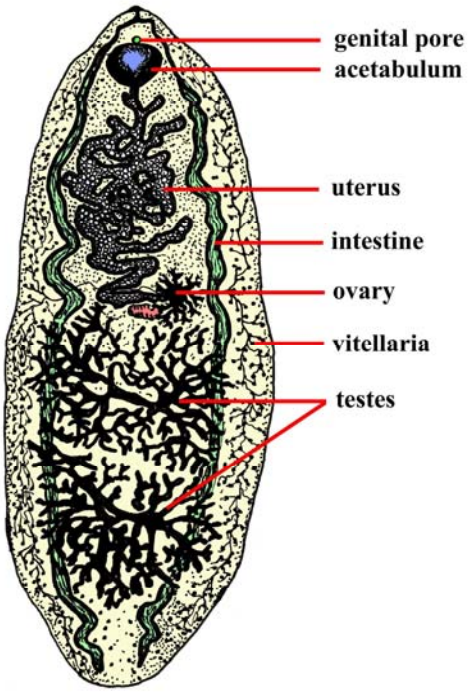
Animal Parasitology, Biology 625

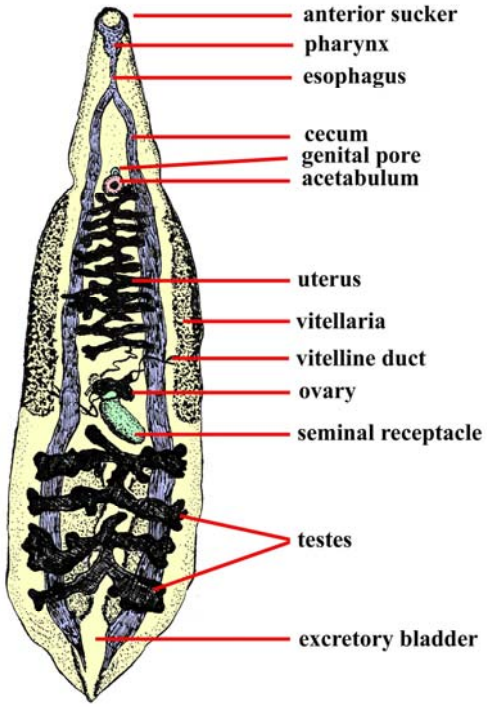
LABORATORY MANUAL *(.pdf version)*

Fall semester 2005

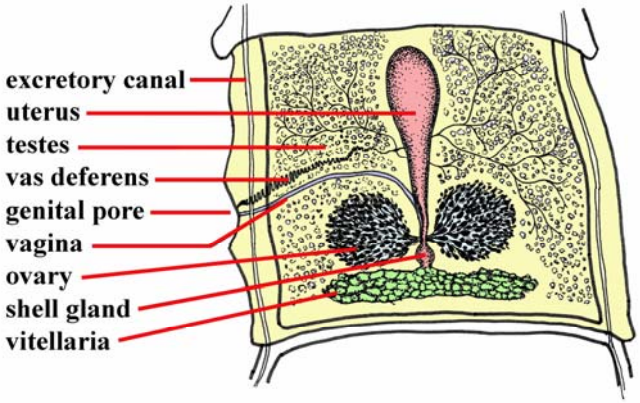
Tuesdays 02:30-5:20; Ackert Rm 226



Fasciolopsis buski



Clonorchis sinensis



Taenia pisciformis **proglottid**

(line drawings by Jarrod Wood)

Introduction to Biology 625

The Animal Parasitology laboratory at Kansas State University is designed to teach students the basics of collecting, preserving, and preparing animal parasites for study. This means that you will need to make a *major* effort to collect fresh host animals and dissect out the parasites. You will then be required to preserve, sometimes stain, dehydrate, and permanently mount on microscope slides different parasite specimens (each a different species) for your laboratory grade. The sooner you get started collecting the better as the cold Fall weather will cause fewer hosts to be available. If you wait until later in the semester to begin your laboratory work, it is likely that you will struggle in this course.

This laboratory manual is a highly condensed version of techniques that parasitologists have found to be both simple and effective for permanent preservation of eukaryotic parasites. There are many other techniques not included, many of which are superior to those described here. However, with limited time and funds, techniques that have proven to be quick and simple for students in this class have been emphasized. Although considerable information is derived from personal experience, some techniques have been acquired from other parasitologists or taken from "The Collection and Preservation of Animal Parasites," by M.H. Pritchard and G.O. Kruse, 1982, University of Nebraska Press, Lincoln, 141 pp as well as "Animal Agents and Vectors of Human Diseases," by P.C. Beaver and R.C. Jung, 5th ed, Lea & Fiebiger, 1985, Philadelphia, 281 pp.

You will be required to hand in <u>one</u> slide of a protozoan, <u>one</u> of a trematode or cestode, <u>one</u> nematode or acanthocephalan, and <u>two</u> arthropods. The other five slides can be comprised of any combination of groups you choose as long as all 10 slides represent *different species of parasites found in Kansas*. Each slide will be graded on a scale of 1-10 (10 being best). Quality of fixation and staining, proper dehydration, quality of clearing, orientation of specimen on the slide, whether or not miscellaneous debris or mounting medium is present, straightness of coverslip and slide label, how well the slide is labeled, and overall appearance (see individual sections below on what to watch for at each step), will be factors affecting your grade. In this laboratory, *neatness counts*. Again, *all specimens must be parasites collected in Kansas and must be handed in no later than 5:00 pm, Wednesday of finals week.* Ten percent of your laboratory grade (i.e. 1 point per slide) will be deducted for each day you are late handing these slides in. Since this class is taught alternate years, no incompletes can be issued in the course except in extreme situations (love life problems are not included in this category).

You will receive a maximum of 100 points for your slide collection (10 points/slide). Since lecture is comprised of a possible 400 points, the laboratory will determine 20% of your grade; a significant portion. You are not required to deposit your slides in the parasitology collection at Kansas State University, but we urge you to do so. Should you wish to take your slides with you, they will be available for you to pick up anytime up until June 1st of the following year (about a 6 month window), after which time I will consider them part of the KSU museum collection.

NOTE: Every time I go over this manual I find errors. The editing is never ending. If you find an error, please let me know.

Table of Contents

What you will need for class

Although most supplies are provided in this course, there are several items that you should purchase to make your life easier. These are as follows:

Laboratory coat. This will prevent your clothing from becoming stained or dissolved by reagents. At the very least, wear old clothing.

Small paint brushes. These are used to delicately manipulate small specimens. Be sure to get 2-3 brushes made of real hair as some of the solvents will dissolve plastic bristles.

Dissecting supplies. We have forceps, probes, and scissors in the laboratory, but these are of relatively poor quality. Sorry, but good dissecting equipment always disappears. I suggest you purchase your own set.

Gloves. A limited supply of disposable gloves are available for dissecting, but we do not have an endless supply nor all sizes. You may wish to purchase a box for yourself.

Fine tipped pen (optional). If you opt not to use a computer to write out your slide labels, you will need to write finely on your slide labels. This will require a 000 rapidograph pen with non-water soluble India ink. However, most students prefer to make their slide labels using a laser jet printer. NOTE: do not use an ink jet printer as the ink is water soluble.

Small staining dishes (optional). We may not have enough staining dishes for everyone at all times. It will help you if you can find 8-10 very small glass dishes that each hold 5-10 milliliters of liquid.

Ziplock bags (optional). After each dissection, animals will need to be bagged prior to disposal. We will supply some bags for such purposes, but it is likely that you will be working during off hours when the TA is not present and some supplies are not accessible.

Getting started

It will be sometime before we talk about all of the parasite phyla in the environment, hosts they infect, and methods of preservation. In the meantime, it is important that you get started collecting. As Fall progresses and the cold weather sets in, parasites will become scarce and it will become more difficult to find hosts. Please keep in mind during the course of the semester that no matter how long you've been in parasitology, trial and error still play important roles in determining which techniques may be effectively employed and which cannot for particular specimens. This means that even the experts sometimes have their prize specimens shrivel up or fall apart during processing. Nevertheless, there are some important points that you should consider as you get started collecting:

Freshness counts. **In general, the fresher the parasites, the better the preparations. Remember that soft internal details are lost rapidly, often within minutes following host death, and best results can be obtained by fixation of a specimen when it is still alive. Frozen specimens are worthless for anything other than a private collection.**

Proper fixation. **Be sure parasites are prepared properly for fixation. Many require a brief interval in distilled water or another solution so that proper relaxation can occur, eggs can be expelled, or that a proboscis can be extruded. Adhering debris should be removed with a clean paint brush. Different parasites will require different fixatives. NOTE: Some of you have contacts at the College of Veterinary Medicine and will acquire pathological specimens placed in 10% formalin. Only a small percentage of these poorly fixed specimens will be suitable for use.**

Cleanliness counts**. Always make sure your glassware and reagents are clean and uncontaminated. Make sure stains are filtered and free of precipitates.**

Mounting media. **The only long term, truly proven mounting media are Canada balsam and Gum damar. Many other mounting media shrink and form bubbles relatively rapidly, or crystalize over long periods of time.** ***Keep the lids on the jars when not dipping out of them*****.**

Be patient. **Never hurry through staining. Under-staining results in a specimen that is not crisp and appears faded. Internal details are poorly differentiated in these specimens. Never hurry through dehydration procedures. Shortening dehydration times often results in enough moisture left behind to eventually turn specimens cloudy and cause deterioration. This initially results in a milky, white color in and around your specimens once they are placed in the mounting media.**

Be gentle. **Always manipulate specimens delicately. Use brushes whenever possible. Microscopically, a heavy hand can result in considerable soft tissue damage.**

Pay attention. **Read and listen carefully. Virtually everything of importance is written out in this manual. I also go over most procedures verbally. Nonetheless, many students remain clueless in class. You should know from the very beginning how to precisely relax, fix, and stain each type of organism mentioned in class, and you will be tested over this on the first exam. If you read the material thoroughly and take good class notes, then the cloud of confusion will disappear.**

Where to get parasites

Every species of animal contains some parasites, although not every animal will have an abundance of specimens. You must get out and collect hosts, examine them thoroughly both externally and internally, and properly relax and fix the specimens according to recommended procedures. Based on past experiences in this class, the following tables provide a sample of some common sources of parasites in the Riley County area that are easily mountable (i.e. of appropriate size/disposition for mounting onto slides).

