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INTRODUCTION

Over 70 different species of parasites, belonging to two major groups (Protozoa and Helminths), can be found in various parts of the human body.

Parasitosis may result from exposure via one or more of the following sources: 1) contaminated soil or water; 2) food containing the immature infective stage of the parasite; 3) a blood sucking insect; 4) a domestic or wild animal harboring the parasite; 5) another person, their clothing, bedding, or the immediate environment that they have contaminated; or 6) oneself.

Competent laboratory work is dependent on several factors; 1) satisfactory specimens; 2) safe and adequate facilities, including a good quality microscope; 3) personnel trained in examining specimens and accurately identifying organisms; and 4) personal trained in safety and protection from stool, body fluid and blood-borne pathogens (Universal Precautions).

The most common types of body material submitted for parasitology examination are stools and blood, however other materials, such as anal swabs, urines, aspirates, abscesses or respiratory specimens, surgical specimens and biopsies may all be submitted in certain cases.

In diagnostic parasitology accuracy is most important, for careless work may result in the lack or delay of treatment for an infected patient or treatment for an incorrectly diagnosed infection. Quality work is based on two principals; quality control (QC) and quality assurance (QA). Quality control ensures that each step of the process is done properly, while quality assurance ensures that the entire process produces the correct result. The definitive diagnosis must rest on positive identification of species present, not on speculation.

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LABORATORY SAFETY GUIDELINES

TREAT ALL SAMPLES AS BIOHAZARDOUS MATERIAL

As a general operating procedure all material should be considered to be a biohazard. It is therefore important that all unpreserved and unfixed samples should be handled with suitable gloves and that appropriate precautions are taken when handling the material (Universal Precautions).

Everyone working in the lab should read and be familiar with *Protection of Laboratory Workers from Infectious Disease (NCCLS Document M29-T2)* (see next page) and should consult the orange covered copy of *Laboratory Safety Manual (WHO)*.

ALL GENERAL GUIDELINES FOR LABORATORY SAFETY APPLY (See TML Safety Manual).

- Wear gloves when required
- Never mouth pipette
- No smoking or consuming food or drink anywhere in the laboratory
- Do not work with uncovered opened cuts or broken skin. Cover with suitable dressing and latex gloves.
- Do not create aerosols. Use extreme care when operating centrifuges, stirrers, pipetters etc. Cool inoculating loops or needles before touching colonies on plates or in liquid material
- Wipe off benches in your working area with suitable disinfectant before and after each day's work.
- Do not wear lab coats outside the lab.
- Do not place personal items such as eyeglasses on workbench.
- Beware of reactive and poisonous chemicals and handle them with respect.
- All fixatives and chemicals should be properly labelled.
- Know in advance where you nearest fire extinguishers are located.
- Always wash your hands before leaving the laboratory.
- Be aware that all specimens may contain biohazardous agents and protect yourself accordingly.
- Clean up any spills (generally with 1% bleach) before proceeding
- Make sure your co-workers are aware of any chemical or biological hazards that exist.

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Correct disposal of material is essential! All samples must be disposed of in the yellow plastic containers (sharps, glass or liquid), or in the designated biohazard disposal bag (non-sharp solids). If in doubt, ask for help. Do not dispose of non-biohazardous materials in the biohazard waste-- this is a waste of space and money.

Any percutaneous injury with sharps or any exposure to blood or body fluids on broken or damaged skin should be reported immediately to the lab director and Employee Health. If unsure always ask.

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TURNAROUND TIMES (TAT)

The Parasitology Laboratory is open on a weekly basis from Monday to Friday. An on-call service is provided – only for malaria requests – on weekends and statutory holidays.

1. STAT SPECIMENS

All malaria bloods are considered STAT. A joint malaria protocol exists between the Hematology Department, Toronto Medical Laboratories and the Toronto Medical Laboratories/Mt. Sinai Hospital Department of Microbiology. All malaria samples are screened by the Hematology Lab or by the Rapid Response Labs at both Toronto General and Toronto Western Hospitals and are resulted within 2 hours. All screening results are confirmed by a qualified Microbiology Technologist within 18 hours.

2. SPECIMENS REPORTED WITHIN 24 HOURS.

- Skin snips for microfilaria
- Urine sediments for *S. haematobium*
- Aspirates/fluids (i.e. liver aspirates, duodenal aspirates, CSF, etc.)
- Bloods for microfilaria and Trypanosomes
- Adult worm identifications
- direct smears from Skin Scrapings for *Leishmania*
- Dark Field requests
- Schisto Hatch
- Pinworm preps

3. SPECIMENS REPORTED WITHIN 48 TO 72 HOURS

- *Acanthamoeba* culture – preliminary reading
- *Strongyloides* agar cultures

4. SPECIMENS REPORTED WITHIN 5 WORKING DAYS

- Stool for Ova and Parasite (including *Cryptosporidium/Cyclospora*)
- Slit skin smears for leprosy

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5. SPECIMENS REQUIRING MORE THAN 5 DAYS

- *Acanthamoeba* cultures held for 7 to 10 days
- *Leishmania* cultures held for a total of 21 days (if negative), checked at 10 and 14 days
- Stool O&P samples requesting microsporidium are sent to the Public Health Laboratory for testing. TAT is approximately 10 days.
- Samples requesting worm/insect identification are also sent to the Public Health Laboratory for testing. TAT is approximately 10 days.

REFERENCES

National Committee for Clinical Laboratory Standards. Protection of Workers from Infectious Disease Transmitted by Blood and Tissue. Proposed Guideline M29-P1 National Committee for Clinical Laboratory Standards, Villanova, PA 1987

National Committee for Clinical Laboratory Standards. Clinical laboratory Waste Management. Tentative Guideline GP5-T National Committee for Clinical Laboratory Standards, Villanova, PA 1991

National Committee for Clinical Laboratory Standards. Protection of Workers from Infectious Disease Transmitted by Blood, Body Fluids and Tissue. Tentative Guideline M29-T2 National Committee for Clinical Laboratory Standards, Villanova, PA 1991

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COLLECTION AND PRESERVATION OF STOOL SPECIMENS

Introduction

The generation of clinically meaningful test results must begin with stringent criteria for specimen acceptance or rejection and specimen handling. Unless specimens are properly labeled, collected and processed, time and reagents will be wasted and the test results may mislead the physician. Ensuring proper specimen collection and processing is part of the laboratory “Continuous Quality Improvement Program”.

PATIENTS SHOULD BE GIVEN WRITTEN AND VERBAL INSTRUCTIONS TO FACILITATE PROPER COLLECTION OF SAMPLES (See Appendix XXII).

Factors Affecting Samples

Fecal samples should be collected in clean specimen containers with tight fitting lids to prevent accidental spillage. The specimens should not be contaminated with water or urine, or retrieved from the toilet bowl because the motile forms of protozoa will be destroyed. In addition, free living organisms may be present in the water and would cause contamination of the specimen. Samples contaminated in this manner are not suitable specimens and would not be accepted by the laboratory. These specimens would be canceled in the computer with a comment stating the reason why they were not suitable.

Criteria for Rejection:

- There is *any* sign of leakage
- They are not correctly labeled
- Requests for more than examination of more than one sample collected on the same day (unless a clinical consult is obtained from lab director)
- Requests for more than 3 stool examinations for a single episode of diarrhea or clinical syndrome (unless a clinical consult is obtained from lab director)
- There is any sign of contamination (water, urine, non-fecal debris)
- There is evidence of barium
- It is known that the patient had been taking nonabsorbable anti-diarrheal drugs, mineral oil based laxatives, or antimicrobials within 1 week.

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- If the sample comes from an inpatient who develops nosocomial diarrhea (has been admitted >3 days prior to onset of symptoms) without a clinical consultation with a Tropical Disease physician or lab director.
- Liquid fecal samples greater than 60 minutes after passage before processing or fixation.
- Formed stools greater than 24 hours after passage before processing or fixation.

Nonabsorbable antidiarrheal drugs and antimicrobials may interfere with the detection of intestinal protozoa. Specimen collection should be delayed for at least 7 days after Barium, mineral oil, or antibiotics. Specimens showing the presence of substances such as barium will result in specimen rejection by the laboratory and the order being canceled in the computer.

Number of Specimens and Collection Time

Because of the intermittent passage of certain parasites, the possibility of finding organisms is increased by examining multiple specimens.

- It is suggested that 3 specimens, collected at 2 to 3 day intervals, should be examined both pretreatment and post treatment (to ensure eradication of documented pathogenic protozoa).
- Post therapy examinations should be performed 3-4 weeks after therapy for protozoa and 5-6 weeks after therapy for Taenia (gut tapeworm) infections.
- Examination of more than 3 stools is rarely useful and requests for > 3 stools should be referred to lab director before processing.

Occasionally specimens may be obtained following the use of a cathartic such as magnesium sulfate or normal saline enemas.

Type and Stability of Stool Specimens

Fresh stools are essential for the recovery of motile trophozoites which are most likely to be found in the order of liquid > soft > formed stools.

- Liquid and soft stools should be examined and/or preserved in SAF fixative within 30 minutes and one hour of passage respectively.
- Formed stools should be examined and/or preserved in SAF fixative within 12 hours of passage.

Fresh stool only is a suboptimal specimen. It will be accepted and processed (provided it is within time limits) but a request should be made for additional SAF preserved specimens.

Preservation of Stools, and Fixatives

Because of the workload within the laboratory or transit distance/time for the specimen to reach the laboratory, most laboratories recommend preservation of the specimens. Sodium acetate acetic-acid formalin (SAF) is the fixative currently used in our laboratory because it is useful

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for both concentration and permanent stains and is relatively safe and easy to use compared to other fixatives. The SAF kit is available from the Central Supply.

Transport and Mailing of Specimens

Double mailing containers should be used in shipping any parasitological specimens other than microscope slides. The specimen vials/tubes in an inner aluminum container should be packed in cotton or tissue papers to absorb any moisture or material that might result from leakage or breakage. The screw-capped inner container is put into an outer cardboard screw-capped mailing container. Patients' and other information sheets may be wrapped around the inner cylinder before it is placed in the outer cardboard mailer. Alternatively commercially available "Saf-T-Pacs" can be used. Prepared slides may be packed in boxes, cardboard slide holders or any container that will prevent damage or breakage.

Health Canada, Transport Canada and Transport companies have regulations concerning the shipment of dangerous goods such as liquid nitrogen, dry ice, and biological samples. It is your responsibility to know the rules and comply with them. Courier companies employ experts in the transport of dangerous goods. Please consult them and the Microbiology Manager to facilitate appropriate and safe shipping.

Sending Samples to Reference Laboratories

Any parasitic specimens that cannot be identified by our staff can be sent to the **Provincial Health Lab, Parasitology Dept.** for identification. The following guidelines should be followed:

- If there is a question about the identification of the parasite, split the sample and send a portion to the Provincial Laboratory for confirmation. If the result is urgently required phone the PHL and inform them.
- Tapeworm segments and other worms: if at all possible submit specimens alive in saline (0.85% NaCl). If a long delay is anticipated (5+ days) submit the specimen in SAF.
- Mites, ticks, fleas, lice, fly maggots, etc. can be sent "dry" in a leak proof double bagged container. Try not to crush the specimen and a live sample may be the best choice (particularly for a fly maggot).
- Microscope slides should be shipped in a cardboard slide container and should be clearly labeled as to their origin.
- Complete a PHL Special Parasitology form 245-44 (84/08), note the sample is being sent in the appropriate log book and arrange for pick up and transport.
- When results are received and entered on-line, the name and address of the testing laboratory must appear on the report.
- In the case of a positive result, the lab number of the referring lab must also appear on the report.

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INTRODUCTION

The usual diagnostic stages of intestinal parasites are helminth eggs and larvae and protozoan trophozoites and cysts. In general, nematodes, such as *Ascaris*, Hookworm and *Trichuris* shed eggs more or less constantly and may be detected daily in feces. Other parasites, especially protozoa, are passed irregularly and possibly for only a few days at a time. In certain helminth infections, particularly Schistosomes, and those caused by *Diphylobothrium sp.* and *Taenia sp.*, eggs may be passed intermittently.

Most specimens will be collected in SAF preservative and the specimens must be handled so that these parasite stages, when present, will be identifiable when the specimen reaches the laboratory. Concentration and staining procedures can be performed on the same preserved sample. Inadequate samples (see criteria for rejection see page 6) are usually of little value in establishing a diagnosis and may lead to erroneous results.

The microscopic examination of the stool specimen consists of three separate techniques:

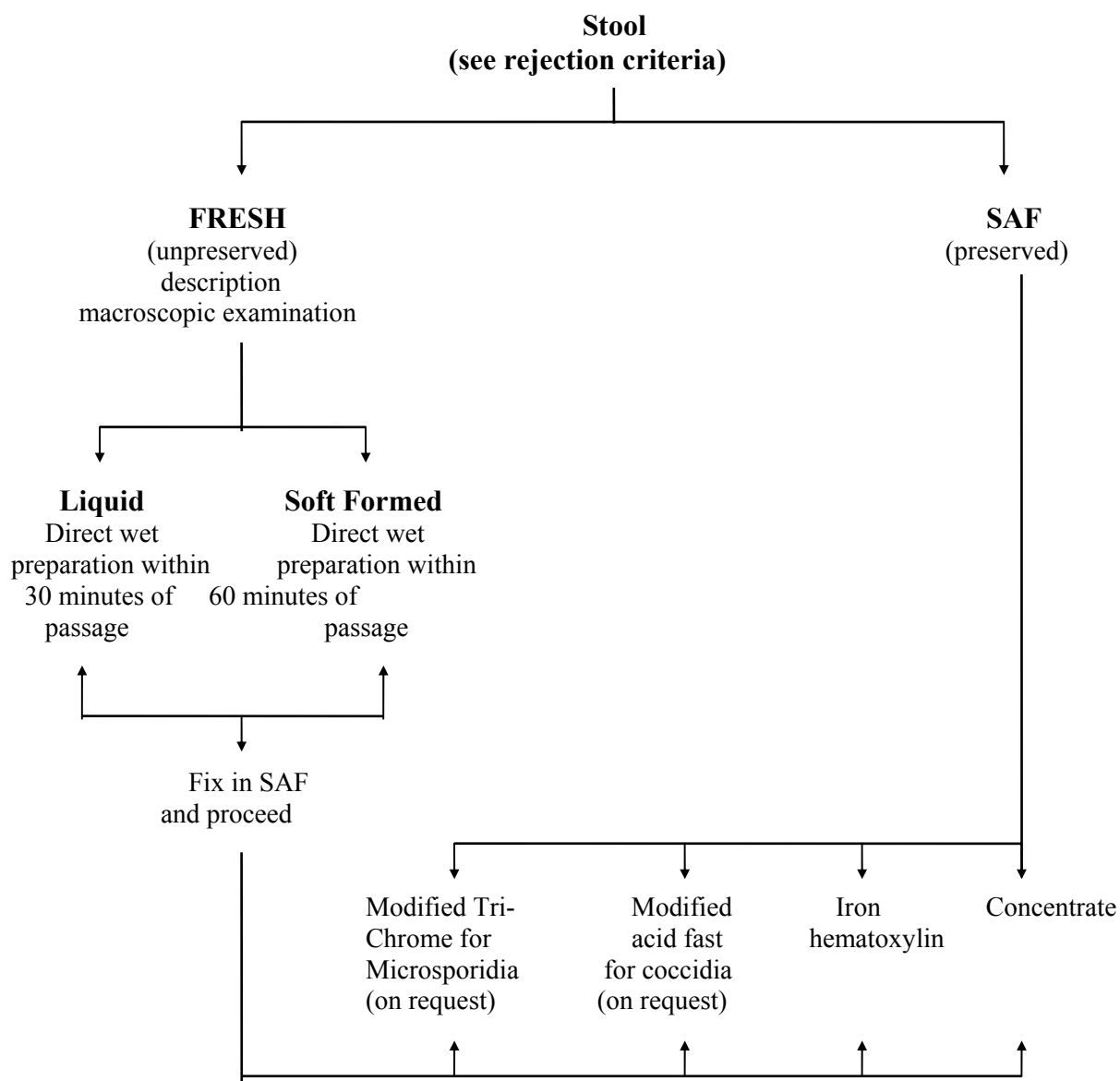
1. The direct wet smear
2. The concentration
3. The permanent stain smears.

SEE FLOW CHART ON NEXT PAGE

Because the great majority of stool specimens are now collected directly into SAF preservative the direct wet smear is no longer a mandatory part of the routine ova and parasite examination. However if fresh fecal specimens are delivered to the laboratory the direct wet smear should be performed particularly on liquid stools.

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Procedure: Examination of Stool



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Direct Wet Preparation Of Fresh Stools

PRINCIPLE

The value of wet preparations lies in the fact that certain protozoa trophozoites retain their motility which may aid in their identification. Definitive identification however may not be possible, especially for amoeba, since the nuclei of trophozoites and cysts are often not clearly visible. Wet preparations on fresh unpreserved liquid stool should be performed and examined as soon as possible (within 30 minutes of passage) and on soft/formed stool within 60 minutes of passage provided that prior arrangements have been made with the lab. Wet preps can also be used to determine schistosome egg viability (“flame” cells). (Wet preps can also be performed on SAF fixed specimens but motility is lost since the fixative kills the parasites.)

SPECIMEN

- Fresh liquid stool (within 30 minutes of passage - by prior arrangement with lab)
- Duodenal or small bowel aspirate in SAF or if fresh - within 30 minutes of collection by prior arrangement with lab.
- Abscess sample
- Respiratory sample
- CSF sample
- Urine
- Stool in SAF (no motility possible)
- External QC samples

MATERIALS

Reagents

Normal saline (0.85%)

Lugols iodine (commercial product—Snap N’ Stain).

Preparation:

potassium iodide	10 gms
powdered iodine crystals	5 gms
distilled water	100 mls

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1. Dissolve potassium iodide and iodine crystals in distilled water in a flask or bottle using a magnetic stirrer.
2. The potassium iodide solution should be saturated with iodine with some excess crystals left on the bottom.
3. Store in a tightly stoppered brown bottle protected from the light.
4. Label the bottle with the expiration date of one year.
5. Dilute a portion 1:5 with distilled water for routine use (working solution). Place this working solution in a dropper bottle and discard when the color lightens (within 14 days).

Equipment:

Pasteur pipettes
Glass microscope slides
Applicator sticks
Glass cover slips, (22 x 22 mm)
Sharps disposal container
Light microscope with ocular micrometer and set for Kohler illumination.

QUALITY CONTROL

For direct smear:

1. check the working iodine solution each time it is used
2. Iodine should be the color of strong Orange Peko tea, discard if it is too light.
3. Protozoan stained with iodine should contain yellow gold cytoplasm, brown glycogen material and paler refractile nuclei. The chromatoidal bodies may not be as clearly visible as in a saline mount.
4. The microscope should be calibrated (within the last 12 months)
5. All QC results should be appropriately recorded and any “out- of-control” results referred to the laboratory director for action.
6. Ensure that reagents and chemicals used are not expired.
Safety note: Universal precautions should be observed.

PROCEDURE

1. Place one drop of 0.85% NaCl on the left side of the slide and one drop of iodine (working solution) on the right side of the slide.

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2. Take a small amount of fecal specimen and thoroughly emulsify the stool in saline and iodine using an applicator stick. The sample should be spread thinly enough that newsprint can barely be read when the slide is placed on top of text.
3. Slide a 22mm cover slip at an angle into the edge of the emulsified fecal drop. Push the cover slip across the drop before allowing it to fall into place.
4. Systematically scan the entire 22mm cover slip with overlapping fields with the 10x objective.
5. Switch to high dry (40X objective) for more detailed study of any suspect eggs or protozoa.

