SECTION 9 Parasitology

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9.1  Introduction

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PART 1

Equipment

If necessary, maintenance procedures can be performed and documented more often than the minimal recommendations presented in this section.

1. MICROSCOPE

Good microscopes and light sources are mandatory for the examination of specimens for parasites. Identification of the majority of organisms depends on morphologic differences, most of which must be seen by using stereoscopic or regular microscopes. An explanation of how to calibrate the microscope can be found later in this section.

A. Stereoscopic

A stereoscopic microscope should be available for larger specimens (arthropods, tapeworm proglottids, various artifacts). The total magnification usually ranges from $\times 10$ to $\times 45$. Some microscopes have a zoom capacity from $\times 10$ to $\times 45$, and others have fixed objectives ($0.66 \times$, $1.3 \times$, $3 \times$) that can be used with $5 \times$ or $10 \times$ oculars. Use the light source either from under the specimen or directed onto the top of the specimen.

B. Regular

The light microscope should be equipped with the following parts.

1. Head

A monocular head can be used, but a binocular type is recommended to reduce fatigue during lengthy examinations. The binocular head should contain a diopter adjustment to compensate for variation in focus between the eyes.

2. Oculars

$10 \times$ are required; $5 \times$ can be helpful but are not essential.

3. Objectives

$10 \times$ (low power), $40 \times$ to $45 \times$ (high power), $97 \times$ to $100 \times$ (oil immersion).

4. Stage

A mechanical stage for X and Y movement is necessary. Graduated stages can be helpful and are recommended for recording the exact location of an organism in a permanently stained slide. This capability is essential for consultation and teaching responsibilities.
I. MICROSCOPE (continued)

5. Condenser
   a. A bright-field condenser equipped with an iris diaphragm is required. The N.A. of the condenser should be equal to or greater than the N.A. of the highest objective.
   b. An adjustable condenser is not required with the newer microscopes.

6. Filters
   Both clear blue glass and white ground-glass filters are recommended.

7. Light source
   a. The light source, along with an adjustable voltage regulator, should be contained in the microscope base. Align this light source as directed by the manufacturer.
   b. If the light source is external, the microscope must be equipped with an adjustable mirror and a condensing system containing an iris diaphragm that can be released or lowered.
   c. The light source should be a 75- to 100-W bulb.

C. Microscope maintenance
   1. Remove dust from all optical surfaces with a camel hair brush.
   2. Remove oil and finger marks on the lenses immediately with a clean soft cloth or several thicknesses of lens tissue. Single-thickness lens tissue may permit corrosive acids from the fingers to damage the lens. Do not use any type of tissue other than lens tissue; otherwise you may scratch the lens. Use very little pressure to clean the lenses in order not to remove the coatings on the external surfaces of the lenses.
   3. Use water-based cleaning fluid for normal cleaning. Use organic solvents sparingly and only if absolutely necessary to remove oil from the lens. Since microscope manufacturers do not agree on a common solvent, the manufacturer of each microscope should be consulted. One recommended solvent is 1,1,1-trichloroethane; it is a good solvent for immersion oil and mounting media, but it will not soften the lens sealers and cements. Do not use xylene, any alcohols, acetone, or any other ketone.
   4. Clean the lamp (cool, in off position) with lens tissue moistened in 70% isopropyl or ethyl alcohol (to remove oil transferred from the fingers to the lamp) after the lamp has been installed in the lamp holder.
   5. Clean the stage with a small amount of disinfectant (70% isopropyl or ethyl alcohol) when it becomes contaminated with fecal material.
   6. Cover the microscope when not in use. In extremely humid climates (a relative humidity of more than 50%), good ventilation is necessary to prevent fungal growth on the optical elements.
   7. Clean and lubricate the substage condenser slide as needed. Petroleum jelly or light grease can be used as a lubricant.
   8. Schedule a complete general cleaning and readjustment at least annually to be performed by a factory-trained and authorized individual. Record all data related to preventive maintenance and repair.