TABLE 1. Collecting suggestions – Common local protozoa

HOST	SITE OF INFECTION	PARASITE SPECIES	PREPARATION
calves (3-4 wk old)	small intestine	*Cryptosporidium parvum*	fecal smear
calves (4-8 wk old)	GI tract	*Entamoeba bovis;* *Giardia bovis*	fecal smear
crickets and grasshoppers	intestine	*Gregarina* spp.	smear
earthworms	seminal vesicles	*Monocycstis* spp.	smear
frogs and toads	cloaca	*Opalina* spp.	smear
frogs and toads	gall bladder	*Myxidium serotinum*	plasmodial mounts
termites	intestine	many commensal flagellate species	gut smear

TABLE 2. Collecting suggestions – Common local digenes

HOST	SITE OF INFECTION	PARASITE SPECIES	PREPARATION
catfish	intestine	*Alloglossidium corti;* *Crepidostomum ictaluri*	whole mounts
catfish	urinary bladder	*Phyllodistomum lacustri*	whole mounts
catfish; bass	skin; fins; musculature	*Clinostomum* spp. metacercariae	whole mounts
frogs	intestine	*Cephalogonimus americanus*	whole mounts
frogs	lung	*Haematoloecus* spp.	whole mounts
frogs	cloaca	*Megalodiscus temperatus*	whole mounts
frogs	urinary bladder	*Gorgoderina attenuata*	whole mounts
lepomids	viscera (esp. liver)	various metacercariae	whole mounts
painted turtle	gut arteries	*Spirorchis parvus*	whole mounts
snapping turtle	lung	*Heronimus mollis*	whole mounts
terrestrial snails	body cavity	*rediae*	whole mounts

TABLE 3. Collecting suggestions – Common local cestodes

HOST	SITE OF INFECTION	PARASITE SPECIES	PREPARATION
bass	intestine	*Proteocephalus ambloplitis*	whole mounts
lepomids	body cavity	*Proteocephalus ambloplitis* (pleurocercoids)	whole mounts
catfish	intestine	*Corallobothrium fimbriatum*	whole mounts
frogs, toads	intestine	*Cylindrotaenia americana*	whole mounts
white sucker	intestine	*Glaridacris catostomi*	whole mounts

TABLE 4. Collecting suggestions – Common local nematodes

HOST	SITE OF INFECTION	PARASITE SPECIES	PREPARATION
bass; lepomids	intestine	*Camallanus oxycephalus*	whole mounts
American cockroaches	intestine	*Hammerschmidtiella diesingi; Leidynema appendiculatum*	whole mounts
field crickets	intestine	*Cephalobium microbivorum*	whole mounts
frogs	lung	*Rhabdias ranae*	whole mounts
frogs and toads	intestine	*Cosmocercoides dukae; Falcaustra catesbeiana; Oswaldocruzia pipens*	whole mounts
terrestrial snails	body cavity	"rhabditids"	whole mounts
toads	lung	*Rhabdias bufonis*	whole mounts

NOTE: Most nematodes are not mounted or stained because they have a thick cuticle that makes fixation, dehydration, and mounting very difficult. Many are also too large to fit onto microscope slides. Thus, most nematodes are simply stored in 70% ethanol with a drop or two of glycerine, and taken out of the bottle only when examined microscopically. However, with some effort, the above nematode species have been found to lend themselves to processing and mounting.

TABLE 5. Collecting suggestions – Common local arthropods

HOST	SITE OF INFECTION	PARASITE SPECIES	PREPARATION
cats; dogs	hair	*Ctenocephalides felis*	whole mounts
children	hair	*Pediculus humanus capitus*	whole mounts
cicadas	body cavity	*Emblemasoma erro*	whole mounts
deer	hair, skin	*Dermacentor albipictus*	whole mounts
deer mice	hair	*Hoplopsyllus affinis*	whole mounts
frogs and toads	reddish skin nodules	*Hannemania penetrans*	whole mounts
grasshoppers	body cavity	dipteran fly larvae	whole mounts
grasshoppers	wings	*Eutrombidium locustarum*	whole mounts
guinea pigs	hair	*Gliricola porcellis; Gyropus ovalis*	whole mounts
honey bees	thorax	*Varroa jacobsoni*	whole mounts
many mammals	skin	*Amblyomma americanum; Dermacentor variabilis; Eutrombicula alfreddugesi*	whole mounts
none; ponds	N/A	mosquito larvae and pupae	whole mounts
swine	hair, skin	*Haematopinus suis*	whole mounts
turtles	neck dermis	*Sarcophaga cistudinis*	whole mounts

TABLE 6. Collecting suggestions – Common miscellaneous parasites

HOST	SITE OF INFECTION	PARASITE SPECIES	PREPARATION
bass	intestine	*Leptorhynchoides thecatus*	whole mounts
turtles	intestine	*Neoechinorhynchus emydis*	whole mounts
turtles	outer surface	*Placobdella parasitica*	whole mounts
under rocks in streams	N/A	*Placobdella parasitica*	whole mounts
white sucker	intestine	*Pomphorhynchus bulbocolli*	whole mounts

Hopefully, the above tables will provide you with a variety of sources for specimens. Perhaps 80% or more of all species of animals on the planet are parasitic, so its generally only a matter of time and effort before you run across them. Common sources of parasites used in this class are from domestic/companion animals (ectoparasites), fish, frogs, and toads. Some students also attend necropsies at the College of Veterinary Medicine and obtain specimens. NOTE: Animal Parasitology has Institutional Animal Care and Use Committee (IACUC) approval for students to necropsy fish and amphibians in the classroom. *These are the only types of vertebrate animals that you are allowed to bring into the classroom and necropsy*. Invertebrates are not covered by regulations and any species of non-dangerous invertebrate found in Kansas is fair game. Collection of some animals may require a State of Kansas permit (available in class), owner permission, or a valid state fishing license (fish). Rabies and tetanus vaccinations should be considered when dealing with wild mammals.

What to do when you find a parasite

Parasites should be collected alive and fixed directly from the living condition. This insures proper preservation of internal and external details, as autolysis begins immediately upon death of the parasite. External parasites such as ticks and fleas leave a dead host as soon as it begins to cool and internal parasites die and begin to degenerate soon after their host. Thus, it is best to collect from freshly killed hosts. As far as road kills go, cestodes and trematodes begin degenerating within a matter of minutes to an hour after host death. Nematodes are tougher and may be alive several additional hours. In general, I recommend that if you don't see the parasite moving, internal details are most likely in poor condition.

***Fixation*, the process by which tissues are arrested from their living state, will be different for the different parasites you encounter. *Relaxation*, a process whereby animals can be artificially kept in their natural appearance, may be required prior to fixation and is also different depending upon the parasite. Although details of fixation, staining, dehydration, and mounting are presented later in this manual, the following provides an abbreviated "quick" guide to a hypothetical necropsy and the fixation process.**

***Arthropoda*. Taking a hypothetical host, one would initially want to look for ticks, lice, mites, and fleas; at least if it's a mammal. If an animal is brought into the laboratory in a bag, check the inside of the bag carefully. Then, examine the host around the ears, neck, and remainder of body. Use a toothbrush to help remove mites and lice from both mammals and birds, and a scalpel or razor blade to scrape the edge of wounds where mange mites are suspected. All arthropods (including dipteran larvae), and pentastomes from the lungs of snakes, should be placed directly into a vial containing 70% ethanol. Some investigators use Boardman's solution (97 ml of 20% ethanol with 3 ml ether) to initially relax the arthropods prior to placing the parasites permanently into 70% ethanol, but this is generally unnecessary for most terrestrial arthropods.**

Monogenes. **These are tiny trematodes most commonly found associated with the gills, skin, and nasal passages of fish. They tend to be Spring/early Summer parasites, but we do find them on the gills of fish in the Fall as well. In general, these tend to be so small and delicate that students are unable to work with them effectively. However, if you wish to try, they must first be relaxed in 1:4000 formalin. Cut out the gills of large fish, or if the fish is small, place the entire fish in 1:4000 formalin for 30 min. Then, shake the fish/gills briskly to dislodge relaxed worms and allow particles in suspension to settle for 5-10 minutes. Using a dissecting microscope, carefully pipet the tiny, transparent worms into 10% neutral buffered formalin or alcohol-formalin-acetic acid (AFA). Few individuals seem to be aware of the 1:4000 formalin technique but the procedure, taught to me by my old fish parasitology instructor Wilmer Rogers at Auburn University, works exceptionally well.**

Digenes. **Digenetic flukes may occur in the intestine, gall bladder, urinary bladder, and even oviduct if you're dealing with birds. Place specimens directly into room temperature tap water. As water begins to cross their tegument, eggs will be expelled from their uterus, which will eventually allow for better viewing of internal details. Once egg expulsion slows, after 2-15 minutes, worms must be fixed before they rupture from osmotic shock. For digenes of fish and amphibians, only limited osmosis will occur and eggs may not be expelled. Small digenes should then be placed directly into AFA whereas larger digenes should be sandwiched between a slide and coverslip (as with cestodes) prior to flooding the interface with fixative. Take special care not to smash or distort the worms by adding too much weight. After 48 hr in fixative, transfer digenes to 70% ethanol for long term storage.**

Tapeworms. **These are segmented and are often found attached to the gut wall. Carefully remove the scolex from the intestinal wall using a paint brush or dissecting needle. Place the entire worm in cold distilled water or cold saline solution, which relaxes the worm. Some parasitologists recommend 5-10% ethanol in water at room temperature. During this time, remove any adhering debris from the scolex with a paint brush. In 5-30 minutes (size dependant), after the worm has slowed in motion but is not yet dead, you can fix the specimen. The most common fixative for cesotdes is AFA (alcohol-formalin-acetic acid). Small tapeworms should be placed directly in the solution whereas larger specimens need to be placed on a glass microscope slide, flattened slightly using coverslips, then flooded with fixative from a pipet at the interface. Use care not to smash or distort the worms by adding too much weight while trying to flatten them. After 48 hr in fixative, transfer worms to 70% ethanol for long term storage.**

Nematodes. **Nematodes less than 2 cm long are often suitable for mounting. Try placing live worms directly into glacial acetic acid for 5-10 seconds, which should kill and straighten them. Transfer them *quickly* to 70% ethanol. Some worms rupture during this process, in which case the remainder of the specimens should be placed directly into steaming (but not boiling) 70% ethanol for straightening and fixation. Many investigators add a few drops of glycerol to the 70% ethanol, which helps retard shrinkage and also keeps the worms moist if the ethanol evaporates. Still, other investigators maintain that nematodes can be both fixed and stored in glacial acetic acid, and eventually transferred through a series of glacial acetic acids directly into toluene/xylene prior to mounting.**

Acanthocephala. **A few students may encounter acanthocephala (thorny headed worms) in turtles, birds, and especially fish in class. These look superficially like nematodes but are much more difficult to fix and process. Most members of this phylum are not mounted onto slides. However, if you wish to mount some, you will first need to remove the proboscis from the gut wall. You may need to cut them out, or spend considerable time with a probe manipulating them. The proboscis (snout) contains many tiny hooks that embed themselves into the gut wall. Once the worm is removed from the gut, place them directly into distilled water for 30-120 minutes so that the proboscis will either extrude or remain extruded. Specimens that fail to extrude their proboscis should be discarded as species identification relies heavily on proboscis *oncotaxy* (hook morphology). You can then place the worms directly into AFA for fixation. You should use a fine insect pin to carefully prick their cuticle in a few places or fixation will not proceed with any rapidity (if at all). After 3-7 days in fixative, transfer the worms to 70% ethanol.**