PROCEDURE NOTES

1. If a fresh or unpreserved sample is received:
 - Only process if the duration from passage is known. This is usually only done by prior arrangement with the lab.
 - perform a wet direct mount on liquid samples if received within 30 minutes of passage and on soft/formed stools within 60 minutes of passage and examine for motile trophozoites.
 - describe the consistency of the specimen, e.g. bloody, watery, loose, soft or formed.
 - request a repeat specimen in SAF if only a fresh specimen was sent.
2. Examine specimens macroscopically for the presence of adult worms, proglottids, scoleces and other abnormal conditions. Use applicator sticks to break up the stools as necessary.
3. The sample on the slide should be spread thinly enough that newsprint can barely be read when the slide is placed on top of text.
4. The microscope light should be reduced for low power observation since most organisms will be overlooked with a bright light illumination should be regulated so that some of the cellular elements and feces should be refractile. Iodine solution will help make the nuclei more visible. However it is not useful for confirming motility since it kills trophozoites. In preserved specimens the SAF replaces the saline and can be used in the direct smear; however no motility will be visible since the organisms are killed in SAF.
5. To prevent contamination of iodine solution, the drops of iodine working solution should be placed on the slide before the specimen is added.
6. Duodenal aspirates can be examined directly with or without added saline.
7. If the slide is to be kept for any period of time, the edges of the cover slip may be sealed with Vaseline in order to prevent evaporation.

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REPORTING

Protozoan stained with iodine should contain yellow gold cytoplasm, brown glycogen material and paler refractile nuclei. The chromatoidal bodies may not be as clearly visible as in a saline mount. Protozoal trophozoites, cysts and helminth eggs and larva can be seen and identified. However results from the direct smear should be considered presumptive and should be definitively confirmed with concentrates and direct smears. Use “*Bench Aids in the Diagnosis of Intestinal Parasites*” or the tables found in the section on Permanent Smears to aid in species identification of the observed parasites.

LIMITATIONS

1. Once iodine is added the organisms will be killed and motility will be lost.
2. Specimens that arrive in the lab already preserved do not require a direct smear examination. Concentration and permanent stain smears should be performed instead.
3. Direct smears are normally examined at low (x100) and high dry (x400) power. Oil immersion examination (x1000) is not recommended since organism morphology is often not clear.

AUTHOR

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Formalin-ether Concentration Method

PRINCIPLE

Fecal concentration is a routine part of the ova and parasite examination and allows the detection of small numbers of organisms that may be missed by using a direct wet smear. Sedimentation methods use centrifugation to concentrate the protozoa, helminth ova and larva in the bottom of the tube. Ether is used as an extractor of debris and fat from the feces.

SPECIMEN

- Stool preserved in SAF
- Preserved duodenal aspirates
- External QC aspirates
- Aspirates from abscesses

MATERIALS

Reagents

Normal saline (0.85%)(Commercial product, PML)

Diethyl ether (Commercial product, PML)

10% neutral buffered formalin (pH 7.0) (Commercial product, PML)

IF MADE IN HOUSE:

Formaldehyde (Commercial product)	1200mls
Na ₂ HPO ₄	10.7gr
NaH ₂ PO ₄	0.23gr
Distilled water	10.8 L
Triton X-100 (commercial product)	12mls

1. mix thoroughly before dispensing. Smaller quantities can be prepared.
2. titrate pH to 7 using concentrated HCl or NaOH.

Normal Saline Wash Solution (commercial reagent, PML):

IF MADE IN HOUSE:

NaCl	34gr
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distilled water 4000mL
Triton X-100 (commercial product, BDH) 4mL

Equipment:

Fume hood
safety centrifuge
Microscope with ocular micrometer and set for Kohler illumination
Funnel filter – disposable (PML)
Applicator sticks
Centrifuge tubes and caps (PML)
Pasteur pipette
Sharps discard
Cotton tip applicator
Glass microscope slides
Cover slips (22 x 40 mm)

QUALITY CONTROL

1. Check all reagents each time they are used and ensure that formalin and saline appear clear without any visible contamination.
2. Ensure that all reagents and chemicals have not expired.
3. The microscope should be calibrated (within the last 12 months)
4. All QC results should be appropriately recorded and “out-of-control” results should be referred to laboratory director for action.
5. Whenever possible, one technologist will read the concentrate and a different one will read the smear. Discordant results will be recorded and resolved with the lab director before reporting.
6. Whenever possible multiple samples from the same individual should be read by a different technologist.

Special Safety Notes

Ether is highly flammable and should only be used in a fume hood and should be disposed of in the appropriate hazardous waste container (see appendix and WHMIS manual). Formalin is hazardous and all processing should be performed in a fume hood. Stool samples should be treated as biohazards and universal precautions are always indicated (see appendix).

PROCEDURE

ALL STEPS SHOULD BE PERFORMED IN THE FUME HOOD.

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FOR SAMPLES IN SAF

1. Invert the preserved specimen several times and ensure sample is uniformly resuspended and well mixed with the fixative. Examine the specimen macroscopically for worms, tapeworm segments and note any unusual features such as minimal amount or mucous specimens.
2. Strain approximately 3-4 mLs of well resuspended SAF fixed stool through a commercial funnel filter into a 15mL disposable plastic centrifuge tube. It may be necessary to use more than 3-4 mLs of a diarrheic stool. **DO NOT filter small samples, aspirates or those with a lot of mucous - SEE SECTION ON PROBLEM SPECIMENS BELOW.**
3. Using a fine stream of normal saline Wash Solution from a wash bottle, wash as much of the specimen as possible through the filter device until the volume in the centrifuge tube is 15mls.
4. Before discarding filter device, carefully examine it for worms or tapeworm segments. Save and place any identified specimens into SAF for identification by a technologist or referral.
5. Centrifuge at 2000 rpm (500x g) for 10 minutes. CHECK that the amount of sediment is ~1.0mLs. The amount of sediment can be adjusted at this stage. If there is excess, mix the sediment and remove the excess. If there is too little sediment, add more strained specimen before proceeding.
6. Decant the supernatant. Add 10mLs of normal saline Wash Solution, mix the sediment well with an applicator stick and centrifuge at 2000 rpm for 10 minutes.
7. Repeat wash step 6. The supernatant should be fairly clear after the second wash, if not it may be repeated one more time.
8. Decant supernatant and drain tube well onto paper towel. The final sediment should be between 0.5-1.0mLs.
9. **Make slides for permanent stain smears. See Permanent smear section.**
10. Add 8mLs of 10% neutral buffered formalin to the remaining sediment. Mix well with an applicator stick and allow specimen to stand for at least 10 minutes.

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11. Add 3mLs of diethyl ether, stopper well and shake vigorously for 60 seconds. DO NOT VORTEX. After a 15-30 sec. delay, carefully open the stopper with the tube pointing away from your face, to let off pressure. RE-CAP tube.
12. Centrifuge at 2000 rpm for 10 minutes. Four layers should result: a small amount of sediment (containing the parasites) in the bottom of the tube; a layer of formalin; a plug of fecal debris on top of the formalin layer; and a layer of ether at the top.
13. Carefully open the stopper away from your face (avoid aerosols). Free the plug of debris by “ringing” the plug with an applicator stick; decant the supernatant fluid and detritus (BUT NOT THE SEDIMENT) into a discard container. DO NOT discard directly into sink or drain.
14. After proper decanting, 1 to 2 drops of fluid remaining on the side of the tube may run into the sediment. Mix this fluid with the sediment. The final sediment volume should be ~0.25 to 0.5mLs. Tubes should be left open in the fume hood for ~ 1hour to let residual ether evaporate. Re-cap the tubes until ready to examine.

EXAMINING SPECIMENS

15. Using a plastic transfer pipette, mix the remaining sediment and transfer 1 drop of the resuspended sediment onto a clean slide and cover with a 22 x 40 mm cover slip. If the sediment is too thick to pipette, 1 or 2 drops of 10% formalin can be added to the sediment. DO NOT ADD TOO MUCH or the sample will be diluted and the concentration will be ineffective. Sterile saline can be used in place of formalin but it must be changed at least daily to avoid contamination of sediment.
16. Systematically scan the entire cover slip with overlapping fields under low power (total magnification x 100) especially examining for helminth ova. Suspicious organisms may be reviewed with the 40x objective. At least one third of the cover slip should be examined under high dry power. Total examination should require at least 10 minutes, more if the technologist has less experience.

MUCOUS OR MINIMAL AMOUNT SPECIMENS

1. If the specimen contains a lot of mucous and/or only a small amount of fecal material (such as that derived in an aspirate or from a neonate), DO NOT FILTER but

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- centrifuge the entire sample in 1 or more centrifuge tubes at 2000 rpm (500x g) for 10 minutes.
2. Wash sample with saline Wash Solution as usual but carefully remove supernatant each time with a transfer pipette rather than by pouring. After the final wash, remove supernatant carefully with a transfer pipette being especially careful NOT TO DISRUPT THE SEDIMENT.
 3. Prepare 2 slides from washed sediment - as above. DO NOT PROCEED WITH formalin-ether concentration. Re-cap tube and retain. The technologist will prepare a direct smear from the remaining sediment.
 4. If washed sediment is mainly mucous, place 1 drop of Mayer's albumen on 2 slides. Remove an equal volume of sediment with an applicator stick, mix with albumen on one of the slides. Invert the other slide onto the first slide and squash specimen between the two slides. Carefully draw slides apart horizontally and allow to dry thoroughly before staining. Both slides should be stained and read. Re-cap tube and retain. The technologist will prepare a direct smear from the remaining sediment.

PROCEDURE NOTES

1. Too much (> 0.5mL) or too little (<0.25mL) sediment will result in an ineffective concentration.
2. Centrifuge time should be calculated after centrifuge reaches full speed.
3. Vernier measurements should be taken if an unusual parasite is identified or if a confirmation is required.

Problem solving

1. Too little sediment:

Possible Problem: - less than 0.5mL of sediment before adding formalin
- too much ether used

Corrective action: - adjust amount of sediment used before adding formalin
- adjust amount of ether added
2. Too much sediment:

Possible Problem: - more than 1.0mL of sediment before adding formalin
- too little ether used

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Corrective action: - adjust amount of sediment used before adding formalin
- adjust amount of ether added

3. No sediment:

Possible Problem: - centrifugation speed too low
- tube “shaken” before “ringing”
-

Corrective action: - check centrifuge speed and time
- remove tubes from centrifuge without shaking

REPORTING

1. Protozoan trophozoites and/or cysts, and helminth eggs and larva may all be seen and identified. Protozoan trophozoites and cysts may or may not be identified to species level and are best confirmed by examination of the permanent stained smear. Oocysts of *Cryptosporidium parvum*, *Isospora belli*, and *Cyclospora* sp. Can be seen but can also be identified on modified acid fast or sequential stains (see Section of Stains for Coccidia). Identification of microsporidia require special techniques (see Section on Stains for Microsporidia).
2. Use “*Bench Aids in the Diagnosis of Intestinal Parasites*” or attached tables to aid in a species identification of the observed parasites.
3. Helminth eggs and larva including *Ascaris lumbricoides* and hookworm larva may be identified and reported.
4. Report presence of Charcot-Leyden crystals (which are breakdown products of the eosinophils and may be increased in certain parasitic infections) but do not quantitate.
5. Report pus cells (as “Pus cells seen”) and erythrocytes (as “Red blood cells seen”) if > 3/oil immersion field, but do not quantitate. Do not report yeast.
6. Do not quantitate parasites.
7. Any atypical parasites or those with unusual morphology should be marked with Vernier coordinates and reviewed with the lab director before reporting.

1. LIMITATIONS OF THE PROCEDURE

1. Results obtained by concentrated specimens should usually be confirmed by a permanent stained smear. Confirmation is particularly important in the case of *Entamoeba histolytica* vs *Entamoeba coli*.

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2. Coccidial oocysts may concentrate better with flotation methods. If PVA-preserved specimens are used, *Giardia lamblia*, hook worm, trichuris and strongyloides larva may not be well concentrated or easily identified.

AUTHOR

Kevin C. Kain

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Permanent Stained Smear Methods

PRINCIPLE

Permanent stain smears are used primarily for the identification of trophozoites, occasionally cysts, and for the confirmation of species. Small organisms missed by other examinations may be found on stain smears. Although experienced microscopists can identify certain organisms on wet prep most identification should be considered tentative until confirmed by a permanent stained slide. Permanent stains include iron hematoxylin for the detection and identification of intestinal protozoa and modified acid fast staining for the identification and detection of coccidia oocysts including *Cryptosporidium*, *Isospora* and *Cyclospora* species.

SPECIMEN

- Stool in SAF
- duodenal aspirates in SAF
- fresh stool (within 30 minutes of passage-- by arrangement only) or aspirate specimens

MATERIAL

Reagents

Normal saline (0.85%) (commercial product, PML)

Mayer's albumin (Commercial product, PML)

If preparing in house:

1. add an equal quantity of glycerine to fresh egg white
2. mix thoroughly and gently
3. add a few crystals of thymol to prevent fungal overgrowth

Equipment:

Fume hood

Safety centrifuge

Microscope with ocular micrometer and set for Kohler illumination

Funnel filter

Applicator sticks

Centrifuge tubes and caps

Clean microscope slides (frosted) are recommended so that identifying information can be written on frosted end with an ordinary lead pencil.

Pasteur pipettes

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Sharps discard
Cover slips (22x40mm)
Cotton tip applicator

QUALITY CONTROL

1. Check all reagents each time they are used and ensure that the formalin and saline solutions appear clear without any visible contamination.
2. Ensure that all reagents and chemicals used have not expired.
3. The microscope should be calibrated within the last 12 months or after any changes in the optics.
4. All QC results should be appropriately recorded (see QC binder) and "out-of-control" results (i.e. failure to recover and identify positive control organisms) should be immediately referred to the laboratory director for action. Even in the absence of reported problems, all QC results will be reviewed by the lab director or associate at least monthly.
5. Whenever possible, one technologist will read the concentrate and a different one will read the smear. Discordant results will be recorded and resolved with the lab director before reporting.
6. Whenever possible, multiple samples from the same individual should be read by a different technologist.

PROCEDURE

1. Proceed with SAF preserved stool formalin-ether concentration method until step 9.
2. Decant supernatant and drain the tube well.
3. Label one microscope slide with the sample. Prepare additional smears for each QMP-LS and any referred specimens for future internal QC.
4. Using a Pasteur or plastic transfer pipette, place 1 drop of Mayer's albumin on a glass slide (place the Mayer's Albumin first to avoid contamination).
5. Mix sediment with an applicator stick and transfer an equal amount of sediment to the slide and mix well with the applicator stick.
6. Spread the mixture over the slide making thick and thin areas for examination (approximately 4 to 6 bands per slide).

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7. Allow the slide to air dry at room temperature THOROUGHLY before staining. You can gently warm slides (37°C for 30 minutes) to aid drying.
8. Proceed with the formalin-ether concentration technique on the remaining sediment.

PROCEDURE NOTES

1. Do not heat slides to speed up drying.
2. If too much Mayer's albumin is used the smears will not dry. If too little is used the smears will not adhere well to the slides.
3. Prepare additional smears for each QMP-LS and any referred specimens to be used for future internal QC.

LIMITATIONS

1. Confirmation of intestinal protozoa (both trophozoites and cysts) is the primary purpose of this technique.
2. Most problems encountered in subsequent staining of permanent smears will result because the specimen is too old, the smears are made too densely or fixation is inadequate.

AUTHOR

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Hematoxylin Stain

PRINCIPLE

Hematoxylin is a natural dye and when used in combination with aluminum and iron is a powerful nuclear stain and a chromatin stain for intestinal parasites. It has polychrome properties. The active colouring agent, hematin, is formed by the oxidation of hematoxylin. This process, “ripening” is achieved by exposure of prepared solutions to the air for several days or weeks preferably in the sunlight. The method employed is regressive staining which is accomplished by overstaining of hematoxylin then removing the stain from other constituents by molecular bombardment with a differentiating agent, picric acid. The following method is quick and reliable.

SPECIMEN

- Properly prepared smears from SAF preserved stool
Fresh unpreserved stool. (Prepare a smear and immediately fix in Schaudinn’s fixative. It is important that smears to not dry before fixation. Leave in fixative for 30 minutes. After fixation, place slide in 70% alcohol to which sufficient iodine has been added to give the alcohol a deep brown color).

MATERIALS

Reagents:

- Ethyl alcohol solutions - 50%, 70%, 95% and 100% (absolute).
- Hematoxylin stock solution A and stock solution B (Commercial product).
- Prepare Hematoxylin working solution:
Mix equal parts of stock solutions A and B
Let stand at room temperature for at least 3 to 4 hours before staining commences. (See Appendix).
- NH₄OH (commercial product)
- Picric acid (saturated aqueous approximately 1.2%w/v).
Working solution is equal volumes of distilled water and Picric acid solution.
Important Note: solid Picric acid should not be stored dry since it is explosive (see Appendix).
- Mayer’s albumin (commercial product).
- xylol (commercial product)
- carboxylol (see appendix)

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Equipment:

Microscope with ocular micrometer and set for Kohler illumination.

Glass microscope slides

Pasteur pipets

Glass cylinders

Staining containers

Covers slips 22 X 40 mm

Slide rack

Sharps container

QUALITY CONTROL

1. Positive control slide of SAF preserved stool containing *Dientamoeba fragilis* and/or *Entamoeba histolytica* trophozoites stained with every rack of slides.
2. All QC results are recorded. All “out-of-control” results are referred to the Lab Director for action.
3. Ensure all reagents and chemicals used are in date.
4. The Hematoxylin working solution should be produced at least 2 to 3 hours before use.
5. If stain is to be reused, it should be checked at regular intervals by adding a few drops of used stain to tap water. If the drops remain blue or blue black that mixture is useable. If it turns brown it should be replaced with fresh stain before proceeding.
6. The microscope should be calibrated (within the last 12 months or anytime the optics are altered).
7. When the stain is thoroughly mixed and staining performed correctly, the cytoplasm of Protozoan trophozoites will be blue gray. Cysts tend to be darker. Nuclei and inclusions (chromatoid bodies red blood cells, bacteria etc. are dark gray blue - almost black).
8. All staining dishes should be covered to prevent evaporation of reagents (use a screw top, or jars with glass lids). If the xylol becomes cloudy or if there is water at the bottom of the dish discard, and replace with fresh reagents.

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PROCEDURE

1. Prepare the hematoxylin working solution at least two to three hours before use.
2. Prepare other required reagents and store in appropriately labeled containers and document all changes on the QC stain control sheet.
3. Place the prepared smears in a slide rack and then into the following solutions for the time indicated.
4. alcohol 70% 10 minutes
5. running tap water 10 minutes
6. hematoxylin stain 10 minutes
7. distilled water 1 minute
8. picric acid 10 minutes
9. running tap water 20 minutes
10. alcohol (70%) with drops of ammonia sufficient to achieve a pH of approximately 8 10 minutes
11. alcohol 95% 10 minutes
12. alcohol 95% 10 minutes
13. carboxylol 10 minutes
14. xylol 10 minutes
15. xylol 10 minutes
16. Using Entellan (Commercial product) mount slides, do not allow the slides to dry before mounting.
17. Store the slides in slide tray until read.

EXAMINATION OF STAINED SMEARS

1. When mounting is dry, examine the control smear first before proceeding to patient samples.
2. Examine the smears microscopically with overlapping fields.
3. Using the low power objective (10X) examine vertically the thick areas of the smear for helminth ova and larva for at least 5 minutes. Scanning is then done with a low power oil objective (~50X) or with a high dry objective (40X) if a low power oil is not available. Switch to the 100X for more detailed study. Using the 50X oil objective, screen horizontally the whole slide for at least 15 minutes. Using the 100x oil objective, examine between two thick bands for small protozoa for at least 5 minutes.
4. Use "*Bench Aids in the Diagnosis of Intestinal Parasites*" or other manual to aid in a speciation of the observed parasites.

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PROCEDURE NOTES

1. This stain gives the best morphological details. Organisms can be missed under bright light, therefore lower light observations may be preferable.
2. The most important step in the preparation of a well stained permanent smear is good fixation. If good fixation has not been successful the protozoa will be distorted or may stain poorly.
3. Once the staining process has started the smear should not be allowed to dry at any of the subsequent steps until they are cover-slipped.
4. Slides should always be drained thoroughly between solutions, touch the end of the slide with a paper towel to remove excess fluid.
5. In the final stages of dehydration the 100% alcohol and xylol should be kept as free from water as possible. Containers must have tight fitting caps to prevent evaporation or absorption of moisture. If xylol becomes cloudy replace with fresh stock.
6. If smears peel or flake off, the specimen may have been inadequately dried on the slide or the smear may be too thick, repeat the smears.
7. 1-2 drops of ammonia are added to bring the alcohol to ~pH 8.
8. Patients' stained slides must be allowed to dry thoroughly, (eg. overnight) before examination.
9. Vernier measurements should be taken for the location of any parasite with unusual morphology so that confirmation can be obtained.