II. CENTRIFUGE

A. Requirements
   1. Either a table or floor model centrifuge, which should be able to accommodate 15-ml centrifuge tubes (for concentration procedures), is acceptable.
   2. Regardless of the model, a free-swinging or horizontal head type is recommended. With this type of centrifuge, the sediment will deposit evenly on the bottom of the tube. The flat upper surface of the sediment facilitates the removal of the supernatant fluid from a loosely packed pellet.

B. Maintenance of the centrifuge
   1. Check the carrier cups, trunnions, and rotor for corrosion and cracks before each run. If found to be defective, replace immediately.
II. CENTRIFUGE (continued)

2. Check for the presence of the proper cup cushions before each run.
3. Check the speed at all regularly used speeds at least quarterly by using a stroboscopic light to verify the accuracy of a built-in tachometer or speed settings. Record results.
4. Use 10% household bleach or phenolic solution to disinfect the centrifuge bowl, buckets, trunnions, and rotor following a breakage or spill and at least monthly. Following disinfection, rinse the parts in warm water, and give a final rinse in distilled water. It is imperative to thoroughly dry the parts with a clean absorbent towel to prevent corrosion.
5. Clean the inside of the cups in mild, warm soapy water, and scrub with a nylon brush. Use fine steel wool to remove stubborn deposits. The part should be rinsed in distilled water and thoroughly dried. This should be done as needed and at least quarterly.
6. Lubricate bearings (if not permanently lubricated) as specified by the manufacturer at least quarterly.
7. Check brushes, and replace if worn to 1/4 in. of the spring. Check semi-annually.
8. Check autotransformer brush, and replace if worn to 1/4 in. of the spring. Check semiannually.
9. Remove the head and dust cover, and add lubricant as specified by the manufacturer to fill the grease cup. Do this semiannually.
10. Replace grease in grease cup as specified by the manufacturer annually.
11. Record all data related to preventive maintenance and repair.

III. FUME HOOD

A. Requirements

Although a fume hood is not required, many laboratories prefer to keep their staining reagents and formalin in a fume hood. Even with the substitution of dehydrating reagents other than xylene, fume hoods may be preferred in order to eliminate odors. In keeping with good laboratory practice, the placement of reagents and equipment into a hood should not interfere with the proper operation of the hood.

B. Maintenance of the fume hood

1. Check the air velocity with the sash fully open and the cabinet empty with a thermoanemometer (minimum acceptable face velocity is 100 ft [ca. 30 m]/min) (3, 8) at least quarterly.
2. Perform a smoke containment test with the cabinet empty to verify proper directional face velocity. Check at least quarterly.
3. Lubricate the sash guides as needed to provide for ease of operation.
4. See Appendix 9.10.7–1 at the end of this section for formalin exposure guidelines.

IV. BIOLOGICAL SAFETY CABINET

A. Requirements

A biological safety cabinet is not required for processing routine specimens in a diagnostic parasitology laboratory. However, some laboratories use class I (open-face) or class II (laminar-flow) biological safety cabinets to process all unpreserved specimens.

B. Maintenance of the biological safety cabinet

1. Disinfect the work area after each use. Do not depend on UV irradiation to decontaminate the work surface. UV irradiation has very limited penetrating powers (21).
2. Clean UV lamps (in the off position) with 70% isopropyl or ethyl alcohol at least weekly.
3. Monitor UV output (UV intensity meter; any major laboratory supplier) quarterly to confirm that the effective radiation (excess of 40 μW/cm² at a wavelength of 253.7 nm) is present at the work surface (8). If not, replace the lamp.

4. Have class I biological safety cabinets certified after installation but before use, after they have been relocated or moved, and at least annually. The certification should include the following.
   a. Measurements of air velocity at the midpoint height approximately 1 in. behind the front opening. Make these measurements approximately every 6 in.
   b. The average face velocity should be at least 75 linear ft/min. Use a thermoanemometer with a sensitivity of ±2 linear ft/min (8).
   c. Perform a smoke containment test with the cabinet containing the routine work items, such as a Bunsen burner, test tube rack, bacteriological loop and holder, etc., to determine the proper directional velocity.
   d. Record the date of recertification and the name of the individual or company recertifying the cabinet.