Leeches. **Place all leeches in distilled water, add a few menthol crystals to the surface of the water, and screw the top down. Leeches are very difficult to relax and sometimes require several hours to relax. A recent correspondent has suggested to me that carbonated soda water also works very well to relax leeches because of the carbon dioxide. Once the leeches become limp to the touch, allow them to remain in the relaxation solution at least 15 additional minutes (leeches are tricky). Leeches can then be sandwiched between two microscope slides and the interface flooded with AFA. After about 10 minutes, transfer the worms to a vial of AFA. Leave the leeches in the vial of fixative for 4-7 days, and then transfer them to 70% ethanol. Leeches are one of the few parasites that can be manhandled.**

Blood. **You may wish to make a blood smear. Dogs sometimes have heartworm microfilariae (*Dirofilaria immitis*), birds haemosporina, rodents trypanosomes, and turtles haemogregarines. Discussing the types of blood smears and how to make them is explained later in this manual. The blood smear should be made very early in the necropsy and before the blood coagulates.**

After you have collected, relaxed, and fixed specimens, you generally have several days or weeks before you need to process the parasites further. Later in this manual are the techniques you will need for further processing of your specimens. *The laboratory will be set up so that you can collect and preserve specimens early in the fall while the weather is still warm, and then process (stain, dehydrate, and mount) your specimens later in the semester.*

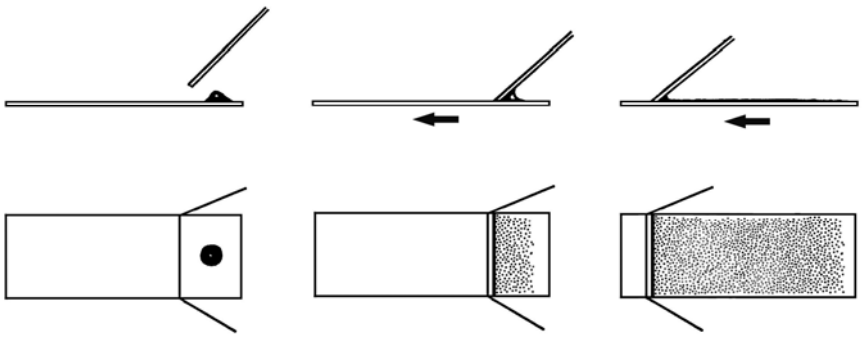
Blood smears

Blood smears are useful for the identification of such parasites as haemosporina, trypanosomes, haemogregarines, some microfilariae, and piroplasms. They can be rapidly stained and examined the same afternoon. *Clean microscope slides are essential* so that the sample of blood adheres to, and spreads along, the slide properly. Slides should be pre-cleaned using 95% ethanol or acetone to remove oils and detergents, and then allowed to air dry for a few minutes. Touch only the very edges of these glass slides since oil and grease will result in clear spots ("fish eyes") in your blood smears. Blood should be taken from an animal before, or very shortly after, death. If any clotting occurs, more serum will be collected than blood cells (and parasites). Two types of blood smears can be performed, both of which are described below:

Thin blood smear. **This is the typical blood smear most of you are familiar with (see figure below). The thin smear is mainly for haemosporina, some trypanosomes, hemogregarines, bacterial infections of red blood cells, and piroplasms. Usually, multiple thin smears are made per animal in case staining times need to be adjusted. *Precaution - never stain all slides at the same time.***

1. **Place a drop of blood near one end of the slide. Touch the end of another slide to the center of the first one and draw it toward the drop of blood until, with contact, the blood flows along the edge of the spreading slide.**
2. **Quickly, push the spreading slide along in a rapid, even motion at a 20-40 degree angle, evenly distributing the blood along the first slide but behind the slide that you are pushing. The thickness of the smear may be controlled by the angle of the slides relative to one another. Increasing the angle thins the smear. Remember, you are pulling (not pushing) the blood.**
3. **Air dry, then fix slide in 100% methanol for 2 minutes.**
4. **Air dry for 30-60 seconds. Slides may be stored fixed and unstained indefinitely.**
5. **Stain in Giemsa or Wright's blood stains for 5-15 minutes.**
   (In our laboratory, we use Giemsa stain because of its ease to use. Giemsa is available commercially and is simply diluted 1:20 with distilled water. It can be buffered with 0.6% (w/v) Na_2HPO_4 and 0.5% (w/v) KH_2PO_4 if desired).
6. **Rinse slide in deionized water (or tap water) and air dry.**
7. **Mount directly with Canada balsam or Damar gum mounting media.**

Mechanics of a thin blood smear



***Thick smear*. The thick smear is not as commonly used, but is very good for organisms that are in low numbers, such as American trypanosomes and microfilariae. It concentrates parasites in a small area and leaves behind both parasites and white blood cell nuclei. The erythrocytes undergo lysis in the process. The following is the procedure:**

1. **Place two or three drops of blood onto a clean slide over an area about 3-4 cm square using a needle, pipette, or toothpick. Allow the blood to clot and dry well. DO NOT FIX the specimen. Slides may be stored this way until staining, which may be hours, days, and sometimes years.**
2. **To stain, soak the slide in deionized water about 20 minutes or until the color disappears. This is to lyse the red blood cells. For slides that are stored for a very long time and the erythrocytes are stubborn, add 2-3 drops of glacial acetic acid to the water to enhance hemolysis.**
3. **Air dry the slide and then fix in 100% methanol (2 minutes).**
4. **Air dry for 30-60 seconds.**
5. **Stain in Giemsa or Wright's blood stains for 5-15 minutes. (In our laboratory, we use Giemsa stain because of its ease to use. Giemsa is available commercially and is diluted 1:20 with distilled water. It can be buffered with 0.6% (w/v) Na_2HPO_4 and 0.5% (w/v) KH_2PO_4 if desired).**
6. **Rinse slide in deionized water (or tap water) and air dry.**
7. **Mount directly with Canada balsam or Damar gum mounting media.**

Tissue impression smears

Protozoa and metazoans that concentrate in viscera organs can sometimes be differentiated using a tissue impression smear. The procedure is especially good for exoerythrocytic stages of malaria, amastigotes of kinetoplastids, and tissue stages of hemogregarines and coccidia. A small piece of organ, for instance liver, lung, heart, spleen, or portion of the intestine, are first excised. Then, the exposed end of the tissue is dabbed onto a kimwipe or paper towel until the tissue becomes "sticky." By pressing the cut surface several times across a clean microscope slide, a 1-3 cell thick layer adheres to the slide. Slides are first air dried and may be stored without further processing (i.e. not fixed) until stained. To stain the slides, the following procedure is useful:

1. **Fix slides in 100% methanol for 2 minutes and then air dry 30-60 seconds.**
2. **Stain in Giemsa or Wright's blood stains for 5-15 minutes. (In our laboratory, we use Giemsa stain because of its ease to use. Giemsa is available commercially and is diluted 1:20 with distilled water. It can be buffered with 0.6% (w/v) Na_2HPO_4 and 0.5% (w/v) KH_2PO_4 if desired).**
3. **Rinse slide in deionized water (tap water works OK) and air dry.**
4. **Mount directly with Canada balsam or Damar gum mounting media.**

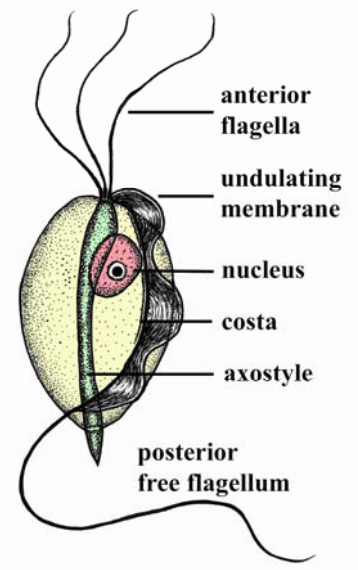
Intestinal protozoa

Protozoan cysts and trophozoites, such as those of of *Giardia* spp., *Entamoeba* spp., and trichomonads, are common in the gut of many animals. Multiple methods of preserving these organisms in feces are available. Perhaps the best and most traditional fixative and stain combination for most intestinal flagellates and amoebae is Schaudinn's fixative followed by an Iron hematoxylin stain. Nuclear details are very well preserved and internal structures appear very well differentiated. Unfortunately, the formulas are somewhat complicated, the reagents expensive, the shelf lives short, and the specimen processing times lengthy. Therefore, trial and error over the years has led our laboratory to utilize, and sometimes devise, quick and simple methods to process protozoa. In effect, the techniques we now utilize in the laboratory are not necessarily superior to the traditional methods, but students usually obtain decent, rapid results.

Giemsa staining procedure for *Tritrichomonas foetus*

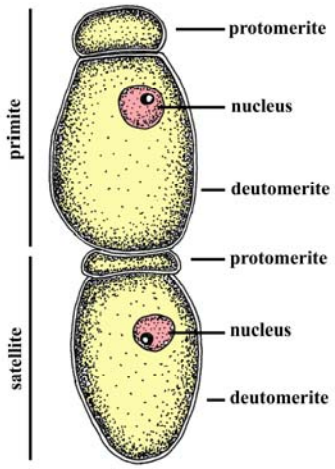
***Tritrichomonas foetus* is a urogenital pathogen of bovids. It represents a major cause of abortion in cattle, but interesting enough can also be isolated from felids. The parasite grows well *in vitro* and trophozoites can be cultured in RPMI 1640 cell culture medium supplemented with 10% fetal bovine serum. During one of the laboratory sessions, a culture of this organism will be made available so that smears can be made by students.**

1. ***Optional step.* Spread a thin layer of egg albumin (1: 5 with water or glycerol) over a clean microscope slide and allow the slide to air dry for at least 30 minutes at room temperature.**
2. **Add a drop of the cultured *Tritrichomonas foetus* to the slide. Allow to completely air dry at room temperature.**
3. **Place slide in 100% methanol for 2-3 minutes.**
4. **Allow to air dry by standing slide on end.**
5. **Place the slide in Giemsa stain (premixed at 1:20 with tap water), 8-10 minutes.**
6. **Rinse slide briefly in tap water, stand on end, and allow to air dry.**
7. **Add a generous drop of Canada balsam or Damar gum (preferred) to the middle of the smeared protozoa. The mounting media should be diluted "slightly" with toluene to enhance fluidity.**
8. **Add a coverslip gently (without introducing bubbles); do not press coverslip down.**
9. **Let dry flattened.**



Acid carmine staining of gregarines – optional procedure #1

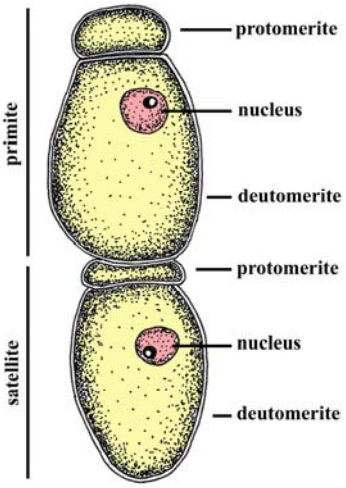
Gregarines can be collected from the intestinal tract or body cavity of invertebrates. Common hosts include grasshoppers, crickets, and damselflies. Around Manhattan, late September through the end of October seem to be the best Fall months for encountering gregarines. Often, late in the season, the intestinal tracts of grasshoppers are teaming with mature *Gregarina* spp.