REPORTING

1. Report all parasites found.
2. Protozoan trophozoites and cysts will be readily seen. Report the organism and stage. Do not use abbreviations. eg. *Entamoeba histolytica* cysts, *Dientamoeba fragilis* trophozoites.
3. Do not quantitate protozoa or organisms.
4. Report but do not quantitate the presence of human cells or products. eg. Leukocytes, red blood cells and Charcot-Leyden crystals.

LIMITATIONS OF PROCEDURE

1. The permanent stain smear is primarily for the identification and confirmation of protozoa. It is not recommended for the detection and identification of helminth eggs or larva since they often retain too much stain or are distorted. However they may be occasionally recognized and identified.

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2. Delayed fixation during this stain preparation may show unusual protozoal characteristics.
3. Coccidial oocysts will not be identified on an iron hemotoxylin stain. This requires a modified acid fast stain.

REFERENCES

NCCLS Procedures for the Recovery and Identification of Parasites from the Intestinal Tract; Approved Guideline M28-A 17(23) December 1997

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Smears For Coccidia

PRINCIPLE

Coccidial infections, particularly those caused by *Cryptosporidium* species are considered a significant cause of acute and chronic diarrhea. In immunocompromised hosts, particularly AIDS patients, these infections can be chronic and life threatening. Oocysts of cryptosporidia, cyclospora, isospora, and sarcocystis are acid fast and will be detected with a modified acid fast stain. They are not reliably detected using a iron hematoxylin stain.

SPECIMEN

- SAF-Preserved stool
- Fresh stool (within 30 minutes of passage-- by arrangement only)
- bile
- duodenal aspirates or small bowel biopsies
- external QC samples
- rarely Cryptosporidium may be identified from respiratory tract samples from compromised hosts.

MATERIALS

Reagents:

- Basic fuchsin (commercial product, VWR)
- Liquid phenol (commercial product, VWR)
- 95% ethyl alcohol (commercial product, VWR)
- 10% Tween 80 (commercial product, VWR)
- Malachite green (commercial product, VWR)
- Decolorizer (commercial product, PML)
- Distilled water
- Carbolfuchsin Stain Solution (PML, or if unavailable use following)
 1. Basic fuchsin 21gms
 2. Liquid phenol (85%) 85.2mls
 3. 95 % alcohol 150mls
 4. 10% Tween 80 7.5ml

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- Gently heat mixture in 45 C incubator for 24 hours to dissolve the crystals. Add distilled water to bring volume to 1.8L.

Malachite Green

- Malachite Green (Commercial Product) 0.5 gms
- Distilled water 100 mls
- Mixed gently and thoroughly until dissolved.

Equipment:

Microscope with ocular micrometer and set for Kohler illumination.
Glass microscope slides
Pasteur pipets
Glass cylinders
Staining containers
Covers slips 22 X 40 mm
Slide container
Sharps container

QUALITY CONTROL

- A quality control slide of *Cryptosporidium parvum* is performed each week or whenever stains are changed.
- Cryptosporidia and other coccidia stain pink-red. Oocysts of Cryptosporidia measure 4 to 6 microns and often 4 sporozoites may be identified internally.
- Record all QC results. Report any “out-of-control” results to Laboratory Director for action.
- Known positive slides and reference books are available to aid in morphologic identification.
- The microscope should be calibrated (within twelve months or when ever the optics are altered).

PROCEDURE

Special Safety Notes: Assume samples are biohazards and use universal precautions. Handle the decolorizer solution with care since it contains sulphuric acid. Always dilute acid into water. Never add water to concentrated acid.

- Fix the smear (from formalin-ether concentrate step 9) in absolute methanol for five minutes.
- Flood the slide with carbolfuchsin and stain for five to ten minutes at room temperature.

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3. Gently wash with running tap water for 60 sec.
4. Decolorize with decolorizer for 30-60 seconds (until no more color runs from the slide).
5. Gently wash with running tap water for 60 sec.
6. Counterstain with 1% malachite green for one minute.
7. Gently wash with running tap water for 60 sec.
8. Air dry slides in vertical position and mount with Entellan.

EXAMINATION OF SMEARS

1. Examine as per permanent stains for iron hematoxylin.

REPORTING

1. The oocysts of *Cryptosporidium* and *Isospora* species will stain pink to red. If Cyclospora oocysts are present they resemble cryptosporidia but are larger and lack definite internal structure.
2. Report the organism and stage. Do not use abbreviations, eg. *Cryptosporidium parvum* oocysts, *Isospora belli* oocysts.
3. Yeast will stain green.

LIMITATIONS OF PROCEDURE

1. Light infections with cryptosporidia and cyclospora may be missed. Immunoassays may be more sensitive.
2. Multiple specimens must be examined since the number of oocysts may vary day to day.
3. Other organisms that stain modified acid fast positive including Nocardia, and Microsporidia may be difficult to identify.

AUTHOR

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Smears For Microsporidia (Modified Trichrome or Chromotrope Stain)

PRINCIPLE

Microsporidia (such as *Enterocytozoon bieneusi*, and *Encephalitozoon intestinalis*) are parasites that are important opportunistic pathogens in immunocompromised patients particularly those with AIDS. It is difficult to recognize Microsporidia in fecal samples because of background debris and their relatively small size (in the order of microns). Scanning electron microscopy is considered to be the gold standard in making a diagnosis, however it lacks sensitivity and is expensive and time consuming. Several staining methods of stool or biopsy samples can be used, however at the current time a modified chromotrope stain with 10X the usual amount of Chromotrope 2R is gaining favor as the most sensitive method to facilitate identification.

SPECIMENS

- Stool in SAF
- Duodenal aspirate in SAF

MATERIALS

Reagents:

Chromotrope 2R (Sigma)
Fast green (VWR)
Phosphotungstic acid (Sigma)
Glacial acetic acid (VWR)
90%, 95% ethyl alcohol (VWR)
methanol (VWR)
xylol (VWR)

Chromotrope stain

- | | |
|---|---------|
| 1. Chromotrope 2R | 6gms |
| 2. Fast green | 0.15gms |
| 3. Phosphotungstic acid | 0.7gms |
| 4. Glacial acetic acid | 3mls |
| 5. Mix ingredients gently and allow to stand for 30 minutes. Then add
100 mls distilled water. | |

Acid alcohol for Chromotrope 2R stain

- | | |
|------------------|----------|
| 1. ethyl alcohol | 995.5mls |
|------------------|----------|

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2. Glacial acetic acid 4.5 mls
3. Mix gently.

Equipment:

Light microscope with ocular micrometer and set for Kohler illumination
 Glass microscope slides
 Pasteur pipets
 Glass cylinders
 Staining containers
 Covers slips 22 X 40 mm
 Slide container
 Sharps container

QUALITY CONTROL

1. Positive control slides should be used every time the staining procedure is performed. If the control slides are not satisfactory, the unknowns cannot be interpreted.
2. All staining dishes should be covered to prevent evaporation of reagents. The reagents should be made up on Monday morning to ensure accurate staining.
3. All “out-of-control” results should be reported to the Laboratory Director for action.
4. When the smear is thoroughly fixed and the stain is properly prepared the spores will be ovoid and refractile with the spore wall being bright pinkish red. Occasionally the polar tube will be seen as a stripe or a diagonal line across the spore.
5. The microscope should be calibrated (within 12 months or anytime there is an alteration of the optics).
6. Known positive control slides, Kodachrome slides, photographs and reference books are available to help with morphologic identification.

PROCEDURE

This is considered to be a non-routine procedure therefore it should only be performed by experienced personnel

Special Safety Notes

Methanol, ethanol and xylol are flammable. The acid alcohol solution is corrosive.

1. Shake the SAF stool to resuspend thoroughly.
2. Prepare thin smear with 10-20 µl of the stool (unconcentrated) on a slide.
3. Air dry the smear thoroughly at room temperature or 60 C.

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STAINING

- | | |
|--|------------|
| 4. Fix the dried smears in absolute methanol | 5 minutes |
| 5. Rinse in tap water | 1 minute |
| 6. Hematoxylin stain | 10 minutes |
| 7. Rinse in tap water | 1 minute |
| 8. Chromotrope stain | 90 minutes |
| 9. Rinse in acid-alcohol | 10 seconds |
| 10. Rinse in 95% methanol | 10 seconds |
| 11. Dehydrate in 95% methanol | 5 minutes |
| 12. Dehydrate in 100% methanol | 5 minutes |
| 13. Dehydrate in 100% methanol | 5 minutes |
| 14. Dehydrate in xylol | 5 minutes |
| 15. Dehydrate in xylol | 5 minutes |
| 16. Drain and coverslip with Entellan mount. | |
| 17. Examine under oil immersion lens. | |

EXAMINATION

Examine smears under oil immersion (x1000) with overlapping fields and examine at least 200 fields.

REPORTS

1. The microsporidial spore wall should stain pink to red and may display a horizontal or diagonal stripe which represents the polar tube. These can be distinguished from spores by the overall morphology and shape.
2. Report the organism. Example: Microsporidia present (*Enterocytozoon bieneusi*, or *Encephalitozoon intestinalis* are probable agents if specimen is feces.)

PROCEDURE NOTES

1. Positive control slides of microsporidia in SAF preserved stool are included every time the staining procedure is performed. If the controls are not satisfactory the patient samples can not be interpreted.
2. Because of difficulty in getting the stain to penetrate the spore wall, prepare thin smears and do not reduce the staining time. Make sure slides are not left too long in decolorizing agents (acid-alcohol).

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3. In the final stages of dehydration, the 100% ethanol and xylol should be kept as free from water as possible. Tight fitting caps to prevent evaporation and absorption of moisture are essential. Change these solutions weekly.

LIMITATION OF PROCEDURE

1. It is preferred to use unconcentrated stool since there are anecdotal concerns that concentration procedures may actually result in a loss of microsporidial spores.
2. Definitive species identification is only possible with electron microscopy or molecular methods.

AUTHOR

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Sequential Haematoxylin/Acid Fast Stain For Routine O & P Including *Cryptosporidium* & *Cyclospora*

PRINCIPLE

Haematoxylin stain is a useful technique, however several organisms of interest such as cryptosporidium and cyclospora do not stain well with this technique. To overcome this limitation an acid fast stain can be ordered on suspected crypto or cyclospora samples, or alternatively a sequential stain can be used. The addition of an acid fast stain causes these organism to stain a bright red, however the depth of stain of *Cyclospora* can be variable. The sequential stain is especially appropriate in situations were it is suspected that crypto and cyclospora levels are elevated in the general population.

SPECIMEN

Prepare fecal smears as in the **permanent stained smear methods**.

SAFETY

Be careful when handling acid solutions and ammonia. Picric acid becomes explosive if the solution is allowed to dry out.

MATERIALS

- See **Permanent Stained Smear** method.
- Kinyoun stain (commercial product, PML)
- Decolorizer (commercial product, PML)
- Hematoxylin stain (commercial product, VWR)
- Picric acid (commercial product, VWR)

PROCEDURE

Place slides into the following solutions for the times indicated; do not stain on a rack:

- | | | |
|----|--------------------------|-------------|
| 1. | 70% alcohol | 6 minutes |
| 2. | Tap water | 1 minutes |
| 3. | Kinyoun stain | 5 minutes |
| 4. | Tap water rinse | 3 minutes |
| 5. | Acid-alcohol decolorizer | 2-4 minutes |
| 6. | Tap water rinse | 2 minutes |

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7.	Haematoxylin stain	8 minutes
8.	Distilled water rinse	20 seconds
9.	Picric acid	3-5 minutes
10.	Running water wash	12 minutes
11.	70% alcohol + ammonia	5 minutes
12.	95% alcohol	3 minutes
13.	100% alcohol	3 minutes
14.	xylol	3+ minutes
15.	xylol	1+ minute

QUALITY CONTROL

- Ensure that the microscope has been calibrated in the last year or any time the optics have been altered and that the results of the calibration are displayed on the microscope base.
- A positive control slide containing *Cyclospora* should be stained at the same time as the unknowns. The unknown slides cannot be read if the control slide is unsatisfactory.

REPORT

- All parasites including *Cyclospora* and *Cryptosporidium*.

PROCEDURE NOTES

1. *H. diminuta* will stain bright red with a clear background. The size of this organism will be reduced because of the stain/fixation process.

LIMITATIONS OF PROCEDURE

2. Light infections with cryptosporidia and cyclospora may be missed. Immunoassays may be more sensitive.
3. Multiple specimens must be examined since the number of oocysts may vary day to day.
4. Other organisms that stain modified acid fast positive including *Nocardia*, and *Microsporidia* may be difficult to identify.

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Hatching *Schistosoma* Eggs

PRINCIPLE

It may be necessary when schistosome eggs are recovered from stool or urine to determine viability. The presence of viable miracidia indicates an active infection that may require treatment. The viability of the miracidia can be determined in two ways: 1) by doing a wet prep on a fresh stool one can examine the egg for actively moving flame cells (primitive excretory cells); and 2) the miracidia can also be released from the eggs by a hatching procedure- the details of which follow below. Schistosome eggs passed in stools develop relatively quickly into miracidia which actively seek out fresh water snails so they can get on with their lives. This method provides all the environmental clues necessary to persuade the eggs to hatch and for the miracidia to concentrate where they can be seen.

SAFETY

Treat fresh stool samples as a biohazard. The miracidia will invade snails, not humans, at this stage so there is no chance of contracting Schistosomiasis by contact with the hatch water.

REAGENTS

0.85% NaCl saline,
dechlorinated tap water

SPECIMEN

Emulsified fresh stool that has not been refrigerated.

PROCEDURE

This is considered to be a non-routine procedure therefore it should only be performed by experienced personnel.

- a) Emulsify stool in 150 ml of normal saline and strain through wet gauze into a 250 ml sidearm flask. Allow to settle for 2 hours.
- b) Decant the supernatant and refill the flask by pouring dechlorinated tap water to the brim.

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- c) Cover the flask with black paper, aluminum foil, black paint or paper bag, except for the side arm which is exposed to light.
- d) The flask is placed near a window or some other form of illumination and left overnight.
- e) After 16 hours, start checking for miracidia and continue for 48 hours. Most hatches will be positive within 24 hours. The miracidia can be seen swimming in the water in the side arm with the use of a hand lens.

QUALITY CONTROL

- As chlorine can kill the miracidia it is necessary make sure that the water has stood long enough for the chlorine in it to dissipate-- usually by leaving at room temperature overnight or spring water/bottled water can be used.
- As it is not possible to have a positive control specimen to use with this procedure, therefore the technologist should review the appearance and size of the schistosome eggs and miracidia to ensure that they match reference material (i.e. *Bench Aids for the Diagnosis of Intestinal Parasites* (WHO).
- Ensure that the microscope has been calibrated in the last year and that the results of the calibration are displayed on the microscope base.

REPORT

- If no miracidia are found report “No miracidia found”
- If miracidia are found report “miracidia found”

LIMITATIONS OF PROCEDURE

- Adult schistosomes settle in the host’s blood system and therefore it can take weeks to months for the eggs to be passed. A negative result may indicate that adults are present but that eggs are not being passed.

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Kato Thick Smear For Egg Count

PRINCIPLE

Most organisms release eggs sporadically and therefore there is no correlation between burden and the number of organisms seen in a stool sample. Schistosome eggs can be released at a steady rate therefore it is desirable to get an accurate measure of the number of eggs in a given amount of sample so that worm burden can be inferred. The Kato thick smear method accomplishes this by depositing a reproducible amount of material on the slide.

SPECIMEN

Any fresh stool sample that has not been refrigerated

SAFETY

Treat fresh stool sample as a biohazard.

REAGENT

Cellophane solution
100mls glycerin
100ml distilled water
3% aqueous malachite green

PROCEDURE

This is considered to be a non-routine procedure therefore it should only be performed by experienced personnel.

- 1) Take approximately 5 g of faeces and sieve it through fine wire mesh.
- 2) Scrape the stool off the underside of the mesh using a tongue depressor.

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- 3) Place a metal plate with a hole at one end over a microscope slide. Fill the hole with the finely sieved faecal material. This calibrated hole holds 0.05g of stool. Remove the metal plate.
- 4) Place a special cellophane strip (cut as a 22x40 mm coverslip) over the feces spread out the sample using an applicator stick. Keep for one hour at room temperature to allow the eggs to clear. The cellophane “coverslips” should be soaked for 24 hours before using.
- 5) Examine this slide for the presence of eggs.

CALCULATION

- Count the number of each type of egg present and multiple by 20 to determine the number of eggs per gram of stool.

QUALITY CONTROL

- Egg counts are subject to errors therefore multiple egg counts on the same specimen should be performed
- Ensure that the microscope has been calibrated in the last year and that the results of the calibration are displayed on the microscope base.
- As it is not possible to have a positive control specimen to use with this procedure, the technologist should review the appearance and size of the organisms present to ensure that they match reference material (i.e. *Bench Aids for the Diagnosis of Intestinal Parasites* (WHO)).

REPORT

- If no eggs found report “No ova found per gram of stool”
- If eggs are found report for example “20 schistosoma ova found per gram of stool”.

LIMITATIONS OF PROCEDURE

- Adult schistosomes settle in the host’s blood system and therefore it can take weeks to months for the eggs to be passed. A negative result may indicate that adults are present but that eggs are not being passed.

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Agar Plate Test for *Strongyloides*

PRINCIPLE

This method is considered to be the most sensitive for the detection of *Strongyloides* since a few larvae can be detected in a relatively large sample. The principle is that larvae will migrate out of the stool sample and will be covered by bacteria. When the larvae migrates across the agar it will leave a bacteria trail that will become observable with time. *Strongyloides* larvae come in two forms, a rhabditiform and an infective filariform, therefore great care must be taken when testing for the presence of *Strongyloides*.

SPECIMEN

Fresh stool sample—reject if it has been refrigerated or if the time limit of 12 hours has been exceeded.

SAFETY

- Assume that the sample contains filariform larvae and wear gloves and take measures to prevent the larvae from migrating out of the dish.
- Warn co-workers about the nature of your work.

PROCEDURE

This is considered to be a non-routine procedure therefore it should only be performed by experienced personnel.

Prepare agar plates (9 ml of 1.5% agar, 0.5% meat extract, 1.0% peptone, 0.5% NaCl per 9 cm x 2.5 cm plate) and store in a cold room until required.

1. Place 2-3 g of unpreserved faeces in the center of the agar plate and then seal the plate by placing tape around the circumference of the plate. (**Very important! We don't want any "escapees"!**)
2. Let the sample incubate for 2-3 day and then examine the agar surface for larva and the "bacteria trails" that have resulted from migrating larva.

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3. Speciation can be confirmed by obtaining a migrating larva and examining it under a microscope.

QUALITY CONTROL

- Do not use old or suspicious agar plates.
- Ensure that the microscope has been calibrated in the last year or after any changes in the optics and that the results of the calibration are displayed on the microscope base.

REPORT

- Bacteria trails on the agar indicate the presence of *Strongyloides*.

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Charcoal Culture for *Strongyloides*

PRINCIPLE

Culture techniques are useful to detect light numbers of larvae not found during normal concentration procedures. They also allow for easier speciation between strongyloides and hookworm due to development of rhabidiform larvae of hookworm. Finally culture techniques allow larvae to develop to the filariform stage to further aid in diagnosis. This method is less sensitive than the agar plate method since it relies on directly observing the larvae. Charcoal is used to maintain pH and to provide a medium in which the larvae can develop.

SPECIMEN

Fresh stool sample that has not been refrigerated.

SAFETY

- Assume that the sample contains filariform larvae and wear gloves and take measures to prevent the larvae from migrating out of the dish.
- Warn co-workers about the nature of your work.