5. Replace the filters as needed.

6. Have a class II biological safety cabinet certified to meet Standard 49 of the National Sanitation Foundation (Ann Arbor, Mich.) when it is installed (8).

7. Recertify the cabinet to meet Standard 49 when the cabinet is moved, after filters are replaced, after the exhaust motor is repaired or replaced, or after any gaskets are removed or replaced (8). Recertify at least annually, and record the date of service recertification along with the name of the individual or company performing the service.

8. Keep all airflow parts completely clear at all times.

V. REFRIGERATOR-FREEZER

A. Requirements

A general-purpose laboratory (non-explosion proof) or household-type refrigerator-freezer is adequate for use in the parasitology laboratory. The temperature should be approximately 5°C. Do not store solvents with flash points below refrigeration temperature even in modified (explosion-proof) refrigerators.

B. Maintenance of refrigerators-freezers

1. Monitor and record the temperature of the refrigerator on a daily basis. Place the thermometer into a liquid to permit stable temperature recording.
2. Monitor and record the temperature of the freezer on a daily basis. Place the thermometer into antifreeze (any brand with a freezing point below that of the freezer, such as ethylene glycol-water solutions, glycerol-water solutions, Prestone) to permit stable temperature recording.
3. Check the operation of the fan (if so equipped) inside the cabinet.
4. Monthly, check the door gasket for deterioration, cracks, and proper seal.
5. Semiannually, clean the condenser tubing and air grill with a vacuum cleaner.
6. Semiannually, check to ensure that the drain tubes are kept open.
7. Annually, wash the interior with a warm solution of baking soda and water (approximately 1 tablespoon/qt [ca. 13 to 14 g/0.946 liter]). Rinse with clean water, and dry.
8. Annually, decontaminate the interior as needed.
9. Annually, wash the door gasket and water collection tray with a mild soap and water. If the gasket accumulates a black mold, scrub with a 50% household bleach solution and a small brush. Rinse with clean water, and dry.
PART 2

Safety

The standard safety considerations of any clinical laboratory (3–7, 12, 14–18, 20, 21) should be practiced in a parasitology laboratory. Specific attention should be given to personal practices, specimen and reagent handling, and equipment (see section 15).

I. PERSONAL PRACTICES

Since many parasitic infections are acquired via the oral route, it is imperative that the parasitologist practice good personal hygiene. Hand washing with soap and water and adherence to laboratory technique standards are two of the most important ways to guard against infection in a clinical laboratory. Gloves, while not mandatory, are recommended, and standard precautions must be followed at all times.

II. IMMUNIZATIONS

In addition to the routine childhood immunizations against polio and rubella, an immunization against hepatitis B is recommended for those individuals processing fresh unpreserved fecal specimens. At present, there are no effective immunizations for parasitic diseases.

III. SPECIMENS

A. Collection

1. Collect fecal specimens and transport them to the laboratory in such a way that no one handling the container comes in direct contact with the specimen. Bag the test request slip separately from the specimen.
2. Usually, the specimen is placed in a waxed cardboard container with a tightly fitting lid.
3. If polyvinyl alcohol (PVA) fixative (containing mercuric chloride) is included in the collection kit, the following is strongly recommended.
   a. The word “Poison,” the skull-and-crossbones symbol, and the statement “Causes Severe Burns” must be included on the specimen container label.
   b. It is also recommended that the following first aid instructions be included on the collection kit insert:

      Contains mercuric chloride. Avoid contact with eyes or mucous membranes or prolonged contact with skin. Wash hands thoroughly after use. If swallowed, give milk or egg whites. Call physician immediately. For contact with eyes or skin, flush thoroughly with water. For eyes, get prompt medical attention. Keep out of reach of children.