1. **Pipette gregarines from intestinal contents into a vial of tap water. Allow parasites to settle to the bottom of the vial. Collect as little debris as possible.**
2. **Once the gregarines have settled, carefully pipette off most of the water.**
3. **Add AFA (alcohol-formalin-acetic acid). They can be stored for weeks in AFA.**
4. **Affix gregarines to slide thinly coated with albumin (1:5 with water or glycerine). Let air dry 30 min.**
5. **Place slide into 70% ethanol, 2-3 min.**
6. **Stain in Semichon's acid carmine, 5 min.**
   - **A. glacial acetic acid (100 ml)**
   - **B. distilled water (100 ml)**
   - **C. carmine (lake) (as needed, about 3 g).**
   - **D. Mix acid and water into bottle; add enough carmine (1-2 g) until no more will go into solution (saturated). Place in hot water bath, heat 15 min (temperature should not go above 95 C). Cool and filter. Add equal amount of 70% ethanol. Stain will keep indefinitely.**
7. **Place slide in 70% ethanol, 2-3 min.**
8. **Place slide in destain (1% 1 N hydrochloric acid in 70% ethanol), 5-10 sec depending upon specimens. Usually, 2-3 "quick" dips.**
9. **Place slide in alkaline ethanol to neutralize acid ethanol and to "blue-up" specimens (1% 1 N sodium hydroxide in 70% ethanol, or some sodium bicarbonate in 70% ethanol), 3 min.**
10. **Place slide in 95% ethanol, 3-5 min.**
11. **Place slide in 100% ethanol, 3 changes, 3-5 min each.**
12. **Place slide in toluene, 2 changes, 5 min each.**
13. **Mount using Canada balsam or Damar gum. Do NOT press coverslip down or you may smash gregarines. Use Balsam or Damar thinned with toluene.**

Acid carmine staining of gregarines – optional procedure #2

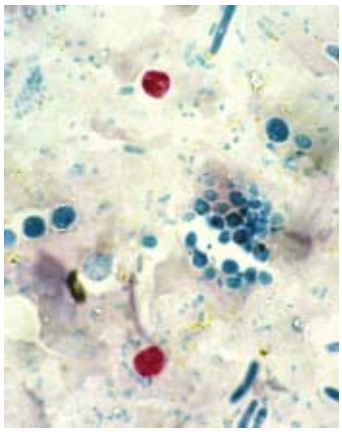
This procedure is performed within the vial following fixation, rather than on a microscope slide. Specimens are mounted onto slides at the end of the procedure rather than at the beginning. Use about 1/2 ml of each solution in the vial, pipetting each solution in and then out. Be sure to pipette out solutions *as completely as possible between each step*.



1. **Replace AFA with 70% ethanol, 2-3 min**
2. **Add Semichon's acid carmine stain 5 min.**
   - **A. glacial acetic acid (100 ml)**
   - **B. distilled water (100 ml)**
   - **C. carmine (lake) (as needed, about 3 g).**
   - **D. Mix acid and water into bottle; add enough carmine (1-2 g) until no more will go into solution (saturated). Place in hot water bath, heat 15 min (temperature should not go above 95 C). Cool and filter. Add equal amount of 70% ethanol. Stain will keep indefinitely.**
3. **70% ethanol, 2-3 min.**
4. **Destain (1% 1 N hydrochloric acid in 70% ethanol), 5 sec**
5. **Alkaline ethanol to neutralize acid ethanol and blue-up specimens (1% 1 N sodium hydroxide in 70% ethanol), 3 min.**
6. **95% ethanol, 3-5 min.**
7. **100% ethanol, 3 changes, 3-5 min each. Make sure 100% ethanol is "slightly" higher level in vial than 95% ethanol was.**
8. **Toluene, 3 changes, 2 min first time and then 5 min each. Make sure toluene is "slightly" higher level in vial than 100% ethanol was.**
9. **Pipette 8-12 gregarines onto a clean microscope slide. Allow toluene to "almost" dry (but not totally).**
10. **Add a drop of dilute Damar gum or Canada Balsam, and coverslip. Do NOT press coverslip down or you may smash the gregarines.**

Acid-Fast stain (modified) for *Cryptosporidium parvum*

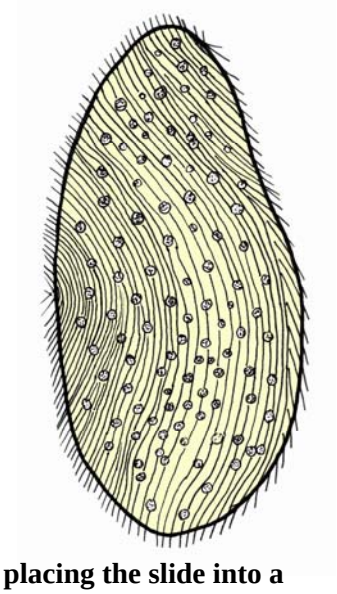
Fecal smears containing coccidia, i.e. *Cyclospora cayetanensis*, and sometimes freshly unsporulated oocysts of *Isospora* spp., as well as oocysts of *Cryptosporidium* spp., can be stained effectively in this stain. It relies on the stain getting through a break in the oocyst wall and staining the cytoplasm within.



1. ***Optional step:*** **Spread a *thin* layer of egg albumin (1:5 with water or glycerol) over a clean microscope slide and allow the slide to air dry at room temperature for at least 30 min.**
2. **Spread a thin film of fresh feces onto the glass slide and allow it to dry thoroughly on a hot plate set for <u>low</u> heat. Once it has *barely* dried completely, remove it from the hot plate. Alternatively, the slide may be flamed until the fecal smear is dry (heating/drying causes the oocyst wall to become compromised so that the stain may enter). DO NOT over-heat or the smear will become brittle and flake off the slide.**
3. **Place slide into 100% methanol, 2-5 min.**
4. **Lay slide flat. Add 3-4 drops Kinyoun's carbol-fuchsin to cover feces (few drops on flat slide), 5 min. The formula for this stain is as follows:**
   - **A. basic fuchsin (4.0 g)**
   - **B. phenol (fresh, 8 ml)**
   - **C. mix fuchsin and phenol into a slurry**
   - **D. add 95% ethanol (20 ml) and mix**
   - **E. add de-ionized water (100 ml) and mix well**
   - **F. filter prior to use**
5. **Wash briefly in tap water (optional) so that next solution doesn't turn color quite so quickly.**
6. **Decolorize in acid alcohol (1% 1 N HCl in 95% ethanol) (45 sec - 1 min). Leave in solution until nearly all reddish color has just left the smear, then remove.**
7. **Wash briefly, but thoroughly, in tap water.**
8. **Place slides in Coplin jar of methylene blue, 30 sec - 1 min (dissolve 1.0 g methylene blue in 100 ml absolute ethanol). Be sure to keep jar well covered at all times so that ethanol does not evaporate.**
9. **Rinse in tap water, drain, and dry. Specimen should have bluish background with reddish tinge mixed in. If reddish tinge is absent, then place specimens back into Kinyon's and try again.**
10. **When totally dry, mount using Damar gum or Canada balsam.**

Iron hematoxylin procedure for Opalinids

Flagellates in the genus *Opalina* and ciliates in the genus *Nyctotherus* are common in the cloaca of ranid frogs. Especially large numbers are common in frogs in Kansas beginning mid-September to mid-October. The flagellates can usually be mounted and stained fairly easily whereas the ciliates usually need a more elaborate staining procedure.



1. **Pipette out the opalinids in water into a vial. Allow the organisms to settle to the bottom, then carefully pipette out as much of the water as possible.**
2. **Add AFA to fix the protozoa. They should be fixed at least 24 hr prior to staining, and may be stored indefinitely in AFA**
3. ***Optional step:* Spread a *thin* layer of egg albumin (1:5 with water or glycerol) over a clean microscope slide and allow the slide to air dry at room temperature for at least 30 minutes. This step helps the protozoa to adhere better to the slide, although be warned that it also picks up some types of stains.**
4. **Add a generous drop of the protozoa in AFA to the microscope slide; typically 12-20 opalinids. Allow to air dry completely at room temperature.**
5. **Stain slide in Hansen's iron hematoxylin (5-6 min) either by placing the slide into a Coplin jar containing the stain or by laying slide flat and adding several drops of stain onto the specimen. The latter method may result in some precipitates settling onto the specimens. (*Hansen's iron hematoxylin:* Dissolve hematoxylin (0.75 g) in 35 ml water. Dissolve ferric ammonium sulphate (iron alum, 4.5 g) in 65 ml water. Combine the two solutions and bring to a boil. Cool and filter). Stain is good for about 1 month.**
6. **Rinse slide with tap water**
7. **Place slide in Coplin jar with acid ethanol (1 ml, 1 N HCl in 99 ml 70% ethanol), 20-30 sec**
8. **Place slide in Coplin jar with alkaline ethanol (1 ml, 1 N NaOH in 99 ml 70% ethanol), 1 min**
9. **Place slide in Coplin jar with 95% ethanol (1-2 min)**
10. **Place slide in Coplin jar with 100% ethanol (2 changes, 2 min each)**
11. **Place slide in toluene (2 changes, 2-3 min each)**
12. **Do not allow specimens to dry out. Mount in Damar gum or Canada balsam.**

Miscellaneous fixatives and stains for protozoa

Although we will not be using any of the procedures explained in this particular section of the laboratory manual, a variety of additional stains and processing procedures have been derived for protozoa. I can't resist presenting a few of my favorites to you, most of which are more complicated than students wish to pursue in this course.

Iron hematoxylin (traditional) staining for protozoa

This is a great stain for many protozoa, including flagellates, amoebae, and gregarines. It represents the classic, old time procedure used by parasitologists in yesteryears. Most individuals no longer use the traditional iron hematoxylin because 1) the process takes longer than most other staining procedures, and 2) the stain is complex to make and relatively short lived. However, Schaudinn's fixative followed by iron hematoxylin is absolutely the best for preserving and staining nuclear details of protozoa. Over the years, multiple variations of the traditional iron hematoxylin formulation have been developed, including Hansen's iron hematoxylin which is quicker and easier to make and employed in another section of this manual.