PROCEDURE

This is considered to be a non-routine procedure therefore it should only be performed by experienced personnel.

1. Mix 20-40 g of fecal material in tap water until a thick suspension is obtained.
2. Add this mixture to a suitable covered dish to which has been added a similar volume of #10 granulated hardwood charcoal.
3. Mix the suspension well using a tongue depressor. Add enough water so that the charcoal will glisten but not enough to allow it to lay at the bottom of the dish. Keep at room temperature.

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4. Check culture daily for sufficient moisture and sprinkle water on the surface, if needed. *As infective filariform larvae could be present at any time during this procedure, caution must be used when any handling of the culture occurs.*
5. After 5-6 days the larvae, if present, can be harvested by using the Baermann technique.

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Baermann Technique for *Strongyloides*

PRINCIPLE

The Baermann technique works on the principal that larvae will migrate out of a fresh stool sample and will subsequently sink to the bottom of their liquid environment. Larvae will therefore concentrate in the lowest point and the contents of a large stool sample can be examined.

SPECIMEN

- fresh stool sample that has not been refrigerated
- charcoal culture.

SAFETY

- Assume that the sample contains filariaform larvae and wear gloves and take measures to prevent the larvae from migrating out of the dish.
- Warn co-workers about the nature of your work.

PROCEDURE

This is considered to be a non-routine procedure therefore it should only be performed by experienced personnel.

1. Firmly attach a 6 inch glass funnel to a retort stand using a ring adaptor. Attach rubber tubing with a secure clamp to the stem of the funnel. Place a collection container under the end of the tubing.
2. On top of the funnel place a wire mesh with two layers of gauze. Make sure that the gauze is trimmed to the size of the funnel, so that none of the potentially infective solution will drip over the side of the funnel and contaminate the surrounding bench area. Fill the funnel with water.
3. Place the charcoal culture on top of the gauze, making sure that it is in contact with the water.
4. Allow the apparatus to stand for 2 hours or longer before draining off a portion of the fluid directly above the clamp. Centrifuge the fluid and examine for the presence of motile larvae under a 10X or 40X objective. *As infective filariform larvae could be*

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present at any time during this procedure, caution must be used when any handling of the culture occurs.

QUALITY CONTROL

- Be aware of temperature variations.
- As it is not possible to have a positive control specimen to use with this procedure, the technologist should review the appearance and size of the organisms present to ensure that they match reference material (i.e. *Bench Aids for the Diagnosis of Intestinal Parasites* (WHO)). Ensure that the microscope has been calibrated in the last year and that the results of the calibration are displayed on the microscope base.
- Free living larvae could be present in the culture. To rule out their presence add 0.3ml of
- conc. HCL per 10ml of water containing larvae. Free living nematode larvae will be killed by the acid, while parasitic species can live for 24hours.
- As larvae of certain species are susceptible to cold, fresh stool samples that have been refrigerated are not acceptable for culture techniques
- Be aware of any leaks or drips from Baermann app.

REPORT

- If no larvae are found report “No larvae found after X days of incubation”.
- If larvae are found report for example “Strongyloides stercoralis filariform larvae found after 7 days of incubation”.

AUTHOR

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Anal Swabs for Pinworm Eggs

PRINCIPLE

Adult female pinworms migrate out of the anus and their deposit eggs in the perianal region of their host. The adult female (8 to 13mm long) can occasionally be found on the surface of a stool specimen or on the perianal skin. Since eggs are usually deposited around the anus, they are not commonly found in feces and must be detected using other diagnostic techniques. The most popular methods of detecting the eggs are to use a plastic paddle with an adhesive surface, or to use a piece of Scotch (TM) tape, which is then stuck to a slide and examined.

SPECIMEN

Specimens should be collected in the morning before the patient bathes or goes to the bathroom. The most widely used diagnostic procedure for pinworm is the cellulose tape method, however several commercial collection procedures are also available. It has been suggested that a swab coated with petroleum jelly is the most appropriate technique since this minimizes the possibility of laboratory workers becoming infected.

To collect a specimen:

1. If a commercial pinworm paddles is available follow the directions supplied with it. If a commercial paddle is not available use the following method.
2. Set up the sampling slide by producing a slide (or tongue depressor) with cellulose tape looped back such that the sticky surface is facing out. To do this place a strip of cellulose tape on a microscope slide, starting 1/2 inch from the unfrosted end of the slide and then run the tape to the unfrosted end of the slide. Loop the tape back (sticky side out) to the frosted end of the slide and then fold the sticky end underneath to secure the tape to the slide.
3. To obtain a sample from the perianal area, grip the frosted end of the slide and press the tape firmly against the right and left perianal folds.
4. Undo the tape at the frosted end of the slide and spread the tape back on the other side of the slide, adhesive side down.

Do not use Magic transparent tape; use regular cellulose tape. If Magic tape has been used a drop of immersion oil can be placed on top of it to facilitate clearing.

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SAFETY

Pinworm eggs are frequently plentiful and can be carried by air currents. Each egg will often contain a fully developed embryo and will be infective within a few hours after being deposited. Wear gloves and consider the use of a face mask if you suspect the sample will allow eggs to become airborne.

PROCEDURE

1. Remove the paddle and apply to a microscope slide, sticky side up. Add a drop of 0.85% NaCl saline and a cover glass for clearer viewing. If tape has been submitted lift one side of the tape and apply one small drop of toluene or xylene and press the tape down on the glass slide. The preparation will then be cleared and the eggs will be visible.
2. Examine for eggs under the low power objective of a microscope.

QUALITY CONTROL

- As it is not possible to have a positive control specimen to use with this procedure, the technologist should review the appearance and size of the organisms present to ensure that they match reference material (i.e. *Bench Aids for the Diagnosis of Intestinal Parasites* (WHO)).
- Ensure that the microscope has been calibrated in the last year or every time an optical element has been changed and that the results of the calibration are displayed on the microscope base.
- Eggs can be missed if too bright an illumination is used.

REPORT

The presence or absence of pinworm eggs and any other parasite seen.

LIMITATIONS OF PROCEDURE

- Swabs must be taken prior to the patient bathing—preferably first thing in the morning.

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Duodenal Aspirates

PRINCIPLE

In infections with *Giardia*, *Strongyloides*, *Clonorchis*, *Fasciola* and hookworm species routine stool examinations may be negative when the organisms are present. It may therefore be necessary to obtain a sample of the contents of the duodenum, either by using an Entero-Test capsule, or by more invasive means.

SPECIMENS

Either an Entero-Test capsule or duodenal drainage should be submitted. If the sample will not be examined within 2hrs add 10% formalin. The sample may vary in volume from <0.5ml to several mls. Often the duodenal fluid may contain mucus; this is where the organisms will tend to be found. Centrifugation of the specimen is important, and the sedimented mucus should be examined. If the amount of duodenal material submitted is very small, then permanent stains can be prepared rather than using any of the specimen for a wet smear examination.

SAFETY

Consider if *Strongyloides* larvae might be present.

REAGENTS

Saline (commercial product, PML)
 If needed 0.85% NaCl (9g/L NaCl)
 phosphate buffer
 Na₂HPO₄ 10.7 g
 NaH₂PO₄ 0.23 g
 Water 11,200.00 ml
 Giemsa stain (commercial product, VWR)
 methanol

PROCEDURE

1. If the sample is an aspirate then centrifuge the specimen at 500g for 5 minutes.
2. If the sample is supplied as an Entero-Test capsule then scrape or “milk “ the string to release material into a tube.

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3. Place a drop of sediment on a microscope slide.
4. A coverslip is added and the preparation is examined under low power. Examination of the slide under 400X may be useful to detect the flutter of a *Giardia* flagella.
5. For speciation the sample can be fixed in methanol and stained with 1:50 dilution of Giemsa stain for 30 minutes.

QUALITY CONTROL

- It may take organisms several minutes to acclimate to their new environment and to start moving again.
- If limited sample is available do the permanent stained smears first.
- Examine 5 or 6 drops of material before declaring the sample negative
- Test pH of the string for alkaline pH (ie. to ensure it was in the duodenum) and look for a yellow-green color on the section that should have reached the duodenum.
- As it may not be possible to have a positive control specimen to use with this procedure, the technologist should review the appearance and size of the organisms present to ensure that they match reference material (i.e. *Bench Aids for the Diagnosis of Intestinal Parasites* (WHO)).
- Ensure that the microscope has been calibrated in the last year and that the results of the calibration are displayed on the microscope base.
- The presence of the “falling leaf” motility of *Giardia* in fresh samples is an excellent indication of its presence.
- Monoclonal FA or EIA detection kits are available for *C. parvum* and *G. lamblia*.
- All QC results should be recorded.

REPORT

Report the organism seen and the stage (trophozoite, cyst, oocyst).

LIMITATIONS OF PROCEDURE

- Many of the parasites will get caught up in mucus, therefore centrifugation may be required.

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- Some organisms are more easily seen in wet preps, others are only visible with staining.

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Urines

PRINCIPLE

Urine samples can be submitted for the detection of *Schistosoma hematobium* or for the presence of *Trichomonas vaginalis*. Adult *Schistosomes* take up residence near the bladder and start to release eggs. The eggs migrate to bladder and are released when the host urinates. *T. vaginalis* lives in the vagina and can produce a frothy secretion which can be released during urination.

SPECIMEN

- For *Schistosoma hematobium*, obtain the last few drops of urine passed about or shortly after noon.
- For *T. vaginalis*, obtain the first portion of voided urine.

PROCEDURE

- 1) Spin urine sample at 500 g for 5 minutes.
- 2) Examine sediment under low power.

QUALITY CONTROL

- Ensure that the microscope has been calibrated in the last year or after any alteration to the optics and that the results of the calibration are displayed on the microscope base.
- As it may not be possible to have a positive control specimen to use with this procedure, the technologist should review the appearance and size of the organisms present to ensure that they match reference material (i.e. *Bench Aids for the Diagnosis of Intestinal Parasites* (WHO)).

LIMITATIONS OF PROCEDURE

- Schistosome eggs hatch when they come into contact with fresh water therefore care should be taken to avoid adding water to a specimen.
- Schistosome egg viability may be affected by chlorinated water sources.

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- *Trichomonis* does not have a cyst form therefore care must be taken to avoid conditions that will damage these organisms and make identification difficult.

REPORT

- Presence of schistosome eggs and if observed report flame cell activity.
- Report the presence of *T. vaginalis*.

AUTHOR

Ian Crandall

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Vaginal and Urethral Swabs

PRINCIPLE

Trichomonas vaginalis can be detected in wet preparations of vaginal and urethral discharges. *T. vaginalis* has an undulating membrane and a characteristic jerky motility.

SPECIMENS

- swab from cervix,
 - vaginal prostatic
 - urethral secretions.
- If examination is to be delayed, smears should be submitted to the laboratory for staining.

PROCEDURE

This is considered to be a non-routine procedure therefore it should only be performed by experienced personnel.

- 1) Dilute the sample with a drop of saline and place on a microscope slide.
- 2) Examine under low power and low intensity light for the presence of jerky motility.

QUALITY CONTROL

- Ensure that the microscope has been calibrated in the last year and that the results of the calibration are displayed on the microscope base.
- Haematoxylin staining can be used to confirm the diagnosis
- See QC comments associated with haematoxylin staining.

REPORT

The presence of *Trichomonas*

AUTHOR

Ian Crandall

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Sigmoidoscopy

PRINCIPLE

Material obtained from sigmoidoscopy can be helpful in the diagnosis of amebiasis that has eluded routine stool examinations. Material from the mucosal surface should be aspirated or scraped and six slides from six different areas should be prepared. Two methods of examination are available; direct mount, and SAF fixative. Samples should be examined for *E. histolytica* and if fresh material is examined live trophozoite forms may be seen.

1) *Direct Mount:*

SPECIMENS

Samples are obtained from the sigmoidoscope and should be carried immediately to the Parasitology laboratory for examination. See stool sample rejection criteria.

REAGENTS

0.85% NaCl saline

PROCEDURE

- 1) A drop of material is mixed with a drop of saline
- 2) A coverslip is added and the preparation is examined under low power.

QUALITY CONTROL

- It may take organisms several minutes to acclimate to this treatment and to start moving again.
- There will be epithelial cells, macrophages, and possibly PMNs and red cells present.
- If limited sample is available do the permanent stained smears first.
- if sufficient material is available some of it can be cultured.
- Ensure that the microscope has been calibrated in the last year and that the results of the calibration are displayed on the microscope base.

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REPORT

Any parasites observed.

2) *Samples submitted in SAF*

SPECIMEN

Specimen should be submitted in a small amount of SAF.

PROCEDURE

- 1) After spending 30 minutes in the SAF solution the specimen should be centrifuged at 500g for 10 minutes.
- 2) Smears from a small amount of sediment (<0.1ml) are made.
- 3) Smears are stained with Hematoxylin Stain (see section on Hematoxylin staining).

QUALITY CONTROL

- Ensure that the microscope has been calibrated in the last year and that the results of the calibration are displayed on the microscope base.
- See QC comments associated with haematoxylin staining.

REPORT

Any parasites present

LIMITATIONS OF PROCEDURE

- Any delay between sampling and diagnosis should be avoided. If samples are being submitted consult with the Parasitology lab to ensure that the sample can be processed immediately.
- This is an invasive procedure and while it produces a superior sample it is used less frequently than a standard stool sample.

AUTHOR

Ian Crandall

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Lung and Liver Aspirates

PRINCIPLE

Aspirates will frequently detect parasites when other methods fail. Organisms that may be found in aspirates include *P. carinii* and *E. histolytica*, from lung and liver respectively.

SPECIMEN

If an amoebic abscess or visceral leishmaniasis is suspected, the aspirate should be placed in a clean and empty bottle and submitted to the laboratory as soon as possible. As the amoebae are generally located near the wall or edge of a liver abscess, it is preferable to place the last part of the aspirate (frequently the reddish “anchovy paste”) in a separate container since it is more likely to contain *E. histolytica*.

REAGENTS

Robinson’s Medium (see appendix)

PROCEDURE

This is considered to be a non-routine procedure therefore it should only be performed by experienced personnel.

1. Start by examining the last part of the aspirate.
2. Examine the sample as a wet mount, or alternatively you can inoculate Robinson’s culture media to grow *E. histolytica* and prepare a permanent stain slide.

QUALITY CONTROL

- Ensure that culture media is not contaminated.
- Ensure that the microscope has been calibrated in the last year and that the results of the calibration are displayed on the microscope base.
- See QC comments associated with haematoxylin staining.

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REPORT

The presence of any parasites.

LIMITATIONS OF PROCEDURE

- An amoebic liver abscess is a necrotic lesion and therefore the chances of detecting amoebae in it are quite low. A lack of trophozoites does not rule out *E. histolytica*.
- Amoebic cultures take several days and there is no guarantee that the parasite will adapt to culture conditions.

AUTHOR

Ian Crandall

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Hydatid Cysts

PRINCIPLE

When the dog tape worm infects humans it creates a hydatid cyst in its host. The cyst can be removed surgically, however great care must be taken to avoid releasing viable daughter cysts. When hydatid cysts are removed a sample is sent for confirmation and a determination of viability.

SPECIMEN

Hydatid cyst, scraping or aspirate from surgery (i.e. liver abscess) in a bottle or bucket.

REAGENTS

0.1% eosin
10% KOH

PROCEDURE

- 1) If the cyst material is fluid centrifuge at 500g for 3 minutes
- 2) Remove some of the sediment and make a wet mount.
- 3) Examine the material with low power and low light levels looking for scolices, hooklets, and calcareous corpuscles. 0.1% eosin can be added to determine viability (living organisms exclude eosin).
- 4) If the cyst material is viscous or solid add 10% KOH and centrifuge at 500g for 3 minutes.

QUALITY CONTROL

- The absence of scolices or hooklets does not rule out hydatid disease.
- Very viscous material can be sandwiched between two glass slides.

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- Ensure that the microscope has been calibrated in the last year or the last time the optics were changed and that the results of the calibration are displayed on the microscope base.
- As it is not possible to have a positive control specimen to use with this procedure, the technologist should review the appearance and size of the material observed to ensure that they match reference material.
- Request that warning be given prior to the arrival of the sample so that it can be processed quickly.

REPORT

The presence of scolices, hooklets, and calcareous corpuscles and viability of cyst.

LIMITATIONS OF PROCEDURE

Only a portion of the sample is routinely examined. Contents of the cyst that “spill” into the body cavities during the procedure can not be examined.

- The sample may be mushed by the time it arrives for evaluation making identification more difficult.

AUTHOR

Ian Crandall

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Skin Biopsy

PRINCIPLE

Microfilariae of *Onchocerca volvulus* and *Mansonella streptocerca* do not appear in the blood stream. Examination of small pieces of skin tissue or aspiration of tissue fluid from a nodule may demonstrate microfilaria. Bloodless skin snips should be submitted in a small volume of saline as soon as possible.

SPECIMEN

A skin snip in a concave well slide with saline added.

REAGENTS

0.85% normal saline

SAFETY

Microfilaria require passage through their insect vector before they become infective, therefore the sample cannot produce a filaria infection from a sharps stick.

PROCEDURE

1. Place the skin snips immediately in a drop of saline and cover and place in a moist chamber so the specimen will not dry out.
2. Examine under low power for microfilaria when first received and at time points of 1, 4, & 24 hours.
3. If microfilaria are found capture and stain with haematoxylin (see following procedure)

QUALITY CONTROL

- Do not subject the sample to extremes of temperature or let it dry out.

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- Ensure that the microscope has been calibrated in the last year and that the results of the calibration are displayed on the microscope base.
- See QC comments associated with haematoxylin staining.

REPORT

Report as negative after 24H or if positive then any microfilaria or other parasites seen. Speciation requires haematoxylin staining of the observed microfilaria (see **Hematoxylin Stain for Microfilaria**).

LIMITATIONS OF PROCEDURE

- Microfilaria will not be evenly distributed in all parts of the skin, therefore a skin sample may be from a region that does not contain microfilaria.
- The size of the skin sample may be too small to detect infections with low numbers.

AUTHOR

Ian Crandall

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Sputum

PRINCIPLE

The organisms that may be found in a sputum include the migrating larvae of *Ascaris*, *Strongyloides*, hookworm, the eggs of *Paragonimus*, *Echinococcus* hooklets, *Pneumocystis**, *E. histolytica*, *E. gingivalis* and *Cryptosporidium*. In a *Paragonimus* infection the sputum may be viscous and tinged with brownish flecks, which are clusters of eggs (“iron filings”) and may be streaked with blood.

SPECIMEN

Two types of sputum may be submitted: 1) expectorated sputum; and 2) induced “deep” sputum. Deep sputum samples should not contain saliva or other products of the mouth. Induced sputa are collected by pulmonary or respiration therapy staff collecting samples. The induction protocol is critical for success of the procedure, and well trained individuals are mandatory for the recovery of organisms. If possible, sputum samples should be submitted immediately after collection. KOH is added if the sputum is thick however it should be avoided if you are looking for *Entamoeba spp.* or *T. tenax*. If examination has to be delayed for any reason, the sputum should be fixed in 10% formalin to preserve helminth eggs or larva or SAF fixative to be stained later for protozoa.

REAGENTS

3% KOH,
phosphate buffer, pH 7.2,
Giemsa and Haematoxylin stains (commercial products, VWR)

PROCEDURE

1. Examine a direct wet preparation (saline or iodine) using low and high dry power.
2. Remove a 1ml portion of the sample and place it in a 15ml tube.
3. Add 1ml of 3% KOH.
4. Incubate at room temperature for 15 minutes
5. Add 2mls of phosphate buffer.

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6. Centrifuge the material for 5 minutes at 500g.
7. Use the sediment to prepare wet mounts and smears for Giemsa staining.

