the EPG is probably the most commonly used method. (First used by the University of Wisconsin’s Parasitology Laboratory, it is a modification of the Stoll technique.) It is the most accurate as it counts all the eggs in 3 grams of feces and because it is a flotation method it has little debris to interfere with the count. However, if the EPG is high, there may be too many eggs to count.

1. Fill a 15 ml test tube with 10 ml of Sheather’s* solution.

2. Weigh 3 grams of feces and place into a cup.

3. Pour the Sheather’s* solution from the test tube into the cup and mix well.

4. Place a funnel into the test tube, place a strainer into the funnel and pour the fecal-sugar solution mixture through the strainer into the test tube. Using a tongue depressor, squeeze the liquid out of the feces that is left in the strainer.

5. Centrifuge the tube for 2 to 4 minutes.

6. Fill the tube to just over the top with more Sheather’s solution and place a cover slip onto the meniscus.

7. Let sit for about 5 minutes, then remove the cover slip and place on a slide.

8. Examine the entire cover slip and count the number of eggs that you find.

9. The number of eggs counted is the number per 3 grams of feces, so divide by 3 to find the EPG.

* Sheather’s Solution: Add 454 gm (1 lb) of table sugar to 355 ml of very hot water. Stir until dissolved and allow to cool. This solution will grow mold if left out, so keep refrigerated and use quickly. Some people add 6 ml of formaldehyde to the solution to preserve it.
Objects Sometimes Mistaken for Helminth Eggs and Protozoan Cysts

- Plant cells
- Plant hair
- Seed fibers
- Starch granules
- Protein particles
- Vegetable cells
- Fat droplets
- Oil droplets
- Air bubbles
- Scratches on slide
- Plant spring cells
- Pollen grains
- Fungus and yeast spores
**Blood**

Collected for two basic parasitologic procedures:

1. Smears - to detect protozoal and rickettsial infections (e.g., *Trypanosoma*, *Babesia*, *Anaplasma*). Smears must be fixed and stained to reveal organisms.
2. Concentration - to detect microfilaria (i.e., *Dirofilaria* and *Dipetalonema*)

If blood is not to be processed immediately upon removal from the patient, an anticoagulant must be added to the sample. Among those commonly used:
   a. Heparin - effect lasts only for a matter of hours
   b. EDTA - effect lasts several days

**Procedure for making Blood smears (thin films):**

a. Clean slide by wiping with alcohol. Handle slides by edges only. (Any grease on the slide will cause the dried blood to flake off during staining).

b. Place a very small drop of blood near the end of a slide.

c. Place the end of another slide (the "spreader") on the sample slide so that the edge of the spreader is just ahead of the drop of blood.

d. Holding the spreader at an angle of about 30° (relative to the sample slide), draw it back until its edge just touches the drop of blood. The blood will then run along the entire edge of the spreader slide.

e. Push the spreader briskly in one fluid motion completely across the sample slide. Note that the blood is being dragged behind the spreader, not pushed in front of it. (There is a video of this procedure on the lab’s web site.)

f. If the correct amount of blood was applied, the smear should end before the end of the slide, and the smear should end in a "feathered edge," a region where the blood cells are well separated.

g. Air dry.

h. Fixation and staining - various methods can be used. Normally a commercial staining kit is utilized following the manufacturer’s instructions.

2. **Procedures for concentration of blood (Knott and Filter tests):**

There are several reasons for using one of the concentration techniques in the laboratory examination of dog blood for microfilariae. Probably the main reason for using a concentration method vs. the direct smear is that more than 25% of the positive cases may be missed if the direct smear is the only method used. Secondly, a concentration method that kills the microfilariae allows easy differentiation between *Dirofilaria immitis* and *Dipetalonema reconditum*. Also, keep in mind that certain anthelmintics can kill heartworm microfilariae in a way that may lead to the death of an infected dog and thus you must be sure the dog is microfilariae free (not just Ag negative) before treating the animal with these drugs.

The two acceptable concentration methods most commonly employed in practitioners’ laboratories are:
A. **Modified Knott Method** (also known as the Knott Test)


1. Add 1 ml freshly-drawn blood to 9 ml 2% formalin (aqueous) in a centrifuge tube.
2. Mix well to lyse red blood cells.
3. Centrifuge for 5 minutes at 1500 rpm.
4. Pour off supernatant fluid. Note: Invert the tube completely when decanting the supernatant. Remember, the blood sample you are using is dilute so you won't see a large pellet.
5. Add a drop of 0.1% aqueous methylene blue. (Adjust the amount to suit yourself; it stains the microfilariae blue and makes them much easier to see.) Then stir or mix up the sediment in the bottom of the tube.
6. Mix again and place a drop of the stained mixture on a microscope slide and add a cover slip.
7. Examine under a microscope.

<table>
<thead>
<tr>
<th>Microfilariae of:</th>
<th><em>Dirofilaria immitis</em></th>
<th><em>Dipetalonema reconditum</em></th>
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<tbody>
<tr>
<td><strong>Numbers</strong></td>
<td>May exceed $2 \times 10^4$ ml$^{-1}$</td>
<td>Usually $&lt; 10^3$ ml$^{-1}$</td>
</tr>
<tr>
<td><strong>Length</strong></td>
<td>$&gt; 300$ microns</td>
<td>$&lt; 300$ microns</td>
</tr>
<tr>
<td><strong>Width</strong></td>
<td>6.7 - 6.9 microns</td>
<td>4.7 - 5.8 microns</td>
</tr>
<tr>
<td><strong>Anterior End</strong></td>
<td>slightly tapered</td>
<td>blunt</td>
</tr>
<tr>
<td></td>
<td>(cone on a cylinder)</td>
<td>(hemisphere on a cylinder)</td>
</tr>
<tr>
<td><strong>Posterior End</strong></td>
<td>straight (usually; may vary)</td>
<td>hooked (usually; may vary)</td>
</tr>
</tbody>
</table>

**NOTE:** As a further modification, a microfilaria count can be made if a measured amount of the stained mixture is counted. Although it is only a generality, *D. immitis* microfilaremias are often characterized by having high concentrations of microfilariae, whereas *D. reconditum* microfilariae are often found in low concentrations.
B) Filtration Method

1. Collect a 1 ml blood sample into EDTA or heparin and add to 10 ml lysing solution within a syringe. Mix thoroughly. (Lysing solution consists of 5.0 ml Triton X-100, 8.0 grams NaCO₃, 1 liter water.)

2. Attach syringe to a filter unit (see drawing). The lysed blood solution is pushed through an 8 µm pore filter membrane.

3. Remove the filter from the filter holder, place it on a microscope slide and add one drop of 1:10,000 Methylene Blue Stain. Cover filter with a cover glass and examine under microscope.
C. Miscellaneous

It is frequently difficult to distinguish microfilariae of *D. immitis* from microfilariae of *D. reconditum* using the morphologic characteristics outlined above. More definitive techniques for differentiation are available, but they are not usually practical for routine use in the practitioner's laboratory.

The first technique employs a histochemical (acid phosphatase) stain of microfilariae. *D. immitis* stain positive in certain zones only and *D. reconditum* stain over the entire microfilariae. See *J. Am. Vet. Med. Assoc.* **158**:601-605, 1971 or consult a parasitologist.

The second technique exploits the fact that *D. reconditum* microfilariae have a cephalic hook and *D. immitis* microfilariae do not. Again, since this technique requires good microscopic capability, it may not be suited for routine use. See *Proc. Helminthol. Soc. Wash.* **32**(1):15-20, 1965, or Bowman’s Georgi's *Parasitology for Veterinarians* or consult a parasitologist.
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