1. **Tissues have already been prefixed in Schaudinn's, and excess fixative should have been removed using iodine/ethanol (see fixative procedure below).**
2. **Hydrate using graded series of ethanols (50%, 30%) and into distilled water; 3-5 min each.**
3. **Place in 2% aqueous iron alum (mordant) for 45 min to 3 hr. This solution will sharpen details of the stain.**
4. **Wash out mordant with distilled water for 1-2 min.**
5. **Stain in 0.5% hematoxylin stain, 2-3 hr.**
6. **Remove excess stain by rinsing in distilled water 5 min.**
7. **Destain and differentiate in 1-2% ferric chloride or 1-2% iron alum until stain is no longer given off in large amounts. Destain until sharp nuclear detail staining remains but stain in cytoplasm is gone. If one destains too long, the specimens can be restained with the hematoxylin and the destaining procedure tried again. This is a tricky art to get the specimen stained precisely.**
8. **Transfer to distilled water to stop destaining process.**
9. **Rinse several minutes in tap water, which should result in the stain turning a dark blue to black tint.**
10. **Wash 20-30 min in 3-4 changes of distilled water.**
11. **Blue up the solution by washing for 5 min in saturated lithium carbonate solution.**
12. **Rehydrate through graded series of ethanols (30%, 50%, 70%, 95%) at 3-5 min each.**
13. **100% ethanol (3 changes, 3-5 min each).**
14. **Toluene or xylenes (2-3 changes, 3-5 min each).**
15. **Mount in Canada balsam or Damar balsam.**

Kohn's Chlorazol black fixative and stain (1960, Ref Med Quart Israel 19: 160-161)

This is a useful, rapid stain for intestinal protozoa in feces where fixative and stain are combined into the same solution. Structures in cysts stain black and can easily be seen and photographed using this stain. I've found that it works particularly well for *Giardia* spp. and *Entamoeba* spp. Few scientists seem to use it, however, and I've never figured out why. NOTE: The fixative and stain are one. DO NOT use other fixatives prior to using this stain.

1. ***Chlorazol black solution:***
   - **A. 90% ethanol (170 ml)**
   - **B. 100% methanol (160 ml)**
   - **C. Glacial acetic acid (20 ml)**
   - **D. Liquid phenol (20 ml)**
   - **E. 1% aqueous phosphotungstic acid (12 ml)**
   - **F. Deionized water (618 ml)**
   - **G. Chlorazol black (5 g)**
   - **H. Mix liquids. Then, grind dye in mortar and add small amounts of the liquids so that a smooth paste is formed. Add more solution and continue to grind. Let settle and pour off supernatant fluid into separate container. Keep adding more solution and repeat grinding, settling, and pouring until all dye is in solution. Allow stain to age 4-6 wks prior to use.**

2. ***Staining:***
   - **A. Make fecal smear on slide with applicator stick. Do not let smear dry.**
   - **B. Add smear to Chlorazol black stain and let sit for 1-2 hours. Trial and error will determine the exact time for each batch of stain.**
   - **C. Transfer to 95% ethanol, 5 min.**
   - **D. 100% ethanol, 5 min. (2 changes)**
   - **E. Toluene or xylene, 5 min. (2 changes)**
   - **F. Mount in Canada balsam or Gum damar.**

Lugol's iodine solution

Iodine stains are effective for fresh "wet mount" preparations of protozoa, especially in fecal smears that need to be examined quickly. Add a drop of iodine solution to a dilute fecal suspension and you can examine the preparation immediately. Trophozoites and cysts pick up the iodine and stain a tan or yellowish color.

Lugol's iodine can be purchased commercially. However, if you opt to make it yourself, first dissolve 2 g of potassium iodide in 100 ml deionized water. Then, add 1 g of iodine so that the latter will go into solution. Store the solution in a brown bottle away from light or it will go bad in a couple of weeks.

Schaudinn's fixative

Schaudinn's fixative is the old, traditional fixative that is superior to most other solutions for preserving nuclear structures and other internal details of protozoa. It is especially superior when iron hematoxylin staining is used. I've tried a variety of other solutions, but nothing beats Schaudinn's for quality of nuclear details. Unfortunately, it contains mercuric chloride, a powerful toxin that also needs to be removed to avoid precipitates. A stock solution is made by preparing a saturated, aqueous solution of mercuric chloride and then mixing that with ethanol. Within 24 hr of use, glacial acetic acid is added. The recipe is as follows:

1. **saturated (ca 5% w/v), aqueous mercuric chloride (66 ml)**
2. **95% ethanol (33 ml)**
3. **glacial acetic acid (5 ml) (add just before use)**

Fecal specimens containing protozoa can either be fixed in bulk or they can first be smeared onto a glass microscope slide or (preferably) glass coverslip. Fixation should proceed for 15-20 minutes. The specimens should first be transferred to 50% ethanol for 5 min, then into 70% ethanol with iodine (Lugol's solution added to the 70% ethanol to form a straw-colored solution). Two 5 min changes of the iodine/ethanol solution are advised, which allows mercurial deposits (black particles) to be converted into mercuric iodide, which is soluble in ethanol. The specimens can be stored indefinitely in 70% ethanol until further processing/staining. Iron hematoxylin is the preferred stain for Schaudinn's fixed material. NOTE: Don't fix specimens in Schaudinn's if you wish to employ the Kohn's Chlorazol black technique.

Wheatley's trichrome stain (1951, Am J Clin Pathol 21: 990-991)

This is a relatively simple variation on the many forms of trichrome stain that one may utilize. It is a very good stain for microsporidia, and can be used for numerous other types of protozoa. The end product is very nice since protozoa stain multiple colors, although working out the staining times so that the colors are balanced can be a tricky endeavor.

1. **Make a fecal smear on a microscope slide or coverslip from unfixed or fixed material. Within 24 hr after making the smear, and using Coplin jars, adhere to the following:**
2. **95% ethanol (1-2 min)**
3. **70% ethanol (1-2 min)**
4. **Wheatley's trichrome stain (5-10 min; the slide or coverslip can be placed flat and a few drops of the stain used to flood the smear; be wary of precipitates, however)**
   - **A. Chromotrope 2R (0.6 g)**
   - **B. Light green (0.15 g)**
   - **C. Fast green FCF (0.15 g)**
   - **D. Phosphotunstic acid (0.7 g)**
   - **E. Glacial acetic acid (1 ml)**
   - **F. Distilled water (100 ml)**
   - **G. Place dry stains into flask. Add acetic acid and swirl to mix. Allow the "goo" to sit for 30 min to ripen. Add the distilled water. Shake to mix. Keeps for many years.**
5. **Deionized water (2 dips)**
6. **95% acid ethanol (5 sec; 95% ethanol with 3-4 drops of acetic acid or HCl for every 10 ml)**
7. **100% ethanol I (1 dip; a sort of wash)**
8. **100% ethanol, two changes (1-2 min each)**
9. **Toluene, two changes (1-2 min then 2-5 min)**
10. **Mount in Canada balsam or Damar gum without allowing specimen to dry out.**

Monogenes

Monogenes are found mainly on fish, although a few species occur on turtles, invertebrates, and even amphibia; rarely reptiles and birds. They are located inside the mouth, on the gills, fins and skin, in gill chambers, urinary bladders, and cloaca. Large monogenes are treated the same as digenes (below); however, most species are small and can be processed as outlined:

1. **Place small fish, gills, fins, or other structures that may contain monogenes in a jar of 1:4000 aqueous formalin. After 30 min, shake tissues and remove them from jar. Whole small fish may be added to the solution. For students, gills generally will yield more monogenes that other areas of the fish.**
2. **Allow the solution to stand for at least 10 minutes to allow monogenes to settle to the bottom of the jar.**
3. **Carefully as to not disturb the bottom sediments, decant extraneous fluid.**
4. **Examine sediment carefully under a dissecting scope. Monogenes tend to be tiny, as small or smaller than gregarines, and nearly transparent. Remove specimens by Pasteur pipet and drop them into AFA (alcohol-formalin-acetic acid fixative).**
5. **Let monogenes fix for one hour, then transfer them to 70% ethanol. Monogenes can be stored permanently in 70% ethanol. However, if permanent mounts are desired, the following double coverglass mount represents a procedure used in our laboratory:**
    - **A. Place a large (22 mm or larger) coverglass on the edge of a warming plate.**
    - **B. Add a drop of melted glycerine jelly in the center of the coverglass and orient specimen within jelly (ventral side down). Be sure that no small air bubbles are present in the glycerine jelly, which tends to be the most difficult part of the procedure.**
    - **C. Slowly lower a second, smaller coverglass onto the specimen. Let assembly cool overnight. Again, be careful as to not introduce air bubbles into the glycerine jelly.**
    - **D. Scrape off excess jelly with razor blade.**
    - **E. Turn double coverslip preparation over so that large glass is now on top. Lower assembly onto a drop of Canada balsam or Damar gum that has been placed on a microscope slide. The media will spread out under the small coverslip and extend to the edge of the large coverslip, forming permanent seal.**

Overall, monogenes tend to be late Spring/early Summer taxa. However, students have found monogenes on the gills of fish in Kansas in the Fall on multiple occasions. The main problem that students have is first finding and then working with the worms. They are very tiny and nearly transparent, difficult for students to identify, and they are difficult for students to manipulate. Unless you are a monogene enthusiast, I would suggest not bothering with this group.

Digenes

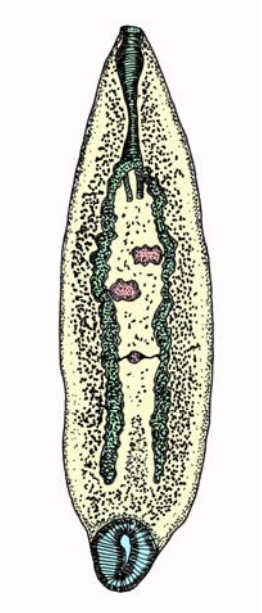
Adult digenes are found commonly in the intestine, although gall bladder, lungs, urinary bladder, oviducts, and veins are not uncommon. Developmental stages (sporocysts, rediae, cercariae) are found in some molluscs, especially some snails. Metacercariae are found encysted on vegetation or in tissues of invertebrates or vertebrates. Over the years, students have had poor luck finding developmental stages in snails, but always a great deal of success finding metacercariae encysted within fish, either in the visceral organs, musculature, or skin. Both adult and developmental stages of digenes tend to be delicate and must be fixed well soon after collection.