QUALITY CONTROL

- Make sure that the saline, phosphate buffer, and 3% KOH are free from contamination.
- Include a control slide if Giemsa Stain is used. Red cells should stain grayish, white cell nuclei stain red-purple and cytoplasm stains bluish.
- Ensure that the microscope has been calibrated in the last year or when ever the optics have been changed and that the results of the calibration are displayed on the microscope base.
- Care should be taken not to confuse *E. gingivalis* which can be found in the mouth/siliva, with *E. histolytica* which could result in an incorrect suspicion of pulmanary abcess. *E. gingivalis* will usually contain ingested polymorphonuclear leukocytes (PMNs), while *E. histolytica* may contain ingested red cells.
- see QC comments associated with hematoxylin staining.
- If *Cryptosporidium* is suspected then acid fast staining or monoclonal antibody techniques normally used for detection in stools can be used.

REPORT

Report the species and developmental stage of any parasites seen.

LIMITATIONS OF PROCEDURE

- Care must be taken to ensure that the sputum sample is not contaminated from environmental sources containing organisms.

AUTHOR

Ian Crandall

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Lymph Nodes

PRINCIPLE

Not all parasites can be detected in the blood and stools. Several organisms find that attacking the white cells that form the immune system is an effective strategy. (The best defense is a good offence!) To detect these organisms lymph nodes can be examined for trypanosomes(African, South American), *Leishmania*, and *Toxoplasma*.

SPECIMEN

lymph node impression smears or aspirates

PROCEDURE

This is considered to be a non-routine procedure therefore it should only be performed by experienced personnel.

- 1) A portion of any fluid material can be examined under low power to check for motile organisms.
- 2) Impression smears can be prepared by sandwiching the sample between two slides and staining with Giemsa stain (5% for 30 minutes).
- 3) Smears can be allowed to air dry before they are fixed in methanol and stained in Giemsa stain (5%) for 30 minutes.
- 4) NNN culture media (see appendix) can be inoculated with any remaining material.

QUALITY CONTROL

- Include a control slide if Giemsa Stain is used. Red cells should stain grayish, white cell nuclei stain red-purple, and cytoplasm stains bluish.
- Ensure that the microscope has been calibrated in the last year or any time the optics have been changed and that the results of the calibration are displayed on the microscope base.

REPORT

Any parasites observed.

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LIMITATIONS OF PROCEDURE

- Samples may be difficult to observe if material is too thick.
- Culture of specimens may take several days for organisms to be detectable.
- Reference material should be consulted before a definitive diagnosis is made since these organisms are infrequently seen in Canadian laboratories.

AUTHOR

Ian Crandall

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Muscle Biopsy

PRINCIPLE

The presumptive diagnosis of trichinosis is based on patient history since the original source of the infection is no longer available. Confirmation of trichinosis is provided by finding encapsulated larval *Trichinella spiralis* in a muscle biopsy specimen. The other source of requests for muscle biopsies comes from the suspicion of cestode larval stages (*Echinococcus granulosus*, *Tenia solium*, *Multiceps*, *Spirometra*, or *Diphyllobothrium*).

SPECIMEN

A muscle biopsy sample.

REAGENTS

Pepsin solution (5g pepsin (Sigma), 7mls HCl, make to 1L).

PROCEDURE

This is considered to be a non-routine procedure therefore it should only be performed by experienced personnel.

1. If trichinosis is suspected, the specimen can be examined by squeezing it between two glass plates before proceeding to the next step.
2. Mince tissue and place in 20 volumes of pepsin solution Incubate at 37°C for 12 to 24 hours in a shaker or with a magnetic stirrer.
3. Add 2 volumes of 37°C water (40 volumes of the original sample size).
4. Pour the mixture into a Baermann funnel and add tap water up to the screen.
5. Allow the mixture to settle for 2 hours and then remove the sediment at the bottom of the funnel.
6. Examine under 10X, if no larvae are seen then centrifuge 50mls and examine the sediment.

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QUALITY CONTROL

- Use only fresh Pepsin solution.
- Ensure that the microscope has been calibrated in the last year or any time the optics have been altered and that the results of the calibration are displayed on the microscope base.
- These organisms are not routinely detected therefore use reference material (Orheil and Asch—Tissue Parasites) to confirm the morphology.

REPORT

Any larva observed.

LIMITATIONS OF PROCEDURE

- The diagnosis is based on limited muscle tissue submitted for analysis.

AUTHOR

Ian Crandall

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Rectal or Bladder Biopsy Smears for Coccidia

PRINCIPLE

If a patient has an old, chronic infection of *S. mansoni* or *S. japonicum* the eggs may not be found in the stool, however, eggs will be present in the rectal mucosa. *S. haematobium* passes in the urine therefore a bladder biopsy is ordered.

SPECIMEN

The rectal or bladder biopsy should be delivered to the Parasitology laboratory quickly.

REAGENTS

4% KOH

PROCEDURE

This is considered to be a non-routine procedure therefore it should only be performed by experienced personnel.

1. The biopsy specimen may be crushed between two microscope slides and examined under low light for *Schistosoma mansoni*, *S. japonicum*, *S. haematobium*.
2. To determine if the eggs are viable switch to the 40X objective and look for active flame cells in the miracidia.
3. If necessary small pieces of tissue can be digested with 4% KOH for 2-3 hours at 60-80°C, the sediment collected and examined under low power for eggs.

QUALITY CONTROL

Ensure that the microscope has been calibrated in the last year and that the results of the calibration are displayed on the microscope base.

REPORT

The presence or absence of Schistosome eggs and if they are viable.

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REPORTING RESULTS OF ENTERIC PARASITOLOGY SPECIMENS

1. The stage(s) of parasite(s) present in the specimen should not be included as part of the physician report. However, this information should be recorded on the laboratory workcard. There are two exceptions to this:
 - a) For *Strongyloides stercoralis*, the stage is clinically important and the report should indicate rhabditiform, filariform or free-living adults.
 - b) Haematophagus (forms containing red cells) trophozoites of *Entamoeba histolytica*.

2. The quantity of parasite(s) present in the specimen should not be included as part of the physician report. This information should be recorded on the laboratory workcard. The exception to this is:
 - a) In repeat testing for disseminated strongyloidiasis, increasing quantities and progressively more mature forms may be clinically significant.

3. Unless reliable tests that differentiate *Entamoeba histolytica* from *Entamoeba dispar* are used to distinguish between these two species (eg serology), laboratories should report *Entamoeba histolytica/dispar* to their clinicians. The report should include the following statement: "Only *Entamoeba histolytica* is capable of causing disease and should be treated. *Entamoeba dispar* is non-pathogenic and does not require additional investigation or treatment. Currently, serological testing is the only available test to aid in the discrimination between these species."

4. The following Pathogenic Protozoa should **always** be reported:
 - Entamoeba histolytica*
 - Giardia lamblia*
 - Cryptosporidium parvum*
 - Cryptosporidium* species
 - Cyclospora cayetanensis*
 - Microsporidia
 - Dientamoeba fragilis*
 - Balantidium coli*
 - Isospora belli*
 - Eimeria* species
 - Sarcocystis* species

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5. The following protozoa that are currently considered to be non-pathogenic should **NOT** be reported when present. However, they should be recorded on the laboratory workcard. The report should state: “No pathogenic parasites present.”

Endolimax nana
Entamoeba coli
Entamoeba dispar
Entamoeba harmanni
Entamoeba polecki
Enteromonas hominis
Iodamoeba buetschlii
Retortamonas intestinalis
Trichomonas hominis
*Blastocystis hominis**

*Inclusion of *Blastocystis hominis* in this group may change as its pathogenic potential is clarified by well-controlled studies. Also it is debatable whether it is a protozoan.

6. All helminth species should be reported to the clinician.
7. The presence of polymorphonuclear (pus) cells and red blood cells should be reported without quantity. Their absence should **NOT** be noted on the report.
8. A therapeutic recommendation is inappropriate as part of a laboratory report.
9. The presence of yeast should not be routinely reported.
10. All facets of the work must be completed before the final result can be released. All final results are viewed by the senior technologist using the O&P review work list before release.
11. One stool sample may produce up to two samples (concentrate and sequential stain) to be read independently of each other. To maintain objectivity the samples should be read with no knowledge of prior results. It is therefore advisable to consult other lab members to aid in identification of an observed organism, however an attempt should be made to prevent a single individual making the identification of an organism in different preparations of the same sample. Samples used to test the proficiency of the laboratory can be discussed after each member views and records their results.

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NOTE: The presence of Leprosy, *Cryptosporidium*, *Cyclospora*, *Entamoeba histolytica*, *Giardia lamblia*, *Trichinella spiralis* and species of malaria are reportable to the Medical Officer of Health by the next working day. For more information, please refer to the General Information Manual, page 34.

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CULTIVATION OF INTESTINAL AND UROGENITAL PROTOZOA

PRINCIPLE

Culturing intestinal protozoa confirms their presence and provides material for our research activities. Culturing in the presence of an antibiotic helps to control the inevitable bacteria present in the stool sample.

SPECIMEN

Fresh stool sample.

Robinson's Culture Technique for *E. histolytica*

REAGENTS

BR (see appendix)
0.5% erythromycin,
BRH (see appendix)

PROCEDURE

This is considered to be a non-routine procedure therefore it should only be performed by experienced personnel.

To each bottle of agar slope, add 1.5 ml "BR", 4 drops (0.12 ml) of 0.5% erythromycin and 10 mg of starch

1. Add about 50 mg of fresh stool to the culture bottle, mix with the overlay and incubate 24 hours at 37°C.
2. On the second day and again on the fourth day examine the culture for *E. histolytica*.
3. If required, further subcultures are made in 3 ml volumes of the culture medium.

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QUALITY CONTROL

- Make sure that the agar slope is not outdated or contaminated and that the correct temperature is maintained.
- Ensure that the microscope has been calibrated in the last year and that the results of the calibration are displayed on the microscope base.

REPORT

The presence of growing entamoeba.

AUTHOR

Ian Crandall

REFERENCES

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Free-Living Amoebae

PRINCIPLE

Free living amoebae can be opportunists and cause infections in humans. *Naegleria fowleri* is rare, but can cause fatal primary amoebic meningo-encephalitis when it is present in the CSF. Acanthamoeba feed on bacteria, however they can also thrive in some body sites, such as the eye. Diagnosis is made by finding the trophozoite or cyst forms of these organisms.

SPECIMEN

CSF sample
contact lens
corneal scrapings.

PROCEDURE

This is considered to be a non-routine procedure therefore it should only be performed by experienced personnel.

1. Place the specimen in the center of two agar plates pre-coated with *E. coli* (ATTC). Incubate one plate at 37°C and the other plate at room temperature.
2. Observe the plates daily for 7 days, using a low power objective of an inverted microscope. Examine the agar surface for bacterial trails or tracks which indicate the presence of migrating amoebae.
3. Amoebae can be stained with Giemsa stain to aid in identification.

QUALITY CONTROL

- Make sure that the agar plates are not outdated or contaminated and that the correct temperature is maintained.
- Ensure that the microscope has been calibrated in the last year and that the results of the calibration are displayed on the microscope base.

REPORT

The species of any amoebae observed.

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AUTHOR

Ian Crandall

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COLLECTION OF BLOOD SPECIMENS

Blood

- a) In suspected cases of malaria, whole blood should be collected (0.5 to 7 mLs) in a lavender top (EDTA anticoagulant) or yellow top (ACD anticoagulant) tube as soon as the diagnosis is suspected. Alternatively malaria smears can be prepared directly from a finger prick blood sample (no anticoagulant) They should be submitted to the laboratory for analysis on an urgent basis. Multiple samples may be required to exclude malaria (one smear collected every 12 hours x 3-4).

- b) All filarial infections of man except *Onchocerca volvulus* and *Mansonella streptocerca* can be diagnosed by the demonstration of microfilariae in the blood. Filaria infections may often be identified by examination of day and night bloods and/or by special filtration procedures. Occasionally other blood-borne parasites such as leishmaniasis and trypanosomiasis may be identified in peripheral blood. Whole blood specimens (0.5 to 7 mLs) should be collected in a lavender top (EDTA) or yellow top (ACD) tube.

Factors Affecting Samples

Blood samples should be collected in Vacutainer tubes (as above) or may be a blood smears prepared from a finger prick blood sample. A sample in any other form is not suitable and will not be accepted by the laboratory. These specimens should be canceled in the computer with a comment stating the reason why they were not suitable. Blood samples should be kept at 4C if they are not delivered within 12 hours.

Blood samples should be rejected if:

- There is *any* sign of leakage
- If the tube is broken
- They are incorrectly labeled
- There are not provided in the correct tube (yellow or lavender top)

For samples with evidence of leakage or container damage: wear gloves, place sample in a separate plastic bag to protect subsequent handlers, place in appropriate biohazard disposal container, wash hands and notify supervisor for corrective action.

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Section: Parasitology Manual	Subject Title: Protocol for Malaria Smears	
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Approved by: Laboratory Director	Revision Date: November 26, 2004	
	Review Date: December 15, 2003	

PROTOCOL FOR MALARIA SMEARS

INTRODUCTION

Malaria is caused by infection with the *Plasmodium* parasite. Of the four species associated with human disease, *P. falciparum* is potentially the most dangerous. Recently there has been a continued marked increase on the number of cases of malaria in travelers and immigrants. There have been two recent fatalities. In addition individuals who appear relatively well can progress in 1-3 days to become critically ill and require intensive care. It is therefore essential to have rapid and accurate diagnosis of this parasite as well as access to expert clinical advice on the diagnosis and management of the disease. The key features which determine treatment are: 1) the species of malaria, and 2) the percentage of red blood cells (RBCs) that are infected (i.e. % parasitemia). Testing for malaria should be done on anyone who has a fever or a history of fever (within 72 hours) and who has traveled to or through a malaria endemic area (most of the tropics and subtropics) within 1 year (and especially within 3 months).

MALARIA SERVICE AND TURN-AROUND-TIME (TAT):

A joint malaria protocol exists between the Hematology Department, Toronto Medical Laboratories (policy #QHE00001.01) and the Toronto Medical Laboratories/Mt. Sinai Hospital, Department of Microbiology (policy # MI\PAR\10\v03). A separate tube of blood is to be collected for the Hematology and Microbiology Laboratories.

All malaria bloods are considered **STAT** and will only be performed and interpreted by properly qualified Medical Laboratory Technologists. Processing of blood samples for malaria is available 24 hours/day, 7 days/week. From 7:00 a.m. until 9:00 p.m., the Hematology Laboratory, 222 St. Patrick's Street (SPS), will screen all malaria samples and report results within 2 hours. After 9:00 p.m., all malaria bloods will be screened by either the Rapid Response Laboratory at Toronto General Hospital or by the Rapid Response Laboratory at Toronto Western Hospital, and the sample(s) will be forwarded to the Hematology Laboratory (SPS) with the first delivery (6:30 am) the following morning. Screening will include a thin film (BFR) examination for the presence of malaria parasites, calculation of the percentage of infected RBCs (% parasitemia) and performance of a dipstick test for the detection of *P. falciparum*. All blood samples, whether positive or negative, will be picked up daily by the Microbiology technician

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and/or technologist from the Hematology Laboratory. The Microbiology Laboratory will prepare thick and thin smears and speciate any malaria species seen. The Microbiology results will be reported under the test procedure “Plasmodium – Micro” within 18 hours of the initial Hematology Laboratory thin film and dipstick report, 7 days a week. It is the responsibility of the Microbiology technician and/or technologist receiving a blood sample for malaria to check with the Hematology Laboratory to ensure that they have also received a blood sample on the same patient. Processing of the blood sample in the Microbiology Laboratory should not be delayed because the Hematology Laboratory has not received a sample or has not yet reported their results.

ORDERING AND SAMPLE COLLECTION

A) University Health Network (UHN):

Two orders must be entered in MYSIS, one for “Plasmodium Screen – Hematology”, which is accessioned and resulted by the Hematology Laboratory and/or the Rapid Response Laboratories and one for “Plasmodium – Micro” which is accessioned and resulted by Microbiology/Parasitology. Therefore two lavender (EDTA) tubes of blood should be collected, one for each laboratory.

B) Scarborough Centenary Health Centre, a site of the Rouge Valley Health Centre:

Two separate orders must be entered in MEDITECH, one for “Malaria Screen” which goes to the Hematology Laboratory and one for “Malaria Thick and Thin Film” which goes to Microbiology/Parasitology. Therefore two lavender (EDTA) tubes of blood should be collected.

C) Other Hospitals:

Blood samples will be accepted from non-client hospitals, which will be billed by the Microbiology Department. Non-client hospital labs are asked to provide the original EDTA tube of blood and at least two unstained Thick and two unstained Thin Films. These samples are processed in the Microbiology/Parasitology Laboratory only following screening by the referring Hematology and/or Core Lab. The result will be phoned and/or faxed to the referring laboratory or physician within 18 hours. **A contact name and telephone number must be available for every malaria blood submitted.**

Specimen Rejection Criteria

Leaking, unlabeled, mislabeled, or broken tubes will not be accepted.

Hours of Service:

During regular operating hours of the Parasitology Laboratory, the Parasitology technician or technologist will pick up specimens and a copy of the screening report (if available) from the Hematology Laboratory. Samples that arrive after the lab is closed will be picked up first thing

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the following morning. Samples that arrive in the final hour of operation of the Parasitology Laboratory can be examined the next morning ***if the Parasitology technician or technologist can confirm that hematology will issue a report within 2 hours.*** If this will not be done, then it is the responsibility of the Microbiology/Parasitology technologist to process the blood and report the results.

On weekends and statutory holidays, a qualified Microbiology technologist will be on-call from **8:00 a.m. to 4:00 p.m.** When a malaria sample is received by Hematology, they will page the Microbiology technologist on-call by contacting Locating **(340-3155)** at Toronto General Hospital. The Microbiology technologist should call back to acknowledge receipt of the page, but will only return to the laboratory to process the malaria specimens once during the day. If no page is received, it is the responsibility of the Microbiology technologist on-call to call the Hematology Lab **at noon** to check if any malaria samples have to be reviewed. Should questions of a clinical nature arise, the physician should be directed to page the Microbiologist-on-call or the Infectious Diseases Service through Locating **(340-3155)** at Toronto General Hospital.

Follow-up smears during and after treatment:

These will be performed immediately upon request where there is a concern that the response to treatment is not adequate. In such a case, a clinical consultation must be obtained from Tropical Medicine or Infectious Diseases Service. Routine follow-up smears should be performed daily until discharge for *Plasmodium falciparum*, then at day 7 and day 28 after the start of treatment. For patients not admitted and for *Plasmodium vivax* infections, follow-up smears are performed on day 2 or day 3, day 7 and day 28.

Processing of specimens:

A thick and thin smear should be prepared and interpreted for all blood samples for malaria. If not already performed by the Hematology or Rapid Response laboratories, or the result is not available, then the blood sample should also be assayed for the presence of *Plasmodium falciparum* using a dipstick (antigen) test. The Makromed dipstick test is performed according to the supplied instructions. **A negative dipstick test does not rule out malaria due to other Plasmodium species.**

REPORTING PROTOCOL

Hematology will issue a preliminary report for the thin smear and dipstick result (policy #QHE0001.01). The Hematology result will print automatically in the virology report printer. It is the responsibility of the Parasitology/Microbiology technologist to check the printer for reports on a regular basis. For UHN patients *only*, the Hematology report will be viewable on-line in MYSIS. For non-UHN patients, or if the report fails to print, the report can be accessed by calling the Call Centre at 416 340-5898. If a hematology result is not available on a patient then perform a dipstick test (PHE14012.01) on the sample and report the result.