A variety of different stains are used to differentiate digenes. Good stains include Mayer's hematoxylin, Semichon's acetocarmine, Van Cleave's hematoxylin, and Malzacher's stain.
Although Van Cleave's is excellent and my favorite, perhaps the simplest for students is Semichon's acetocarmine. It's easy to make, has a long shelf life, and the processing steps are none too complicated. There will always be a bottle in the laboratory. However, if you ever wish to make or use Semichon's acetocarmine on your own, the following steps should be followed:

1. **Specimens in 70% ethanol should be placed directly into *Semichon's acetocarmine* stain. The staining times will vary depending upon the size of the specimen and concentration and age of the stain. Generally, staining time takes 2-4 hours for medium to large worms. The formula for Semichon's is as follows:**
   - **A. Glacial acetic acid (100 ml)**
   - **B. Deionized water (100 ml)**
   - **C. Carmine (lake) (as needed, about 3 g).**
   - **D. Mix acetic acid and water in a bottle; add enough carmine (1-2 g) until no more will go into solution (saturated). Place mixture in hot water bath and heat 15 min (temperature should not go above 95 C). Cool and filter stain. Add an equal amount of 70% ethanol. Stain will keep indefinitely.**
2. **After staining, place specimens into 70% ethanol (15-30 min)**
3. **Destain briefly in 70% acid ethanol (70% ethanol with 2-5 drops concentrated HCl/10 ml). The more acidic, the faster destaining will occur. This may take 15 seconds to 10 minutes, depending upon the specimen. You want the specimen to gradually fade in color, but not to become too pink.**
4. **Blue up the specimen in 70% basic ethanol (70% ethanol with 2-5 drops 1 N NaOH or ammonia). The more basic, the faster the bluish tint will appear. Here again, 15 seconds to 10 minutes.**
5. **70% ethanol (5 min)**
6. **95% ethanol (15-30 min)**
7. **100% ethanol, 2-3 changes (15-30 min each; for large specimens 3 changes)**
8. ***Optional step:* 100% terpineol (lilacin) (15 min) (for difficult specimens that may not clear well)**
9. **Xylene or toluene, 2 changes (10-20 min each)**
10. **Mount in Canada balsam or Damar gum.**

Processing and staining adult digenes using Semichon's acetocarmine

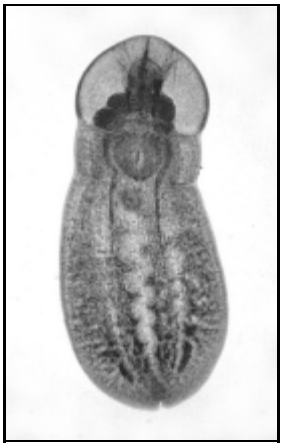
Most adult digenes should be carefully removed from the definitive host and placed directly into distilled water. Osmosis tends to result in expulsion of most eggs from the uterus, and slows worm movement. Once specimens slow and relax, they should be placed into AFA. (NOTE: digenes of fish and amphibian are in hosts adapted to aquatic environments; little osmosis may occur and no eggs may be expelled). All specimens should be kept in AFA at least 48 hr prior to processing to insure proper fixation. Between 2-7 days after collection, worms should be transferred into 70% ethanol for long term storage. Too long in AFA and worms will become brittle.



1. **For staining, transfer specimens from 70% ethanol directly into Semichon's acetocarmine stain (2-3 hr average). This can be accomplished either by transferring the worms into different small staining dishes containing the various solutions, or by pipetting solutions in and out of a single vial containing the parasites. Length of staining time will vary depending upon the size of the worms, and the age and concentration of stain.**
2. **70% ethanol (20-30 min)**
3. **Destain briefly in 70% acid ethanol (70% ethanol with 1 ml 1 N HCl/10 ml). The more acidic, the faster destaining will occur. Specimens will become pinkish-red, but do not destain long enough for them to become pale pink. The time will vary from 30 sec to 15 min, depending upon the stain, the size and type of worm, and the acidity of the ethanol.**
4. **Blue up in 70% basic ethanol (70% ethanol with 1 ml 1 N NaOH or ammonia/10 ml). The more basic, the faster the bluish tint will appear. Times will vary from 30 sec to 15 min.**
5. **70% ethanol (10 min)**
6. **95% ethanol (20-30 min)**
7. **100% ethanol, 2-3 changes (20-30 min each)**
8. **Toluene, 2 changes, 20-30 min each**
9. **Place specimen on microscope slide along with a drop of toluene. Do not allow specimens to dry out. Add a generous drop of Damar gum or Canada balsam. Make sure the mounting medium is not to thick and not too runny. Do not press down on coverslip or you may smash specimens.**

Staining metacercariae using Semichon's acetocarmine

Metacercariae of many species are common in fish in Kansas. They appear as tiny white or transparent dots embedded throughout the viscera, particularly liver. One genus of digene (*Clinostomum*) has very large metacercariae that appear yellowish and can be found embedded within the skin, fins, and musculature of catfish and bass.



1. **Specimens should be carefully removed from their gelatinous encasement, fixed for at least 48 hr in AFA, and then transferred into 70% ethanol for long term storage. Too long in AFA and specimens will become brittle.**
2. **Transfer specimens from 70% ethanol directly into Semichon's acetocarmine stain (2-3 hr average). This can be accomplished either by transferring the metacercariae into different small staining dishes containing the different solutions, or by pipetting solutions in and out of a single vial containing the parasites. Length of staining time will vary depending upon the size of the metacercaria, and the age and concentration of the stain.**
3. **70% ethanol (10-15 min)**
4. **Destain briefly in 70% acid alcohol (70% ethanol with 1 ml 1 N HCl/10 ml). The more acidic, the faster destaining will occur. The specimens will become pinkish-red, but do not destain long enough for them to become pale pink. The time may vary from 30 sec to 10 min, depending upon the stain, the size and type of metacercariae, and the acidity of the ethanol.**
5. **Blue up in 70% basic alcohol (70% ethanol with 1 ml 1 N NaOH or ammonia/10 ml). The more basic, the faster the bluish tint will appear. The time may vary from 30 sec to 10 min.**
6. **70% ethanol (5 min)**
7. **95% ethanol (15 min)**
8. **100% ethanol, 2-3 changes (10-15 min each)**
9. **Toluene, 2 changes, 10 min each**
10. **Place 5-10 metacercariae on a microscope slide along with a drop of toluene. *Do not allow specimens to dry out or they will be ruined.* Add a generous drop of Damar gum or Canada balsam. Make sure the mounting medium is not to thick and not too runny. Do not press down on coverslip or you may smash specimens.**

Cestodes

Adult tapeworms must first be removed carefully from the gut of an animal in such a way as not to damage the scolex (head of the worm). Often, simply by gentle coaxing of the worm with the hairs of a fine paintbrush will result in release of the specimen. Other times, scraping the host tissues deeply will result in worm release, whereas other times the worm and a small amount of surrounding tissue must be placed in ice water to induce release. Be patient.

Cestodes must also first be relaxed before fixation. Different investigators utilize different techniques, and there are several easy methods that usually work:

1. **If the worms are relatively small and free from host tissue, simply put them in water at room temperature. Most worms will gradually slow due to the osmosis, and they can be placed in fixative once their movements cease. Generally, 5-15 minutes. Too long and osmotic lysis of various internal organs may occur.**
2. **Worms may be placed in hot water or saline for 5-10 min. Here again, too long will cause osmotic lysis of various internal organs.**
3. **Place worms in 5% ethanol at room temperature.**

Larval stages (cysticercoids and cysticerci) and small cestodes may be handled and processed as digenes. However, many are larger. If the worm is medium in size, place it between two microscope slides and pipette fixative along edges of the slides. Allow to stand for 30 min, remove specimen, cut strobilae into 3-4 cm lengths, and transfer segments to a dish of fixative. Very large tapeworms must be wound around a bottle so that they do not overlap, pour hot fixative over the worm quickly, unwind and cut into 3-4 cm lengths, and transfer segments to a dish of AFA.

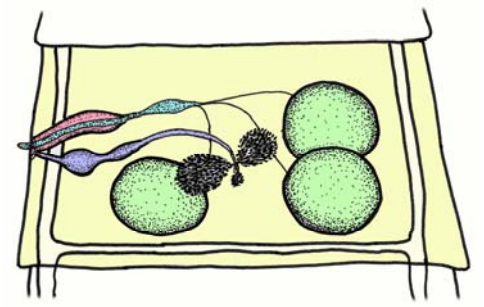
After 24-72 hr of fixation, tapeworms may be transferred to 70% ethanol and stored in ethanol permanently. Too long in AFA will result in brittle specimens. If permanent mounts are desired, stain and process the worms as with digenes. And, like digenes, good stains include Mayer's hematoxylin, Semichon's acetocarmine, Van Cleave's hematoxylin, and Malzacher's stain. Because of its simplicity, Semichon's will be used in the laboratory. The main difference in processing cestodes over digenes is the size of the specimens. Thus, it is important for medium to large specimens that the scolex/neck region (2-3 cm), several mature proglottids (2-3 cm), and several gravid segments (2-3 cm) are represented on each slide.

As per the digenes, the formula for Semichon's acetocarmine is as follows:

1. **Glacial acetic acid (100 ml)**
2. **Deionized water (100 ml)**
3. **Carmine (lake) (as needed, about 3 g).**
4. **Mix acetic acid and water in a bottle; add enough carmine (1-2 g) until no more will go into solution (saturated). Place mixture in hot water bath and heat 15 min (temperature should not go above 95 C). Cool and filter stain. Add an equal amount of 70% ethanol. Stain will keep indefinitely.**

Processing and staining cestodes using Semichon's acetocarmine

Once adult cestodes slow and relax, they should be fixed using AFA. Sometimes the entire specimen may simply be placed in the fixative, whereas other times the worm needs to be sandwiched between a glass microscope slide and a series of coverslips to flatten the specimen. Too much weight and the cestode will rupture. The interface is then flooded with fixative, after which time the partially fixed worm can be transferred to a vial of AFA (after about 10 minutes). Between 2-3 days after collection, worms should be transferred into 70% ethanol for long term storage. Too long in AFA and worms will become brittle.



1. **Transfer cestodes from 70% ethanol into Semichon's stain (2-4 hr average). This can be accomplished either by transferring worms into different staining dishes containing the various solutions, or by pipetting solutions in and out of a single vial containing the parasites. Length of staining time will vary depending upon worm size and age of the stain.**
2. **Transfer to 70% ethanol (20-30 min)**
3. **Destain briefly in 70% acid ethanol (70% ethanol with 1 ml 1 N HCl/10 ml). The more acidic, the faster destaining will occur. Specimens will become pinkish-red, but do not destain long enough for them to become pale pink. The time will vary from 30 sec to 15 min, depending upon the stain, the size and type of worm, and the acidity of the ethanol.**
4. **Blue up in 70% basic ethanol (70% ethanol with 1 ml 1 N NaOH or ammonia/10 ml). The more basic, the faster the bluish tint will appear. Times will vary from 30 sec to 15 min.**
5. **70% ethanol (10 min)**
6. **95% ethanol (20-30 min)**
7. **100% ethanol, 2-3 changes (20-30 min each)**
8. **Toluene, 2 changes, 20-30 min each**
9. **Place specimen on microscope slide along with a drop of toluene. Do not allow specimens to dry out. Add a generous drop of Damar gum or Canada balsam. Make sure the mounting medium is not to thick and not too runny. Do not press down on coverslip or you may smash specimens.**

Acanthocephala

Adult acanthocephalans are generally found only in the intestinal tract of vertebrates. Fish and turtles are your best hosts. Cut out a small square of tissue around any embedded worms, carefully tease away the host tissue from the proboscis if it's still everted, and place worms into cold distilled water. As water enters the worm by osmosis, the proboscis will evert (which may take 15 min to 3 hours) for about 50% of the specimens. An extended proboscis is essential for acanthocephalan identification and a good mount.

To kill the worms, place them directly into AFA. At the same time, puncture the tegument 3-4 times with a very thin pin (near the midbody and base of the proboscis) to allow better fluid exchange. After 3-4 days, transfer the worms to 70% ethanol for long term storage. If you plan to process the worms further, try to leave them in the 70% ethanol at least 48 hours or longer. Acanthocephalans may be stored permanently in 70% ethanol or stained and dehydrated for permanent mounts, as described below.

Acanthocephalans of appropriate sizes that fit on microscope slides may be either stained or unstained. If they are stained then Van Cleave's, Mayer's hematoxylin, or Semichon's acetocarmine are all suitable. Our laboratory will have Semichon's available. Incubation times for all steps should be double to triple of that used for digenes and cestodes.

1. **Transfer worms from 70% ethanol into Semichon's stain (2-4 hr average). This can be accomplished either by transferring worms into different staining dishes containing the various solutions, or by pipetting solutions in and out of a single vial containing the parasites. Length of staining time will vary. (To make Semichon's, mix glacial acetic acid (100 ml) and 100 ml of deionized water in a bottle. Add 1-3 g of carmine (lake) until no more will go into solution (saturated). Place mixture in hot water bath and heat 15 min (temperature should not go above 95 C). Cool and filter stain. Add an equal amount of 70% ethanol. Stain will keep indefinitely).**
2. **Transfer to 70% ethanol (60 min)**
3. **Destain briefly in 70% acid ethanol (70% ethanol with 1 ml 1 N HCl/10 ml). The more acidic, the faster destaining will occur. Specimens will become pinkish-red, but do not destain long enough for them to become pale pink. The time will vary from 1 min to 30 min, depending upon the stain, type of worm, and the acidity of the ethanol.**
4. **Blue up in 70% basic ethanol (70% ethanol with 1 ml 1 N NaOH or ammonia/10 ml). The more basic, the faster the bluish tint will appear. Times will vary from 1-30 minutes.**
5. **70% ethanol (30 min or more)**
6. **95% ethanol (60 min)**
7. **100% ethanol, 3 changes (30-60 min each)**
8. ***Optional step.* Clearing of acanthocephalans can be difficult. Transfering from 100% ethanol to terpineol, toluene or xylene often results in worm collapse. To help avoid this, first pour in clearing agent (terpineol) into a dish and add an overlay of 100% ethanol. Next, transfer worm from 100% ethanol to the bi-layered solution. It may first float at the interface of the fluids where exchange of fluids will occur gradually. Eventually, the worm will sink. Although many specimens can be placed in xylene or toluene directly without using terpineol, terpineol tends to penetrate the cuticle well and allow for a better specimen. After the specimen has sunk, pipet off the top layer of alcohol and replace the clearing agent with fresh toluene.**
9. **Toluene, 2-3 changes (30 min each)**
10. **Place in 3:1 solution of toluene and Canada balsam (30 min)**
11. **Place specimen on microscope slide along with a drop of toluene. Do not allow specimens to dry out. Add a generous drop of semi-dilute Canada balsam and mount specimen.**

Nematodes

Nematodes will normally contract when added to fixatives or ethanol. Therefore, most live worms should be placed directly into cold glacial acetic acid for 5-10 seconds for straightening; then place them into 70% ethanol. However, there are some species that rupture when placed in acetic acid. For these, add them to very warm (not boiling) 70% ethanol and they usually relax.

Nematodes may be stored permanently in a solution of 70% ethanol with 5% glycerol. The glycerol helps soften and clear the worm, plus keeps the specimen moist in case the ethanol evaporates from the container. If permanent mounts are desired, however, try the following procedure (NOTE: not all nematodes have a cuticle that allows preservatives to pass through easily. Thus, they may prove difficult or impossible to process):

1. **Place nematodes back into 70% ethanol (at least 30 min) if they aren't in 70% ethanol already.**
2. **95% ethanol (30 min)**
3. **100% ethanol (30 min)**
4. **100% ethanol (30 min)**
5. **Xylene or toluene (15 min)**
6. **Xylene or toluene (30 min)**
7. **3:1 mixture of xylene or toluene and Canada balsam (30 min)**
8. **Mount in Canada balsam**

Again, due to the cuticle, nematodes can be difficult to mount permanently. Some may prove impossible. The following represents some trouble shooting tips:

Worm collapse ("shrivel"). **Solutions are rushing out faster than solutions are rushing in. This is due to the tough cuticle. Perhaps place specimens in the 3:1 interface solution for a longer period of time, or prick the cuticle in 1-2 places with a very fine needle. The shriveled specimen may or may not be salvageable.**

Worm, or area around worm, turns cloudy (milky) either immediately or within a few days. **Whoops! Some water was still within the specimen and has reacted with the toluene/xylene/terpineol. Run worm back to 100% ethanol, wait until all of the milky color disappears, and then either process more slowly or/and prick the cuticle in a couple places where it won't be noticed; then, re-process.**

Worm turns dark. **Vacuoles formed. Xylene/toluene exited specimen faster than Canada balsam could enter and a void is left behind. Run worm back to the 3:1 interface solution and allow specimen to stay in this solution longer. Some specimens may need to be pricked with a pin before beginning again.**

Leeches

Leeches are commonly found in lakes, ponds, and streams where they may either be associated with a fish or turtle, or be free under rocks and logs. The most commonly encountered leech in Kansas seems to be *Placobdella parasitica*, a common turtle leech. We've encountered some great specimens over the years, many with dozens of offspring attached. You do not have to stain these leeches, although small leeches can be stained just like digenes. Large leeches are never stained and usually are not mounted. Whether you are dealing with a small leech or a large leech, you'll note that they respond to various solutions quite differently than do the Platyhelminths. The end result is that they often shrivel up into a ball and die if fixed without proper relaxation. Luckily, there are several ways that leeches can be relaxed prior to fixation so that the end product is a natural, life-like specimen.

1. **First, place leeches into a small or medium size bowl of tap water.**
2. **Next, leeches need to be relaxed. In our laboratory, we float some menthol crystals over the surface of the liquid. During the next 15-60 minutes, the leeches should become limp and will not respond to touch. Other methods that have been described to me include dilute solutions of ethanol mixed in the water, and simply placing the leeches in carbonated soda water where the carbon dioxide gradually narcs the specimen to submission.**
3. **Remove the leeches from the water**
4. **Place leeches between two microscope slides, clamp slides together if necessary, and flood the interface with AFA. Allow to partially fix for 15-30 minutes.**
5. **Remove microscope slides and place worms in AFA for 48 hrs**
6. **Transfer leeches to 70% ethanol and either store indefinitely or stain as for digenes. If you opt to stain the small specimens, lengths of dehydration and staining times will need to be increased to 60 or more minutes each).**

Placobdella parasitica
(from Manhattan, KS)

Arthropods and Pentastomes

The phylum Arthropoda contains a variety of parasites, most of which occur externally. The pentastomes are an off-shoot group of arthropods and exist in the respiratory tract of some vertebrates, especially reptiles. Virtually all specimens are simply placed directly and live into a solution of 70% ethanol for fixation, and then later processed as below:

1. **Transfer arthropods from 70% ethanol into 10% (w/v) aqueous potassium hydroxide solution (KOH) or lactophenol. Leave specimens in the solution for a matter of minutes (i.e. some mites), an hour or so (i.e. some tick larvae), or 72 hours or longer (some adult ticks, dipteran larvae). The length of clearing time depends upon size and type of specimen, and takes a bit of practice. DO NOT process all of your specimens at once in case you make a mistake and over-clear. The exoskeleton should become a bit more transparent and lighter in color, but stop the process before specimens become clear. Warm (but not boiling) KOH is faster and will clear specimens in 10-15 minutes. Watch mites carefully, since they clear fast and may disappear all together. I do not recommend clearing mites (other than *Varroa jacobsoni*, ca 20 min) because its such a tricky process with these guys. NOTE: pentastomes are not cleared.**

2. **Dipteran larvae, for instance bot flies or larvae from the body cavity of grasshoppers, are usually not mounted. However, small ones can be injected by syringe with AFA or 10% formalin until they swell a bit (but do not rupture) at same time they are being immersed in the same fixative. Fix for at least 48 hr. The larvae can be cleared for one to several days in 10% KOH solution as above. You may have to prick the cuticle in several cryptic places first to allow solutions to enter.**

3. **When you are ready to mount specimens, remove then from the 70% ethanol and place them in 95% ethanol (15-30 min).**
4. **100% ethanol, two changes minimum (15-30 min each).**
5. **Toluene or xylene, two changes minimum (15-30 min each).**
6. **Mount in Canada balsam or Damar gum.**

Although we generally use KOH to clear arthropods, a common clearing agent that is used prior to dehydration is lactophenol. This solution is less harsh on the exoskeleton and is preferred by arthropodologists. Mites and larval ticks fare better in lactophenol since KOH may damage some of the surface bristles, which are important diagnostic features. Lactophenol contains phenol, however, which is highly toxic so be careful if you opt to use this solution. The raw formulation is simply lactic acid (2 parts), phenol crystals (1 part), and distilled water (1 part).

Mounting specimens onto microscope slides

For permanent mounts, a variety of resinous media are available. These include Permount, Histoclad, Kleermount, Damar gum, and Canada balsam. The latter two are the only ones that have been shown to satisfactorily withstand the test of time without cracking or forming bubbles. The major disadvantages of Canada balsam are that it yellows with time and takes years to harden.

Care must be exercised to orient the specimen properly. You should use a small paint brush to manipulate the parasite so that it won't be damaged. Specimens should be placed in the middle of the slide relative to top and bottom. However, when considering the left and right aspects of the slide, remember that a slide label will be on the *left* side. Therefore, place the specimen approximately 2/3 of the distance from the left and 1/3 of the distance from the right of the slide. *Take care, since placing the specimen too close to the right will mean that the coverslip will rub in the groove of the slide box and may eventually break.* The coverslip should be 5-8 millimeters from the right margin of the slide.

Thick specimens will require coverslip support. If you do not use support for the coverslip corners, then the coverslip will rest atop your specimen and often become slanted to one side during drying. Old methods of elevating the coverslip included placing stacks of small pieces of broken coverslips in the balsam in the corners under the intact coverslip. Pieces of capillary tubes were sometimes also used. More recently, however, tiny vinyl prop coverslip supports (my favorites) have become available. These can be stacked to any height desired in the four corners under the coverslip and have proven to be superior to other methods.