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A) If the results from Parasitology are consistent with the preliminary report from Hematology then report as follows:

- If the **thick and thin smears are negative**, report "NEGATIVE for *Plasmodium* by microscopy". Add the comment "Malaria cannot be ruled out as a diagnosis on the basis of a single result. If malaria remains as a diagnostic consideration three serial blood samples at 12-24 hour intervals should be submitted".
- If the **smear is positive** for *P. falciparum* report "POSITIVE for *P. falciparum* malaria by microscopy" and report the parasitemia and stages.
- If a **mixed infection is present** that includes *P. falciparum* report "POSITIVE for a mixed infection that includes *P. falciparum* and _____ malaria by microscopy" and report the presence of the additional species, parasitemia and stages.
- If the **dipstick performed in the Parasitology laboratory is positive** and the **smear is negative** report "POSITIVE for *P. falciparum* by HRP2 antigen detection assay, no malaria parasites seen on blood film". **If it is a follow-up blood** add the statement "Dipstick tests can continue to give positive results for up to 4 weeks after successful treatment." (Refer to **Discrepant Results** below)
- If the **smear is positive** for a species other than *P. falciparum* and the **dipstick is negative** report "POSITIVE for *P. _____* by microscopy"
- All results must be phoned to the appropriate ward or attending physician. All positive results for UHN patients must also be phoned to the Infectious Disease Service.
- All new positive Malaria smears for UHN patients will be phoned to the requesting physician, the Infectious Disease resident on-call, Dr. Jay Keystone and/or Dr. Kevin Kain.
- All results are to be signed out by a qualified technologist without queuing for review by the charge/senior technologist. A list of all Malaria testing is available on-line using the lab information system.

B) If the final report from Parasitology is discrepant with the preliminary report issued by Hematology, proceed as follows:

- Notify Hematology immediately of the discrepancy.
- Otherwise, follow the reporting procedure outlined above.

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Discrepant results:

Discrepant results may occur if:

- The individual has been successfully treated for *P. falciparum*. The HRP-2 antigen may continue to circulate for up to 28 days after the infection has been treated.
- The antigen detection test is known to cross react with rheumatoid factor and thereby may produce a false positive result if this is present in the sample.
- In rare circumstances, the HRP-2 protein produced by *P. falciparum* may not be recognized by the antigen detection assay. It is therefore possible that the assay will completely miss a significant parasitemia.
- If a mixed infection is present, the antigen assay will detect the presence of *P. falciparum*, however another species may be present in larger numbers.
- After successful treatment, the thick film smear may remain positive for a longer period of time as it is more sensitive than the thin film.

If further testing is required to resolve whether *Plasmodium* is present, consult Dr. Ian Crandall or Dr. Kevin Kain to discuss further testing.

NOTE: The presence of Leprosy, *Cryptosporidium*, *Cyclospora*, *Entamoeba histolytica*, *Giardia lamblia*, *Trichinella spiralis* and all species of malaria are reportable to the Medical Officer of Health by the next working day. For more information, please refer to the General Information Manual, page 34.

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Malaria - *Plasmodium* spp. and *Babesia* spp.

PROCEDURE

Preparation and staining of thick and thin smears.

PRINCIPLE

A number of parasites may be recovered in a blood samples, either whole blood, buffy coat preparations or various methods of concentration. These include *Plasmodium*, *Babesia*, *Trypanosoma* sp., *Leishmania donovani* and microfilaria.

The simplest method for detecting malaria and babesia continues to be the blood film.

The thick film provides the greatest sensitivity and should be performed on all malaria requests. Thin films have a lower sensitivity and are primarily used to make the species identification.

SPECIMEN

- whole blood in EDTA or ACD anticoagulant
- fingerprick whole blood sample

MATERIALS

Reagents:

absolute methanol

Giemsa stock solution (commercial product) (see appendix)

phosphate diluent buffer (see appendix)

Field's stain (commercial product)

Equipment:

Light microscope with ocular micrometer and set for Kohler illumination

Pasteur pipettes

Glass slides

Sharps container

Staining jars

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Important safety note: It is important to remember that universal precautions should be used at all time when handling blood or body fluids.

QUALITY CONTROL

1. A QC slide of *P. falciparum* or *P. vivax* should be included with a run of stain slides at least monthly and whenever a new batch of Giemsa stain is acquired.
2. The stock solution of Giemsa is stable for many years but must be protected from moisture. The aqueous working solution of Giemsa stain must be prepared fresh for every staining procedure.
3. A Giemsa stock solution must have a screw lid and be adequately protected from moisture and oxygen.
4. When the smear is properly prepared and the stain correct, the background, red cells, white cells, and protozoan parasites will be as described in results.
5. The identification of Shuffner's dots in *Plasmodium ovale* and *Plasmodium vivax* depend on the pH of stain solution. Verify that the buffer is pH 7.0 to 7.2.
6. Record all QC results and report any "out-of-control" results to lab director for action.
7. The microscope should be serviced annually.
8. The biological safety cabinet (BSC) should be serviced annually or after any movement of the BSC and service dates should be recorded on the BSC.

PROCEDURE

Special Safety Note: *Blood tubes should only be opened in a running biological safety cabinet.* Any spills should be cleaned up immediately. Gloves must be worn when preparing samples and any contact with contaminated sharps or contact of blood or body fluids with broken skin should be reported immediately to the lab director and Employee Health. Malaria, Babesia, and blood-borne viruses can be transmitted from blood samples, therefore follow up of exposure is important.

Prepare thick and thin blood films using pre-cleaned grease free frosted slides.

GIEMSA-thin films

- a. A thin film is prepared exactly as one used for differential count. There should be a thin feathered end (at least 2 cm long) containing evenly distributed red blood cells with no overlap and occupying a central area of slide with margins free on both sides.
- b. Allow film to air dry. **DO NOT APPLY HEAT.**
- c. Fix the blood film in absolute methanol for one minute.
- d. Place into Giemsa stain solution for 50 minutes (1:50 dilution--see appendix)
- e. Wash by gently dipping into buffer pH 7.0 -7.2 two to three times. Note: excessive washing will decolorize the film.

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- f. Drain thoroughly in vertical position and allow to air dry.

GIEMSA thick-film

- a. Apply two or three small drops of fresh whole blood onto an alcohol clean slide
- b. With the corner of another slide using a circular motion spread the drops to cover an area approximately 2cm in diameter (Note: you should just be able to read newsprint through a thick smear).
- c. Allow the film to completely air dry (room temperature). **DO NOT APPLY HEAT.**
- d. **DO NOT FIX THICK FILM.** Place film into GIEMSA stain solution for 50 minutes (1:50 dilution-see appendix).
- e. Wash gently in buffer for one to two minutes.
- f. Air dry in vertical position.

EXAMINATION OF BLOOD SMEARS

- a. Thick and thin blood films should initially be reviewed at low power (100 x magnification) particularly at the edges of the thick and thin film where microfilaria, malaria parasites and trypanosomes may be concentrated.
- b. Thick film should then be examined systematically beginning in the center of the film and moving in a defined fashion out from the center. At least 200 oil immersion fields should be reviewed (magnification x 1000).
- c. Thin films should be examined systematically back and forth across the feathered end of the film for at least 300 oil immersion fields (magnification x 1000).

Expected Stain Results

A. Thin film

- a. The background should be clean and free of debris; the color of the erythrocytes is a pale grayish pink.
- b. Neutrophil leukocytes have a deep purple nuclei with well defined granules.
- c. The chromatin of malaria parasites is a deep purplish red and cytoplasm is a clear purplish blue
- d. Stippling should show up Schuffner's dots in erythrocytes containing *Plasmodium vivax* or *P. ovale* and Mauer's spots in erythrocytes containing the larger ring forms of *Plasmodium falciparum*.

B. Thick film

- a. The background should be clean and free of debris with pale mottled gray color derived from the lysed erythrocytes.
- b. Leukocyte nuclei are a deep purple

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- c. Malaria parasites are well defined with deep red chromatin and a pale purplish blue cytoplasm.
- d. In *P. vivax* and *P. ovale* infections the presence of Shuffner stippling in the ghost of the host erythrocyte can be seen.

REPORTING

The presence of malaria parasites, the species identified and the level of parasitemia should be reported immediately to the attending physician and the laboratory director. High levels of parasitemia (>1% or >50,000 parasites/ul)) are critical and should be reported immediately to the attending physician and the laboratory director.

Method of Determining Parasitemia in thick blood films:

- a. Count parasites and leukocytes separately
- b. If after 200 leukocytes have been counted, 10 or more parasites have been identified, record the results in the record form indicating the number of parasites seen per 200 leukocytes.
- c. If after 200 leukocytes have been counted nine or less parasites have been counted, continue counting until 500 leukocytes have been counted and record the parasites observed per 500 leukocytes counted.
- d. Report the parasite count in parasites per microlitre in relationship to the leukocyte count by the following formula; the parasites per microlitre is equal to:

$$\frac{\# \text{ of parasites X white blood cell count per ul}}{\# \text{ of leukocytes counted}}$$

If a white blood cell count is not available assume a white cell count of 6000/ul.

LIMITATIONS

1. It may take several thick and thin blood smears to exclude the diagnosis of malaria, particularly in semi-immune individuals or on individuals on chemosuppressive therapy.
2. The sensitivity of the thick smear is estimated to be 10-100 parasites/ul and therefore low parasitemias may be missed.
3. It may be difficult to determine the species identification in cases with low numbers of circulating ring forms and in cases of mixed infections.

If blood samples are old or if patients have received partial therapy the morphology of the parasites may be altered making species identification difficult.

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Field's Stain

Alternative Stain for Thick Smears

- a) Place thick smear in Solution A for 5 seconds.
- b) Wash by gently rinsing in tap water for 10 seconds.
- c) Gently shake the slide to remove excess water.
- c) Dip in Solution B for 5 second.
- d) Wash as in b) for 10 seconds.
- e) Stand on end and air dry.

EXAMINATION OF BLOOD SMEARS

- Thick and thin blood films should initially be reviewed at low power (100 x magnification) particularly at the edges of the thick and thin film where microfilaria, malaria parasites and trypanosomes may be concentrated.
- Thick film should then be examined systematically beginning in the center of the film and moving in a defined fashion out from the center. At least 200 oil immersion fields should be reviewed (magnification x 1000).
- Thin films should be examined systematically back and forth across the feathered end of the film for at least 300 oil immersion fields (magnification x 1000).

Expected stain results:

A. Thin film

- The background should be clean and free of debris; the color of the erythrocytes is a pale grayish pink.
- Stippling should show up Schuffner's dots in erythrocytes containing *Plasmodium vivax* or *P. ovale* and Mauer's spots in erythrocytes containing the larger ring forms of *Plasmodium falciparum*.

C. Thick film

- The background should be clean and free of debris with pale mottled gray color derived from the lysed erythrocytes.
- Leukocyte nuclei are a deep purple
- Malaria parasites are well defined with deep red chromatin and a pale purplish blue cytoplasm.
- In *P. vivax* and *P. ovale* infections the presence of Shuffner stippling in the ghost of the host erythrocyte can be seen.

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REPORTING

The presence of malaria parasites, the species identified and the level of parasitemia should be reported immediately to the attending physician and the laboratory director. High levels of parasitemia (>1% or >50,000 parasites/ul) are critical and should be reported immediately to the attending physician and the laboratory director.

Method of Determining Parasitemia in thick blood films:

- Count parasites and leukocytes separately
- If after 200 leukocytes have been counted, 10 or more parasites have been identified, record the results in the record form indicating the number of parasites seen per 200 leukocytes.
- If after 200 leukocytes have been counted nine or less parasites have been counted, continue counting until 500 leukocytes have been counted and record the parasites observed per 500 leukocytes counted.
- Report the parasite count in parasites per microlitre in relationship to the leukocyte count by the following formula; the parasites per microlitre is equal to:

$$\frac{\# \text{ of parasites} \times \text{white blood cell count per ul}}{\# \text{ of leukocytes counted}}$$

If a white blood cell count is not available assume a white cell count of 6000/ul.

LIMITATIONS

- It may take several thick and thin blood smears to exclude the diagnosis of malaria, particularly in semi-immune individuals or on individuals on chemosuppressive therapy.
- The sensitivity of the thick smear is estimated to be 10-100 parasites/ul and therefore low parasitemias may be missed.
- It may be difficult to determine the species identification in cases with low numbers of circulating ring forms and in cases of mixed infections.
- If blood samples are old or if patients have received partial therapy the morphology of the parasites may be altered making species identification difficult.

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***Leishmania* spp.**

PRINCIPLE

Leishmania is found in macrophages around the point of infection.

SPECIMEN

Scrapings from infected areas.

SAFETY

Blood tubes should only be opened in a running biological safety cabinet. Any spills should be cleaned up immediately. Gloves must be worn when preparing samples and any contact with contaminated sharps should be reported. *Leishmania* can be transmitted from blood samples, therefore follow up of exposure is recommended.

REAGENTS

Absolute methanol
Giemsa stain.
NNN media (see appendix)

PROCEDURES

This is considered to be a non-routine procedure therefore it should only be performed by experienced personnel.

1) Smears:

- a) Prepare smears - from touch preps of biopsy material or from an aspirate from the edge of a lesion.
- b) Dry and fix in methanol.
- c) Stain with Giemsa (as for malaria smears) and examine under oil immersion (X1000) for the presence of amastigotes.

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***In Vitro* Cultivation**

- a) Inoculate biopsy material or aspirate from the lesion to prewarmed (room temperature) NNN media culture tubes and incubate at room temperature. Attending physician inoculates specimen at bedside.

- b) Examine culture using a wet prep at 10, 14, and 21 days for the presence of promastigotes.

QUALITY CONTROL

- Make sure that media is not outdated or contaminated.
- Ensure that the microscope has been calibrated in the last year and that the results of the calibration are displayed on the microscope base.
- Include a control slide if Giemsa Stain is used. Red cells should stain grayish, white cell nuclei stain red-purple and cytoplasm stains bluish.

REPORT

Report the presence of *Leishmania* sp. promastigotes from culture and amastigotes from biopsies or aspirates. Definitive species identification requires isoenzyme analysis or molecular methods.

AUTHOR

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Microfilaria in Blood

PRINCIPLE

Male and female filaria invade tissues and then produce offspring that are the means of transmission of the disease. The offspring, microfilaria, circulate in the blood and frequently display peak circulation times that match the biting habits of the insects responsible for their transmission. Several methods exist to detect the presence of microfilaria. A Knott concentration destroys the red cells and concentrates the remaining material, including any microfilaria present. The Nucleopore filter method also destroys the red cells and then filters out any remaining debris.

SPECIMEN

Blood sample in a yellow or purple top tube

SAFETY

The usual precautions when handling blood should be observed. Microfilaria require passage through their insect vector before they become infective, therefore the blood sample cannot produce a filaria infection from a sharps stick.

REAGENTS

2% buffered formalin.

PROCEDURE

This is considered to be a non-routine procedure therefore it should only be performed by experienced personnel.

Knott Concentration

- a) Mix 1 ml of blood with 10 ml of 2% buffered formalin.
- b) Centrifuge at 1,500 rpm for 5 minutes.
- c) Decant supernatant and examine the sediment.

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NOTES

Microfilaria come in two varieties, sheathed and unsheathed. There are also a large number of artifacts that can be mistaken for microfilaria. Microfilaria should have the following characteristics: 1) they should have a smooth surface; 2) they should have rounded or tapered ends; 3) they should contain internal structures; 4) they are frequently motile if they have not been exposed to preservatives; 5) they should be between 200 - 300 μ M in length; and if multiple microfilaria are present they should be very similar to each other.

QUALITY CONTROL

- Ensure that the formalin solution is not outdated.
- Ensure that the microscope has been calibrated in the last year and that the results of the calibration are displayed on the microscope base.

REPORT

The presence of microfilaria or other parasites. Haematoxylin staining is required to determine the species (see **Hematoxylin Stain for Microfilaria**). The sheath may not be visible if Giemsa is used.

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Microfilaria Isolation on Nucleopore Filters

PRINCIPLE

The Nucleopore filter method is gaining in popularity because large volumes of blood can be examined (many mls) and therefore it can detect the presence of microfilaria down to less than one organism per ml.

SPECIMEN

Blood sample in a yellow or purple top tube

SAFETY

Make sure that the filter unit is assembled correctly and never force fluid through the filter unit at a high pressure-- this is an invitation to spraying the sample.

PROCEDURE

This is considered to be a non-routine procedure therefore it should only be performed by experienced personnel.

1. Place a nucleopore filter membrane (25 mm, 0.3 µm) in a Swinex filter being careful not to damage the membrane. (The membrane is pale white, the wax paper between is pale blue.)
2. Assemble the filter unit and tighten until snug.
3. Place a 12 ml syringe, a glass slide, a beaker of water and a beaker for waste material in a sterile cabinet.
4. Draw 1 ml of blood into the syringe, followed by 10 ml of water.
5. Swirl the contents of the syringe for 30 seconds to lyse the red cells.
6. Connect the syringe to the filter unit and gently press the contents of the syringe through the filter. Refill the syringe with 10 ml of water and gently push that through the filter. If the filter alone is being examined for microfilaria, then try to push about 2 ml of air through the filter. If the microfilaria will be examined after release from the filter, omit the air step since it "sets" the microfilaria onto the filter and makes

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dislodging them difficult.

7. Disconnect the water filter from the syringe, and open the filter unit.
If the filter alone is being examined, then remove the membrane from the unit and place it on a glass slide. A "wet mount" examination of the filter can be made at this time. Place a generous drop of 100% methanol on the filter, wait 30 seconds and then let the methanol run off the slide. Stain the filter with Giemsa (30 minutes/5% stain works well), gently rinse with tap water and examine. Finer microfilaria require higher magnification and slide can be mounted to permit oil immersion.

If the microfilaria are to be removed from the filter, then the filter can be agitated with a minimal amount of water to release the microfilaria. The liquid sample can then be transferred to a slide to check for the presence and species of microfilaria. Not all of the microfilaria release with this method so it is important to examine the filter (as above) to determine if microfilaria are present.

8. Properly dispose of the liquid waste and the syringe and wash the filter unit.

QUALITY CONTROL

- White cell debris should be present on the filter membrane. The integrity of the filter unit can be determined at the point in the assay where air is forced into the filter unit-- if air passes through the membrane easily then the procedure needs to be repeated with a new filter unit.
- Ensure that the microscope has been calibrated in the last year and that the results of the calibration are displayed on the microscope base.

REPORT

Any microfilaria or other parasites seen. Speciation requires haematoxylin staining of the observed microfilaria (see **Hematoxylin Stain for Microfilaria**).

AUTHOR

Ian Crandall

REFERENCES

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Hematoxylin Stain for Microfilaria

PRINCIPLE

In order to determine the species of microfilaria isolated by other methods it is necessary to perform a hematoxylin stain on the specimen. Giemsa staining is faster and easier, however the staining of the sheath, an important feature for determining the species can be variable with Giemsa stain.

SPECIMEN

A microfilaria isolated using an isolation method.

REAGENTS

Hematoxylin A & B (commercial product)
Xylol
Methanol
Entellan (commercial product)

SAFETY

Microfilaria require passage through their insect vector before they become infective, therefore the sample cannot produce a filaria infection from a sharps stick.

PROCEDURE

This is considered to be a non-routine procedure therefore it should only be performed by experienced personnel.

1. If a thick film is being examined dehemaglobinize the slide in tap water for 10 minutes and then air dry the specimen.
2. If a thin film is being examined fix in absolute methanol for 15 minutes and then allow the specimen to air dry.
3. Place in running tap water for 5-10 minutes.
4. Place in hematoxylin-mordant solution for 10 minutes (30 minutes if *M. perstans* is suspected).
5. Rinse in tap water 1 minute
6. Rinse in 50% methanol + 10 drops of ammonia for a few seconds
7. Rinse in 70% methanol + 10 drops of ammonia for a few seconds

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8. Rinse in 85% methanol for a few seconds
9. Rinse in 95% methanol for a few seconds
10. Place in absolute methanol for 5 minutes
11. Place in Xylol for 5 minutes
12. Place in Xylol for 5 minutes
13. Mount in Entellan

PROCEDURE NOTES

1. This stain gives the best morphological details. Organisms can be missed under bright light, therefore low power observations may be preferable.
2. The most important step in the preparation of a well stained permanent smear is good fixation. If good fixation has not been successful the microfilaria will be distorted or may stain poorly.
3. Once the staining process has started the smear should not be allowed to dry at any of the subsequent steps until they are cover-slipped.
4. Slides should always be drained thoroughly between solutions, touch the end of the slide with a paper towel to remove excess fluid.
5. In the final stages of dehydration the 100% alcohol and xylol should be kept as free from water as possible. Containers must have tight fitting caps to prevent evaporation or absorption of moisture. If xylol becomes cloudy replace with fresh stock.
6. If smears peel or flake off, the specimen may have been inadequately dried on the slide or the smear may be too thick
7. Patient's stained slides must be allowed to dry thoroughly, (eg. overnight) before examination.
8. Vernier measurements should be taken for the location of any parasite with unusual morphology so that confirmation can be obtained.
9. The species of the microfilaria should be determined using reference material such as "Bench Aids to the Diagnosis of Microfilaria (WHO).