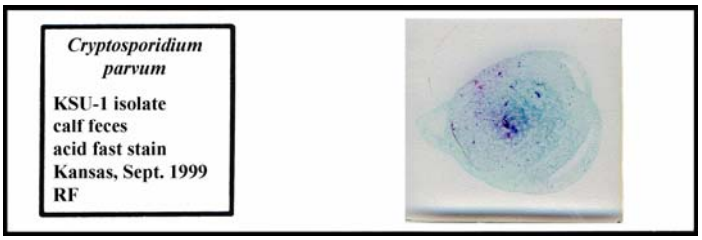
Specimens should be oriented properly. Organisms to be viewed under a brightfield microscope should be oriented upside down and backwards, since the microscope will reverse the light paths and present the specimens in the proper orientation when viewed. Specimens viewed with dissecting scopes should be oriented as you would view them without a microscope. Some specimens have traditional orientations written in stone by the old classical parasitologists. For instance, fleas are always mounted facing right and upside down so that when they are viewed in the microscope they can be seen facing left and right-side up. Ticks are mounted dorsal side up, with the head towards the top of the slide.

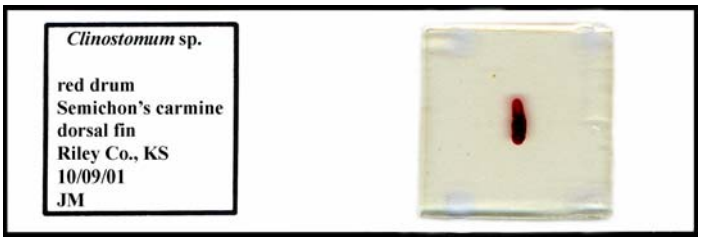
Dried or semi-dried mounting medium can be later cleaned from slides and coverslips using a cotton-tipped applicator stick (i.e. Q-tip), kimwipe, or kleenex moistened in xylene or toluene. Rub lightly and you will eventually be able to dissolve all of the excess mounting media. Be careful so to not break or displace the coverslip. Large amounts of excess medium on the slide itself can also be scraped away using a razor blade once dry. Do not contaminate clean toluene or xylene with used kimwipes or cotton-tipped applicator sticks that have adhering medium.

The slide label should be placed on the left side of the clean slide, but at least a few millimeters from the edge of the slide. Make sure that all excess mounting media is cleaned from the slide prior to adding the slide label. The label should have, in the following order top to bottom, the following information:

1. **The species of parasite (binomial name whenever possible)**
2. **Common name of parasite (optional)**
3. **Species of host (binomial name or common name)**
4. **Site of infection**
5. **Type of stain used, if any**
6. **Geographic location where collected and date of collection**
7. **Collectors name or initials**

Sample slides





(note the spherical slide risers in some of the corners AND the fact that the coverslips are an acceptable distance from the right hand margin of the slides)

Miscellaneous techniques

Although in most cases the average student will not utilize any of the following techniques during the course of the semester, some of the listed techniques might prove useful later. Therefore, I've included some handy procedures for the record.

Formalin-ether sedimentation technique.

Sometimes a sedimentation technique is desired which eliminates much of the debris associated with feces plus it concentrates protozoan cysts and helminth ova into a smaller volume. It allows the researcher to find parasites, if present, much more readily than a straight fecal smear alone. However, if the number of parasites in feces is low enough to warrant such a technique, then generally the sample contains too sparse of parasites for a classroom slide. The following represents the procedure:

1. **After you have fixed the feces containing ova in whatever formalin-based fixative you wish, pour the fecal suspension through 2 layers of cheesecloth. You normally want a 2-4 ml volume in the tube; then, top off with additional fixative until total volume is 10-12 ml.**
2. **Centrifuge at 1000-1500 rpm, 10 min.**
3. **Decant (pour off) off supernatant.**
4. **Add 10 ml of fresh fixative and mix sediment into it well.**
5. **Add 2-3 ml ether.**
6. **Stopper the tube and shake or vortex well.**
7. **Remove stopper and centrifuge 1500 rpm for 1-2 minutes.**
8. **After centrifugation, four layers should be evident:**
   - **A. a top layer of ether.**
   - **B. a thin layer of debris.**
   - **C. the formalin based fixative**
   - **D. the sediment in bottom with parasites.**
9. **Carefully ring band of debris with applicator stick and carefully pour off ether, debris, and formalin. Add a few drops of fresh fixative and resuspend sediment for examination or prolonged storage.**

Pepsin-HCl digestion technique.

Although most of you will not need this technique, tissue digestion is often used to obtain larval digenes and especially nematodes. This is the accepted technique used to survey for trichinosis larvae in wildlife, where heavy concentrations of larvae occur in tongue. The following represents the formula:

1. **Saline (Ringer's, Locke's, or PBS) (198 ml)**
2. **HCl (concentrated) (2 ml)**
3. **Pepsin (2 g)**

Simply add a small piece of tissue. The above solution works well for digestion of larvae from diced tongue samples (3-4 hrs). Using a simple wet mount, live larvae can be seen thrashing around under the microscope. Dilution of this sample in half with saline allows for digestion of skin and viscera for more delicate worms (ca 1 hr).

Sucrose flotation technique.

The sucrose flotation technique is useful when you wish to concentrate protozoan cysts from feces and see if an infection is present. The technique also works well for some nematode eggs, but is poor for many cestode and most trematode ova as they tend to sink.

1. **In a 15 ml conical centrifuge tube, mix small amount of feces (1/2 to 1 gram) with 1-2 ml water, fixative, or other liquid.**
2. **Add sucrose solution (sp. gr. 1.18-1.3) to tube until level is barely above top of tube. When viewed sideways, the liquid should barely be seen rising above tube.**
3. **Place a 22 mm square coverslip atop the tube so that it touches liquid. A small air bubble may form under coverslip, which is OK. If over 1/2 of area of coverslip is taken up by bubble, you have not added enough sucrose solution.**
4. **Centrifuge at about 1,000-1,500 g for 8-10 minutes and parasites will rise to the surface atop the sucrose solution.**
5. **Remove coverslip. Many parasites will stay within the small amount of liquid at the coverslip interface. Place coverslip liquid side down on microscope slide. Examine microscopically. Any parasites in feces should be concentrated well enough to be seen.**

Reclaiming specimens.

Old, dried-out or shriveled specimens stored in ethanol can sometimes be reclaimed. Use a 0.25%-0.5% w/v aqueous trisodium phosphate solution (Na_3P0_4) until specimens regain shape. For microscope slides where the slide is damaged or the mounting medium is crystalizing, specimens can generally be rescued. Two procedures can be used:

1. **Place damaged slide in freezer for 20 minutes or more and, immediately upon removal from the freezer, flick off the coverglass with forceps or dissecting needle. Chip away old mounting media with razor blade and then brush away any small particles. Add a small drop of toluene and then Canada balsam or Damar gum. Add a new coverglass.**
2. **Alternatively, place entire slide into Coplin jar or horizontally in a dish containing toluene. Coverslip will slide off eventually, depending upon amount and age of mounting media, and specimen can be remounted in Canada balsam or Gum damar**

Air in whole mounts.

Sometimes when transferring specimens into Canada balsam or Damar gum, the terpineol, xylene or toluene leaves the specimen faster than the mounting media can replace it. Opaque or dark spots (voids) may appear. You may try several avenues to help remedy the situation. First, try leaving the specimen in the terpineol and xylene/toluene solutions longer. Next, try using xylene in the Canada balsam or Damar gum instead of toluene, which has a different exchange rate. Try a more dilute mounting media, where exchange should occur faster. You may also wish to place specimens through several changes of xylene/mounting medium mixtures (1:1 - 1:6) for a slower exchange. Some authors prick the cuticle of the worm in a place where it won't be noticable, but this requires a very fine needle. Alternatively, other oil clearing agents besides terpineol can be used in place of xylene or toluene. Oils do not allow air bubbles to form because they are removed more slowly than toluene. Examples include beechwood creosote and methyl benzoate; however, these are difficult to obtain since few companies manufacture them anymore.

Miscellaneous solutions not described in text

Although most solutions are pre-made in the laboratory, you may occasionally run out with no one around to help. The following represents some additional formulas that may prove to be useful:

Alcohol-formalin-acetic acid (AFA) fixative:

1. **95% ethanol (30 ml)**
2. **formalin (stock solution) (10 ml)**
3. **deionized water (50 ml)**
4. **glacial acetic acid (add within one month of use) (10 ml)**

1:4000 formalin solution:

1. **formalin (stock solution) (0.25 ml)**
2. **deionized water (99.75 ml)**

10% formalin:

1. **formalin (stock) (10 ml)**
2. **deionized water (90 ml)**

Carnoy's II fixative:

1. **100% ethanol (60 ml)**
2. **chloroform (30 ml)**
3. **glacial acetic acid (10 ml)**

Crystal violet solution (1% aqueous):

1. **crystal violet stain (1 g)**
2. **deionized water (100 ml)**

Davidson's fixative:

1. **95% ethanol (30 ml)**
2. **37% formaldehyde (20 ml)**
3. **glacial acetic acid (10 ml)**
4. **deionized water (30 ml)**

Eosin Y counterstain solution (1% aqueous):

1. **eosin Y stain (1.0 g)**
2. **glacial acetic acid (0.2 ml)**
3. **deionized water (100 ml)**

Gram's iodine solution:

1. **iodine (1 g)**
2. **potassium iodide (2 g)**
3. **deionized water (300 ml)**

Mammalian saline solution:

1. **sodium chloride (0.85 g)**
2. **deionized water (100 ml)**

Methylene blue working solution:

1. **methylene blue (0.5 g)**
2. **glacial acetic acid (0.5 ml)**
3. **deionized water (100 ml)**
4. **always filter stains prior to use**

Neutral buffered formalin:

1. **37% formaldehyde (100 ml)**
2. **deionized water (900 ml)**
3. **sodium phosphate monobasic (anhydrous) (6.5 g)**
4. **sodium phosphate dibasic (6.5 g)**

Reptilian saline solution:

1. **sodium chloride (0.5 g)**
2. **deionized water (100 ml)**

Potassium hydroxide solution (10% aqueous KOH):

1. **potassium hydroxide (10 g)**
2. **deionized water (90 ml)**

Sucrose solution for sucrose flotations:

1. **pour a 4 lb or 5 lb bag of table sugar into a container capable of withstanding heat.**
2. **add 1160 ml deionized water (4 lb bag) or 1450 ml deionized water (5 lb bag).**
3. **heat mixture until sugar just barely goes into solution; just as it comes to a boil.**
4. **remove from heat immediately and allow solution to cool.**
5. **add 8 ml liquid phenol (4 lb bag) or 10 ml liquid phenol (5 lb bag) as preservative.**
6. **store in glass, stoppered container.**