REPORT

Presence and species of microfilaria.

LIMITATIONS OF PROCEDURE

- Species determination is a desirable outcome, however sometimes the numbers or condition of the microfilaria in the sample makes this difficult.

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AUTHOR

Ian Crandall

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Toxoplasma gondii

PRINCIPLE & SPECIMEN

The majority of samples submitted for Toxoplasma detection are CSF samples. Toxoplasma can be present in two forms, either tachyzoites or bradyzoites and tachyzoites are the form that you expect to see in CSF.

SAFETY

Assume that all samples are biohazards.

PROCEDURE

This is considered to be a non-routine procedure therefore it should only be performed by experienced personnel.

- a) Prepare smears from spun sediment.
- b) Fix in absolute methanol and stain with Giemsa for 50 minutes.

QUALITY CONTROL

- Ensure that the microscope has been calibrated in the last year and that the results of the calibration are displayed on the microscope base.
- Include a control slide if Giemsa Stain is used. Red cells should stain grayish, white cell nuclei stain red-purple and cytoplasm stains bluish

REPORT

All parasites seen including *Toxoplasma*. Bradyzoites are pyriform with no kinetoplast, tachyzoites will be crescent-shaped especially when released from cells.

LIMITATIONS OF PROCEDURE

AUTHOR

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Introduction

PRINCIPLE

Estimation of the number of acid fast bacteria, together with the recording of the morphological and tinctorial appearance of the bacilli are essential requirements in the assessment of the type of the infection, the severity of the infection and the response of the infection to treatment.

SPECIMEN

The skin smear is a method that permits such estimation, but it is to be used only as a supplement to a biopsy, which is required for a definitive diagnosis and classification of the type of leprosy.

SAFETY

Leprosy is not highly contagious however the samples should be treated as biohazards.

NOTES

1. Skin smears are taken from appropriate locations on the body as designated by the patient's physician.
2. Smears are routinely taken from both earlobes of newly admitted patients.
3. To give a more reliable index of progress, repeat smears are obtained from sites previously tested.
4. Time intervals between repeat smears are determined by the physician. Suggested minimal intervals:
 - (a) for active patients: every 3-6 months
 - (b) for inactive patients: yearly
 - (c) for discharge: as required by the laws of local government.
5. All microscopic slides on which skin smears are made should be pre-cleaned in 70% alcohol, acetone or alcohol-acetone to remove amorphous debris found on such slides. The slides are wiped dry with a clean hand towel. Blades used in smear-taking are likewise cleaned.

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Procedure for Obtaining Smears

This is considered to be a non-routine procedure therefore it should only be performed by experienced personnel.

1. The patient is seated with his back to the technician to minimize his reaction to the procedure.
2. The skin area is cleaned with 70% alcohol, after which it is wiped dry with cotton. Zepharin tends to make the skin too slippery.
3. A fold of skin is made relatively avascular by pinching. If the skin cannot be grasped by pinching, it is instead compressed. A glove worn on that hand may aid in grasping.
4. The smear location is tested for anesthesia with a sharp pin. If there is inadequate decrease in sensation, local anesthesia with 1T Xylocaine or ethyl chloride spray be carefully applied. The compression of the skin by pinching aids in the anesthesia.
5. An incision 5 mm long and 4 mm deep is made with an alcohol-cleansed single-edge razor blade. (A scalpel may also be used.) Pressure is continuously applied to the area until an adequate smear has been taken.
6. If blood exudes, it is wiped away. Blood must be excluded to obtain a satisfactory representative smear. The hand of the operator which does the scraping contains a swab of sterile cotton in addition to the blade (see illustration below). This permits that hand to wipe away any blood, allowing the other hand to maintain relative avascularity.

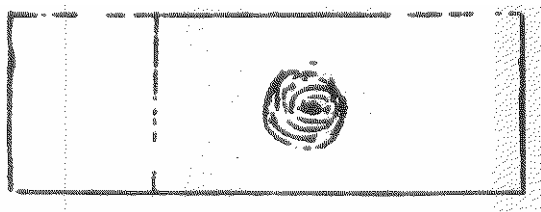


Cotton is held between the third and fourth fingers and the blade between the thumb and second finger of the same hand. This hand is thus able to both scrape the area and wipe away any blood while the other hand maintains constant pressure and relative avascularity at the site.

7. The edges of the wound are scraped with the blade held at a right angle to the incision. Upon scraping, tissue juice and dermal tissue are obtained.

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8. The material is transferred to a clean microscopic slide. A moderately thick smear, without gross blood, with a visible uniform opacity is made. The smear is made in a circular manner (see illustration below) on the slide encompassing an area of some 5-6 mm.



The smear is made in a circular manner on the slide ending in the center and leaving a central button which can be easily focused upon with the microscope.

9. The wound is treated and the edges are opposed to obtain minimal scarring.
10. A single technician takes all smears to provide for more uniform and meaningful results.
11. The smears are then fixed and stained. This is done on the same day that the smears are obtained (see method below).
12. The stained smears are examined under a quality microscope. Numbers and morphology of bacilli are reported per examination under the oil immersion objective. Koehler light illumination and best obtainable lens quality are essential to definitive examination. A single trained technician reads all smears.

Staining of Skin Smears

REAGENTS

10% formalin, carbol-fuchsin, acid-alcohol, alkaline methylene blue (see next page)

PROCEDURE

This is considered to be a non-routine procedure therefore it should only be performed by experienced personnel.

1. Dry the slide with smear in air at room temperature or in an incubator.
2. Place slides in 10% formalin for 15 minutes for fixation.
3. Rinse in tap water.

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4. Stain in Ziehl-Neelsen carbol-fuchsin for 20 minutes at 25 °C, flooding the dye on the slide.
5. Wash off excess dye in tap water.
6. Rinse in acid-alcohol for one minute for decolouration.
7. Rinse slides thoroughly in tap water.
8. Counterstain with alkaline methylene blue for 10 seconds, rinse in tap water and dry in air. For permanent preparation, mount under coverslip in "Permout".

Z-N Carbol Fuchsin

Basic fuchsin	1.0 g
Phenol crystals	5.0 g
95% alcohol	10.0 cc
Water, to make	100.0 cc

Label as "Z-N Carbol Fuchsin" and date.

Acid Alcohol

Hydrochloric acid, concentrated	2.0 cc
95% alcohol, to make	100.0 cc

Label as "Acid Alcohol" and date.

Alkaline Methylene Blue

NaOH 10%	0.06 cc
Methylene Blue	0.35 g
95% alcohol	16.00 cc
Water, to make	100.00 cc

Label "Alkaline Methylene Blue" and date.

Microscopic Examination of Skin Smears

PRINCIPLE

The stained smears are examined under a quality microscope. Total numbers and morphology of bacilli are reported per examination under the oil immersion objective. Koehler illumination and best obtainable lens quality are essential for definitive examination. A single technician reads all smears. The smear will generally have similar numbers of bacilli throughout. However, four separate quadrants of the smear are examined for total numbers of bacteria (bacterial index). The morphology of the bacteria in each of the four quadrants is also noted. Results are averaged and reported.

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Reporting of Total Numbers of Bacteria (Bacterial Index, BI)

Reporting is done using either a descriptive phrase or a numerical code on a semi-logarithmic scale.

Very numerous	-	over 1000 bacilli per oil immersion field
Numerous (5 plus)	-	100-1000 bacilli per oil immersion field
Moderate (4 plus)	-	10-100 bacilli per oil immersion field
Few (3 plus)	-	1-10 bacilli per oil immersion field
Very few (2 plus)	-	from 10-100 bacilli per entire slide (100 fields)
Rare (1 plus)	-	from 1-10 bacilli per entire slide

Reporting of Bacterial Morphology (Morphological Index, MI)

PRINCIPLE

Mycobacterium leprae is a rod-shaped bacterium usually having a length of 1-7 microns and a width of 0.3-0.5 microns. It is said to be **solid staining** only when there is dense uniform staining of the entire bacillus with even sides and rounded ends; the length of the solid bacillus is at least five times the diameter (width) of the bacillus. Only bacilli visualized in their entirety are counted as regards morphology; bacilli which overlap are counted for the bacterial index only.

A **non-solid** staining bacillus shows one or more of the following features: uniform dense staining short form (with rounded or fragmented ends) less than five times as long as in width, failure to stain uniformly, faint staining, the presence of beading and/or fragmentation. Odd shaped forms are considered non-solids.

Morphological Index is reported as the number of solid forms per 100 total bacilli examined. When there are less than 100 total bacilli per smear, they are reported as solid forms per total number of bacilli found. As with the bacterial index, all four quadrants of the smear are examined for the morphology of the bacilli. Results are averaged and reported.

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A few bacillary forms are illustrated below.

- A solid staining bacillus
- B non-solid, at end of bacillus
- C non-solid, at side of bacillus



- D-E morphology cannot be determined due to overlap - counted only in regard to bacterial index
- F shorter solid form than A
- G short form - too short to be counted as solid - length is not 5x or more width
- H fragmented bacillus (non-solid)
- I beaded bacillus (non-solid)
- J club form (non-solid)

Tissue Fixation

REAGENTS

Neutral Buffered Formaldehyde Solution (pH 7.0)

PROCEDURE

It is recommended that specimens be fixed in neutral buffered formalin. This solution lends itself to good fixation and use in different climates, as well as use in shipping tissue long distances. Formalin "neutralized" with calcium or magnesium carbonate added to excess becomes acid readily as tissue is fixed and is not recommended.

Neutral Buffered Formaldehyde Solution (pH 7.0)

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37-40% Formaldehyde solution	100.0 ml
Distilled water	900.0 ml
Acid sodium phosphate-monohydrate (NaH ₂ PO ₄ ·H ₂ O)	4.0 mg
Anhydrous disodium phosphate (Na ₂ HPO ₄)	6.5 g
Label as “Neutral Buffered Formaldehyde Solution (pH 7.0)”	

Staining Procedure for the Leprosy Bacillus Tissues

As used at the U.S.P.H.S. Hospital, Carville, LA

MATERIALS	Xylene	2 parts
	Peanut or mineral oil	1 part
	1% alcohol:	
	Hydrochloric acid, concentrated	1.0 cc
	Alcohol 70%	99.0 cc
	Acid, carbolic, melted crystals	2.5 cc
	Alcohol, absolute	5.0 cc
	Basic fuchsin	0.5 g
	Distilled water, to make	50.0 cc
	Keeps well at room temperature.	
	Working Methylene Blue Solution:	
	Methylene blue	0.5 g
	Glacial acetic acid	0.5 cc
	Tap water	100.0 cc

PROCEDURE

This is considered to be a non-routine procedure therefore it should only be performed by experienced personnel.

1. Deparaffinize sections with Xylene-peanut oil or Xylene-mineral oil solution - two changes, six minutes each.
2. Rinse with running tap water for one minute. Then drain slides for some 45 seconds. **Do not remove all of the oil.** There should be a thin film of oil on the slide at all times during the procedure. The oil hastens acid-fast staining without the use of heat. Do not blot slides.
3. Stain with carbol fuchsin for 20 minutes at **room temperature.**

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4. Wash in running tap water for 3 minutes.
5. Decolorize in 1% acid alcohol until the slide is faint pink. This takes approximately one minute for most slides and slightly longer for larger sections. The acid alcohol should be changed regularly and never used when it is more than a pink color. This is the most critical step.
6. Wash in tap water for 5 minutes.
7. Counterstain with methylene blue for 1-2 minutes according to desired background.
8. Wash in tap water for 20 seconds.
9. Allow to air dry. After the slide has air dried thoroughly, wipe excess stain with oil off the slide with a clean cloth dampened with water. Do not blot or wipe tissue section.
10. Mount in permount. This slide should be mounted in permount as soon as possible and never allowed to remain open to the air overnight.

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ACTION PLAN

For quality control samples that fall out of expected range.
Do not process any patient samples until the problem is rectified.

- 1) Report problem to the Lab Director.
- 2) Document the problem in the QC Log and the Action Log.
- 3) Review procedure with technician/technologist who did procedure.
- 4) Check the expiry date and obvious evidence of contamination on all reagents and solutions used.
- 5) Ensure storage of reagents and solutions is appropriate.
- 6) Check the log of freezer, fridge and other to ensure within expected performance range. Check certification and log and pass service record of centrifuge and microscopes.
- 7) Remake reagents in appropriate solutions.
- 8) Repeat appropriate stain on QC samples. Discuss repeated QC results with Lab Director.

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QUALITY ASSURANCE PROCEDURES FOR THE PARASITOLOGY LABORATORY

1. Equipment

Microscopes:

- The microscopes located in Parasitology are used exclusively by those technologists and are under a service contract under which all microscopes are serviced yearly.
- It is the responsibility of the technologist using the microscope to check the Kohler illumination before starting the day's work and to record and initial this procedure in the Kohler illumination binder.
- It is essential that objectives not be moved between microscopes since it may affect the calibration of the microscopes.
- The condenser's numerical aperture should be equal or greater than the highest objective numerical aperture.
- Ensure that spare light bulbs are available for all microscopes
- use a camel hair brush to clean eyepieces and all glass surfaces
use disinfectant (70% isopropyl alcohol) to clean the stage
- keep microscopes covered when not in use.
- report any frayed or broken electrical wires.

Centrifuges:

- before each run visually inspect the device for loose or damaged parts
- inspect any seals and O-rings on buckets and lids
- inspect the interior for signs of leaking on a previous run and clean with disinfectant as required
- disinfect the centrifuge monthly
- the speed can be checked with a stroboscope or tachometer quarterly
- check the brushes every six months
- record any problems encountered

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Fume Hood:

- at least yearly check to make sure the hood draws air in at a velocity of 100ft/min with the sash fully open
- lubricate the sash guides
- ensure that the minimum amount of material is being stored in the hood
- ensure that stored material does not impede air circulation

Biological Safety Cabinet:

- The BSC is under contract to be cleaned and recertified once a year (see sticker on the cabinet).
- after each use disinfect the work area (no UV light is provided, use bleach solution)
- if the cabinet is moved it must be decontaminated and recertified.
- do not open any section of the cabinet
- check the air pressure gauge on the front of the cabinet. Do not use if the gauge indicates a pressure outside of the operating limits (see gauge) and record reading daily on sheet on side of cabinet.

2. Control slides

- Control slides are used in the various staining procedures performed in the lab.
- Control slides are to be used with each rack of slides to be stained for the Sequential Stain (routine lab stain), Hematoxylin Stain, Modified Kinyoun Stain and Modified Trichrome Stain (Chromotrope Stain).
- The control slides are read by the technologist and the results are recorded and initialled on record sheets maintained in the Laboratory Manual.
- Specimens received for testing such as QMP-LS are handled in a routine manner and are read by the assigned technologist.
- Staining control slides are used whenever QMP-LS specimens are stained.
- The following quality control organisms are available from the ATCC :
 - ATCC 30010 *Acanthamoeba castellanii*
 - ATCC 30133 *Naegleria gruberi*
 - ATCC 30925 *Entamoeba histolytica*
 - ATCC 30001 *Trichomonas vaginalis*
 - ATCC 30883 *Leishmania mexicana*
 - ATCC 30160 *Trypanosoma cruzi*
 - *Escherischei coli* can be obtained from the Microbiology Dept.

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3. Participation in Quality Assurance Programs:

- We participate in QMP-LS, which provides stool samples for preparation, staining and reading.
- All positive malaria bloods are kept and are frozen for PCR testing. Malaria speciation can then be confirmed by this accepted gold standard.
- Monthly meetings are held to discuss laboratory related issues and to determine if an evaluation of a new method is warranted.
- Participation in both the CAP Parasitology and the CAP Blood Parasite surveys.

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PERFORMANCE STANDARDS PARASITOLOGY LABORATORY

Based on current figures the following standards are expected of an MLT working in the Parasitology Laboratory at The Toronto Hospital per day:

- Inexperienced – 15-20 O&P stools (concentration and/or stain)
reading time ranges from 19-26 minutes/specimen
- Moderate experience – 25-30 O&P stools (concentration and/or stain)
reading time ranges from 13-16 minutes/specimen
- Experienced – 40 - 50 O&P stools (concentration and/or stain)
reading time ranges from 8 -10 minutes/specimen

These figures are calculated using a 6 ½ hour working day.

It should be noted that these figures are the accepted standard for laboratories only performing stools for Ova and Parasite examinations. Our laboratory performs many other tests requiring the technologist's time on any given working day. The most important are malaria bloods. Malaria bloods are almost always STAT and must be processed and reported within 45-60 minutes from receipt of specimen. Therefore the standards listed above the MLTs reading O&P stools must be reduced as follows if malaria bloods are received:

- Inexperienced – total read reduced by 2 - 3 O&P stools/malaria blood received
- Moderate experience – total read reduced by 4 - 5 O&P stools/malaria blood received
- Experienced – total read reduced by 6-8 O&P stools/malaria blood received

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RETENTION TIMES

Occasionally a question may arise as to a diagnosis made on a submitted sample. It is therefore necessary to retain specimens and records to allow an examination of any material that is queried.

Retention Times

- All records will be retained for at least one year
- Discontinued laboratory methods (manual sections) will be archived for 2 years.
- Blood samples will be retained for 2 days after examination and reporting
- All positive malaria smears and samples, all positive filaria smears and samples, all positive Leishmania smears and samples, and other smears and samples of interest will be saved indefinitely.
- Stool samples will be retained until after they are reported and will then be disposed of the following week
- Any specimen sent to PHL for confirmation will be saved until the result is received from PHL.
- Stained slides will be retained until after they are reported and will then be disposed of the following week
- Any positive *E. histolytica* samples or any unusual or interesting positives will be saved and added to the teaching collection.

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APPENDICES

I. Buffered Neutral Formalin Solutions

1. 10% formalin, buffered

Na ₂ HPO ₄	10.7 g
NaH ₂ PO ₄	0.23 g
Formalin (commercial)	1200 ml
Water	10,800ml

Mix the formalin with water and then add the buffer salts.

Mix thoroughly before dispensing.

Smaller quantities can be prepared but this solution is very stable.

The pH should be approximately 7.0.

Label as "10% formalin, buffered" and date.

2. 5% formalin, buffered

Na ₂ HPO ₄	10.7 g
NaH ₂ PO ₄	0.23 g
Formalin	600.00 ml
Water	10,800.00 ml

Prepare as for 10% buffered formalin.

Label as "5% formalin, buffered" and date.

It is suggested that each buffer salt is weighed as listed above and mix the two thoroughly.

Store the mixture in a tightly closed bottle.

Prepare one litre of either 10% or 5% formalin and add 0.8 g of the buffer salt mixture.

Phosphate salts are purchased from VWR.

II. Phosphate Bufer

Na ₂ HPO ₄	10.7 g
NaH ₂ PO ₄	0.23 g
Water	11,200.00 ml

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Label as “Phosphate buffer” and date.

It is suggested that each buffer salt is weighed as listed above and mix the two thoroughly.

Store the mixture in a tightly closed bottle.

Phosphate salts are purchased from VWR.

III. Lugols Solution

Iodine	5.00 g
Potassium iodide	10.00 g
dissolve in distilled water	100.00 ml

Label as “Lugols Solution” and date.

IV. Modified Schaudinn's Fixative (working solution)

Glacial acetic acid	5.00 ml
Glycerol	1.50 ml
Schaudinn's stock solution (two parts saturated mercuric chloride solution and one part 95% Ethanol)	93.50 ml

label as “Modified Schaudinn’s Fixative” and date.

V. SAF Fixative

Sodium acetate	1.50 g (1.5%)
Acetic acid, glacial	2.00 ml (2.0%)
Formaldehyde, 40% commercial solution	4.00 ml (1.6%)
Water	92.50 ml (94.9%)

Label as “SAF Fixative” and date.

Remarks

As 40% formaldehyde is used, to calculate the percentage in the final solution, one has to divide by 2.5.

VI. Iodine Alcohol

Prepare a stock solution by adding enough iodine crystals to 70% alcohol to make a dark, concentrated solution. Label as “Iodine Alcohol” and date. For use, dilute some of the stock with 70% alcohol until a strong coloured solution like "tea" is obtained.

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VII. Haematoxylin

Stock Solution 'A'

Haematoxylin crystals	10. g
95% ethyl alcohol	1000. ml

Label as “Haematoxylin Stock A” and date.
Allow to ripen in the light for one week (the solution may require filtration).
Solution A is purchased from VWR (R03439).

Stock Solution 'B' - (Mordant)

Ferrous ammonium sulfate	10. g
Ferric ammonium sulfate	10. g
Concentrated hydrochloric acid	10. ml
Distilled water	1000. ml

Label as “Haematoxylin Stock B (Mordant)” and date.
Solution B is purchased from VWR (R03439)

Working Solution

Solution 'A'	25. ml
Solution 'B'	25. ml

This should be prepared at least 3-4 hours before staining commences.

VIII. Picric Acid Solution

Saturated aqueous picric acid	25. ml
Distilled water	25. ml

Label as “Picric Acid Solution” and date. Make sure bottle is sealed.
Saturated aqueous picric acid is purchased from VWR (3347-1).

IX. Carboxylol

Phenol, liquified in a warm water bath	250. ml
Xylol	750. ml

Label as “Carboxylol” and date.
Add the phenol to the xylol, keeping the phenol warm so that it does not start to crystallize.
Phenol (B29477-34) and Xylol (B30575-86) are purchased from VWR

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X. Kato Thick Smear - Cellophane Solution

Glycerin	100. ml
Distilled water	100. ml
3% aqueous malachite green	1. ml

Label as “Kato Thick Smear- Cellophane Soln” and date. Soak the cellophane for at least 24 hours in this solution.
Malachite Green is purchased from VWR (Gurr B34045).

XI. Field's Stain

Solution 1

Methylene blue	0.80 g
Azure B	0.50 g
Disodium hydrogen phosphate (anhydrous)	5.00 g
Potassium dihydrogen phosphate (anhydrous)	6.25 g
Distilled water	500.00 ml

Label as “Field’s Stain- Soln 1” and date.

Solution 2

Eosin (yellow, water soluble)	1.00 g
Disodium hydrogen phosphate (anhydrous)	5.00 g
Potassium dihydrogen phosphate (anhydrous)	6.25 g
Distilled water	500.00 ml

Label as “Field’s Stain- Soln 2” and date.

Note:

Solutions 1 and 2 are purchased commercially from VWR (35056 3E & 35057 3G).
The stains may be kept for several weeks and used over and over, if they are in covered taining jars. When the eosin solution turns greenish, the stains should be renewed.

XII. Giemsa Stain

Giemsa stain powder	600. mg
Methyl alcohol (acetone free, neutral)	50. ml
Glycerin (neutral, from freshly opened bottle)	50. ml

Label as “Giemsa Stain” and date.

Note: Giemsa stain is purchased commercially from VWR (GURR R66 35086).

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XIII. Robinson's Medium for *E. histolytica* Culture

Materials

- 1) Saline agar slope. Prepare 1.5% agar in 0.7% NaCl, distribute in 2.5 ml lots in quarter-ounce screw capped glass bottles, autoclave and slope.
- 2) Erythromycin solution. Prepare 20% in 70% ethanol in a sterile tube and leave for 2 hours at room temperature. Further dilute to 0.5% in sterile distilled water (0.5 ml of 20% + 19.5 ml distilled water).
- 3) Bactopeptone. 10 g in 50 ml distilled water, autoclave and aliquot in 5 ml lot.
- 4) Rice starch (BDM brand).
- 5) "R" medium for growing *Escherichia coli*, strain B

Stock solution

NaCl	25.00 g
Citric acid, monohydrate	10.00 g
Pot. dihydrogen phosphate	2.50 g
Magnesium sulfate heptahydrate	0.25 g
Lactic acid (BDH 90.08%)	20.00 ml
Distilled water	500.00 ml

Working Solution

Stock solution	100.00 ml
40% NaOH	7.50 ml
0.04% bromothymol blue solution	2.50 ml
Distilled water	1,000.00 ml

Adjust pH to 7.0 and autoclave.

- 6) Pot phthalate (0.5 M) dissolve 20.4 g in 10 ml of 40% NaOH and add distilled water to 200 ml. Adjust pH to 6.3 and autoclave.

Working Solution

Dilute the 0.5 M phthalate to 0.05 M in sterile distilled water and adjust pH to 6.5.

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- 7) Basal amoebic medium ("BR"). *E. coli* is grown for two days at 37°C in shallow layers of "R" medium in sealed flat bottles and then store at room temperature up to two months. pH should not exceed 7.3.
- 8) Horse serum. Heat inactivate at 56°C for 30 minutes each on successive days and store at 4°C.
- 9) Complete medium ("BRH"). Equal volume of horse serum and "BR" are mixed, incubated 24-48 hours at 37°C and stored at room temperature up to one month.

XIV. NNN Medium (Novy-MacNeal-Nicolle)

Bacto-agar	7. g
Sodium chloride	3. g
Distilled water	450. ml
Rabbit, defibrinated blood	150. ml

- 1) Add the Bacto-agar and sodium chloride to the distilled water in a flask and bring to the boil.
- 2) Autoclave at 12 lb. pressure for 15 minutes.
- 3) Cool the mixture to 52°C, add 150 ml rabbit blood and mix.
- 4) Pipette 5 ml amounts into sterile screw-capped test tubes and slant to produce a long slant. Label as "NNN Medium" and date.
- 5) Incubate tubes at 37°C for 24 hours to test for sterility. Antibiotics can be added here if necessary.

Preparation of Agar Plates

1. Remove the plates from the refrigerator and place in a 37°C incubator for 30 minutes.
2. Add 0.5 ml of Page's medium to a slant culture of *E. coli* or *E. aerogenes*. Gently scrape the surface of the slant with a sterile bacteriological loop (do not break the agar surface). Using a sterile Pasteur pipette, uniformly suspend the bacteria by gentle pipetting. Add two to three drops of this suspension to the middle of a

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warmed agar plate and spread the bacteria over the surface of the agar with a bacteriological loop.

XV. Page's Medium for Free-Living Amoebae

Sodium chloride (NaCl)	120. mg
Magnesium sulfate (MgSO ₄ . 7 H ₂ O)	4. mg
Calcium chloride (CaCl ₂ . 2 H ₂ O)	4. mg
Disodium hydrogen phosphate (Na ₂ HPO ₄)	142. mg
Potassium dihydrogen phosphate (KH ₂ PO ₄)	36. mg
De-ionized water	1000. ml

Label as "Page's Medium" and date.

This is dissolved and autoclaved at 15 lb./in.² for 15 minutes. The solution may be stored in the refrigerator for up to six months.

XVI. Non-Nutrient Agar Plates

Agar	1.5 g
Page's amoebae medium	100. ml

Dissolve agar in the saline with heat and sterilize by autoclaving at 15 lb./in.² for 15 minutes. Cool to 60°C and aseptically pour into plastic petri dishes: 20 ml for 100 x 15 mm dish, 5 ml for 16 x 15 mm dish. Label and date plates. Plates may be kept in canisters for about three months when stored at 4°C. The plates may be kept at 4°C for about three months.

XVII. Carbol Fuchsin Stain Solution

Basic fuchsin	21.0 g
Liquid phenol (85%)	85.2 ml
95% ethyl alcohol	150.0 ml
10% Tween 80	7.5 ml

Label as "Carbol Fuchsin Stain" and date.

Heat mixture in 45°C incubator for 24 hours to dissolve the crystals. Add distilled water to bring volume up to 1,800 ml.

XVIII. Malachite green

Malachite green	0.5 g
Distilled H ₂ O	100.0 ml

Label as "Malachite Green Stain" and date.

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XIX. Chromotrope stain

Chromotrope 2R	6.00 gm
Fast green	0.15 gm
Phosphotungstic acid	0.70 gm
Glacial acetic acid	3.00 ml

Label as “Chromotrope Stain” and date.

Mix ingredients and allow to stand for 30 minutes. Then add 100 ml distilled water.

XX. Acid alcohol for Chromotrope 2R Stain

90% ethyl alcohol or 95% methyl ethanol	995.5 ml
Glacial acetic acid	4.5 ml

Label as “Acid-Alcohol Soln.” and date.

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XXI. Proper Use of a Microscope

1. Place the microscope on a firm bench so that it does not vibrate. Do not place it in direct sunlight.
2. Switch on the source of light.
3. Place the specimen on the stage.
4. Select the objective to be used. The 10x objective can be used for adjusting the illumination.
5. Fully rack up condenser with top lens swung in.
6. Focus on specimen with objective. For this, rack the objective carefully downwards, while looking at it from the side, until the lens is near the specimen but not touching it. Then, while looking through the eyepiece, rack the objective slowly upwards until the image comes into view and is sharply focused.
7. Choose down lamp field stop in microscope base (Köhler illuminating base).
8. Focus the condenser by slightly lowering condenser until stop image is in optimum focus.
9. Use the two condenser centering screws to center field stop in field of view.
10. Open up field stop far enough to clear entire field of view. The higher objectives require the field stop to be opened more widely than 10x.
11. Adjust the aperture of the condenser iris to control image contrast. For this, remove eyepiece and look through the tube. Adjust the aperture of the iris so that it fills about three quarters of the lens with the light.
12. Adjust image brightness by means of filter or by varying lamp voltage.
13. After changing objectives, just adopt lamp field stop to size of visual field and condenser diaphragm to objective aperture.

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A. Calibration of the Microscope

1. Remove one eyepiece from the microscope and unscrew the top eye-lens. Place the micrometer disc on the diaphragm without the ocular so that the engraved side is underneath. Screw back the eye-lens and insert the ocular in the microscope. Be careful to keep both micrometer and lens clean.
2. Place the stage micrometer on the stage and focus on some portion of the scale.
3. Looking through the microscope, examine the ruling of the stage micrometer so that you can distinguish between the large (0.1 mm) and the small (0.01 mm) divisions.
4. Adjust the field so that the 0 line on the ocular micrometer is exactly superimposed upon the 0 line on the stage micrometer.
5. Without moving the stage micrometer, find another point at the extreme right where two other lines are exactly superimposed. This second set of superimposed lines should be as far as possible from the 0 line, but the distance will vary with the objective and microscope.
6. Knowing that each of the large divisions of the stage micrometer equals 0.1 mm, determine the total distance (in millimeters) between the two points of superimposition and the number of small ocular units necessary to cover the same distance.

For example: Suppose 27 ocular units (small) equal 2 stage units (large) or 0.2 mm.

7. Calculate the number of millimeters that is measured by one small ocular unit.

Example: 27 ocular units = 0.2 mm
 1 ocular unit = 0.2 mm/27 or 0.0074 mm

8. Since measurements of parasites are usually given in microns, the above determination must be converted to microns:

$$0.0074 \text{ mm} \times 1000 = 7.4 \text{ } \mu\text{m}$$

9. Record the calibrations with each of the objectives. With high and oil magnifications, the thinner ocular micrometer line must be centered on the broader stage micrometer line for more accurate measurement.

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- Keep the calibrations handy so that the size of any organism can be quickly determined by measuring it with the ocular micrometer and noting the particular lens combinations.

B. Use of Verniers and Scales

Most mechanical stages are fitted with two scales and verniers - one running north and south (Y-axis) and the other east and west (X-axis). [Note that these are NOT for measurements.] They are to be used as a position finding device.

Suppose that a particular organism in a slide is of such interest that you may want to re-examine it or show it to someone else at a later date. For locating and relocating the organism, the verniers and scales are very useful. The main scale has a series of lines at 1 mm intervals and a short vernier with 10 lines corresponding to 9 divisions of the main scale.

For finding the coordinates of an organism in a slide, proceed as follows:

- Make sure the slide is seated properly in the specimen holder on the microscope stage and no longer moves when the slide is gently tapped from the sides.
- If the slide was not seated properly, relocate the organism or structure.
- Determine the position of the 0 line of the vernier with respect to the main scale -- for example, the 0 line lies between 93 and 94 on the main scale (in other words, the value for the x-coordinate is more than 93, but less than 94). The first part of the x-coordinate therefore is 93. To determine the decimal figure for the x-coordinate, examine the vernier scale. The number of the vernier line which is in closest coincidence with one of the lines of the main scale (all other vernier lines will be more separated from the main scale lines) gives the decimal. For example, if the vernier line happens to be 7, then the location of the organism along the x-axis is 93.7.
- In the same way, determine the position along the Y-axis (north-south). For example: 12.3.
- The complete coordinates of that particular organism are therefore: 93.7/12.3.
- Record the orientation of the slide (label to the right or left).

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For relocating that same organism, the following steps apply.

1. The same microscope used originally must be used (since vernier scales differ between microscopes, even for the same model).
2. Orient the slide in the same way as it was when coordinates were originally determined (label to the right or left).
3. Seat the slide properly.
4. Seat both scales and verniers to the recorded figures (e.g. 93.7 on the X-axis; 12.4 on the Y-axis).
5. The organism should now be in the field. If not, check orientation of the slide, seating of the slide and proper setting of the recorded figures.

Since the recorded figures determined above are applicable and usable only to one particular microscope, **further information is required if the organism is to be relocated on another microscope** (e.g. if the slide is to be sent to a reference laboratory). For this, proceed as follows:

1. Seat the slide tightly in the specimen holder.
2. Record whether the label end of the slide is to the right or to the left.
3. Move the slide until the upper right hand corner of the cover glass (or another easily identifiable mark such as a chip, etc.) is in the exact center of the field. Determine the readings of verniers and scales, A_1/B_1 .
4. Move the slide until the lower left hand corner (or an easily identifiable mark) is in the exact center of the field. Again, determine the readings of scales and verniers, A_2/B_2 .

With this information (orientation of slide, readings of upper and lower corners and readings of the organism), the readings of scales and verniers of the organism can theoretically be recalculated for any other microscopes equipped with scales and verniers. Since "play" between the scales, slightly inaccurate readings, etc. may result in the recalculated readings being slightly "off", each submission should be accompanied by as complete a description as possible to ensure that the same organism is being examined.

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For calculating the scale and vernier readings applicable to another microscope, proceed as follows:

1. Orient the slide as usual.
2. Set the slide tightly.
3. Determine the readings for upper right hand corner or for the mark supplied with the slide; A_1/B_1 .
4. Determine the readings for the lower left hand corner, A_2/B_2 .
5. On graph paper, determine the "A values - microscope 1" along either the vertical or the horizontal axis, and the "A values - microscope 2" along the other axis. (Please note: the larger the scale on the graph paper, the more accurate determinations become.)
6. Then plot the values corresponding to A_1 , A_2 and $A_2 A_1$. (These are the readings of the corners.)
7. On the same or separate sheet of graph paper, plot the $B_1 B_2$ and $B_2 B_1$ values (i.e. the Y readings of the corners).
8. After drawing lines through the corresponding points thus located, the readings for any point applicable to the second microscope can be determined from these lines.

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XXII. Parasite Kits: Specimen Collection Instructions for Patients

1. The fluid in this container is a poison. Make sure the lid is on tightly and do not drink it.
2. Do not use laxatives, anti-diarrheal drugs, mineral oil, barium, bismuth or antibiotics within one week prior to collecting sample.
3. First pass urine into the toilet.
4. Pass the stool on a dry clean surface, such as a bed pan, plastic cup or onto saran wrap placed just under the toilet seat.
5. Place 1 ounce (walnut-sized) portion of stool into the container with the fluid fixative using one of the applicators provided.
6. Break up the stool into the fluid. MIX WELL.
7. Screw the cap on tightly. Make sure it does not leak.
8. Return the bottle to the zip lock bag.
9. Wash your hands immediately.
10. Label with your name, sex and the date. Return the specimen container with the complete information to the laboratory as soon as possible.
11. Store at room temperature. DO NOT FREEZE.

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XXIII. Isolate Codes

The following SOFT computer codes are to be used to correctly report a parasitic finding on the Isolate Screen in SOFT.

<i>Acanthamoeba</i> species	acaspp
Amoeba species	amospp
<i>Ancylostoma duodenale</i>	ancludo
<i>Ascaris lumbricoides</i>	asclum
<i>Babesia</i> species	babspp
<i>Blastocystis hominis</i>	blahom
<i>Brugia malayi</i>	brumal
<i>Chilomastix mesnili</i>	chimes
<i>Clonorchis sinensis</i>	closin
<i>Cryptosporidium parvum</i>	crypar
<i>Cryptosporidium</i> species	crysp
<i>Cyclospora cayetanensis</i>	cyccay
<i>Cyclospora</i> species	cydsp
<i>Dicrocoelium dendriticum</i>	dicden
<i>Dientamoeba fragilis</i>	diefra
<i>Diphyllobothrium</i> species	diphyo
<i>Diphyllobothrium latum</i>	diplat
<i>Dipylidium caninum</i>	dipcan
<i>Echinostoma</i> species	echisp
<i>Echinococcus</i> species	echsp
<i>Encephalitozoon</i> species	encapp
<i>Endolimax nana</i>	endnan
<i>Entamoeba</i> species	entasp
<i>Entamoeba coli</i>	entcol
<i>Enterocytozoon</i> species	entcyt
<i>Entamoeba hartmanni</i>	enthar
<i>Entamoeba histolytica</i>	enthis
<i>Entamoeba histolytica/dispar</i>	enthd
<i>Enteromonas hominis</i>	enthom
<i>Entamoeba polecki</i>	entpol
<i>Enterobius vermicularis</i>	entveo

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<i>Fasciolopsis buski</i>	fasbus
Fasciola hepatica	fashep
Fasciola hepatica/Fasciolopsis buski	fashbu
Flagellate species	flagsp
Fluke species	fluspp
<i>Giardia lamblia</i>	gialam
Heterophyes heterophyes	hethet
Hookworm species	hooksp
<i>Hymenolepis diminuta</i>	hyndim
<i>Hymenolepis nana</i>	hymnan
<i>Iodamoeba buetschlii</i>	iodbut
<i>Isospora belli</i>	isobel
Leishmania species	leispp
<i>Loa loa</i>	loaloe
<i>Mansonella ozzardi</i>	manozz
<i>Mansonella perstans</i>	manper
Mansonella streptocerca	manstr
Metagonimus yokogawai	metyok
Metorchis conjunctus	metcon
Microfilaria species	micspe
Microsporidium species	mirsp
<i>Necator americanus</i>	necame
Nematode species	nemspp
<i>Onchocerca volvulus</i>	oncvol
<i>Paragonimus westermani</i>	parwes
Parasite(s) seen – referred for definitive identification	parref

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<i>Plasmodium falciparum</i>	plafal
<i>Plasmodium malariae</i>	plamal
<i>Plasmodium ovale</i>	plaova
<i>Plasmodium species</i>	plaspp
<i>Plasmodium vivax</i>	plaviv
<i>Retortamonas intestinalis</i>	retoin
<i>Sarcocystis sui/hominis</i>	sarsh
<i>Schistosoma haematobium</i>	schhae
<i>Schistosoma japonicum</i>	schjap
<i>Schistosoma mansoni</i>	schman
Small flagellate	smafla
<i>Strongyloides stercoralis</i>	strstl
<i>Taenia saginata</i>	taesag
<i>Taenia solium</i>	taesol
<i>Taenia species</i>	taespo
<i>Toxoplasma gondii</i>	toxgon
Trematode species	trespp
<i>Trichomonas hominis</i>	trihom
<i>Trichinella spiralis</i>	trihot
<i>Trichostrongylus species</i>	trispp
<i>Trichuris trichiura</i>	tritro
<i>Trichomonas vaginalis</i>	trivag
<i>Trypanosoma cruzi</i>	trycru
<i>Trypanosoma gambiense or rhodesiense</i>	trygrh
<i>Trypanosoma species</i>	tryspp
Unidentified microfilaria species	unmisp
<i>Wuchereria bancrofti</i>	wucban