4. Blood specimens should be collected in a manner consistent with standard precautions (18).

B. Handling

1. The unpreserved stool specimen should be considered potentially infectious; gloves are recommended. All of the following may be infective: protozoan cysts; Cryptosporidium oocysts; microsporidial spores; eggs of Taenia solium, Enterobius vermicularis, and Hymenolepis nana; and larvae of Strongyloides stercoralis.
2. The fresh specimen may also contain Salmonella spp., Shigella spp., or other bacterial pathogens, and bloody stools may pose a special hazard as potential carriers of hepatitis A and B viruses, human immunodeficiency virus, and enteric non-A, non-B viruses (18).
3. Because the unpreserved specimen may contain these infectious agents, the specimen must be collected, handled, and shipped to the laboratory so
III. SPECIMENS (continued)

the handlers are prevented from coming in direct contact with the specimen.

4. A 5% solution of formalin may permit Ascaris lumbricoides (6, 7, 11) eggs to become embryonated in stool specimens and thereby become infective. A 10% solution of formalin reduces this potential hazard. Personnel are urged to use standard precautions at all times.

C. Processing

1. In processing the unpreserved specimen, avoid direct contact between the specimen and the parasitologist and/or the equipment being used. Use of a biological safety cabinet is often recommended.
   a. For example, use a 3- by 2-in. microscope slide for preparing direct wet mounts.
   b. Sealing the stool preparation with clear nail polish or petroleum jelly will reduce the possibility of direct contact, although this approach is often not practical for routine clinical laboratories.
   c. Be careful when handling the preserved specimen. Maintain pressure on the stopper while shaking the specimen in the defatting step of the concentration procedure (use of ethyl acetate). Built-up pressure may cause an uncontrolled release of the contents.

D. Disposal

1. Unpreserved fecal and blood specimens can be safely discarded into a sanitary sewage system if local health codes permit (18).
2. Specimens preserved in formalin or PVA fixative may pose a unique disposal problem because of regulations regarding formalin and mercury-based compounds. PVA fixative contains Schaudinn’s fluid (mercuric chloride base) to which PVA plastic powder is added. Consult state public health officials for proper disposal guidelines.

IV. REAGENTS

Know what chemical hazards are in the workplace (2). One way to make the hazards known is to make available the MSDS on the chemical(s) in use. The availability and location of these sheets should be well known by all laboratory personnel. Usually they are kept filed in a notebook rather than being posted.

A. Use

There are certain basic considerations when chemicals are used in the laboratory.

1. Use the smallest container of flammable solvents (flash point below 37.8°C) compatible with the work.
2. Do not use flammable solvents near open flames or microincinerators.

B. Storage

1. Because the parasitology laboratory is usually one of many sections within the clinical laboratory, the storage of reagents must be considered in terms of the complete clinical laboratory.
2. Be sure that the quantities of flammable solvents stored comply with the Code of the National Fire Protection Association, Quincy, Mass., and the relevant regulations of the OSHA (1), and store solvents so that incompatible chemicals are segregated.
3. Do not store flammable solvents having a flash point below 4°C in a refrigerator.
   a. Evaporation can occur in these reagents even at this low temperature and cause dangerous levels of vapors to accumulate inside the refrigerator.
   b. Refrigeration temperatures also cause solvents to pick up atmospheric moisture faster than does room temperature, and refrigeration does not retard the formation of dangerous peroxides in ethers.
IV. REAGENTS (continued)

c. It is better to store these solvents in stoppered containers on well-ventilated open shelves away from open flames.
d. Some laboratories have elected to store these solvents in an explosion-proof fume hood.

4. As in the general laboratory, do an inventory of reagents at least annually, and discard all reagents that show signs of deterioration or that are beyond their expiration dates.

C. Disposal
Most water-soluble chemicals can safely be discarded in the sanitary sewer.
1. Neutralize acids and bases to within a pH range of 2 to 12.5, and then dilute them 1:20 with tap water prior to disposal (14).
2. Dilute small quantities of flammable solvents similarly prior to disposal in the sewer.
3. Since permissible substances and quantities will vary, contact state and local authorities for proper disposal guidelines.

D. Substitution of less hazardous reagents
The financial impact of various state and federal regulations governing the use and disposal of hazardous laboratory reagents serves as the impetus to search for less hazardous reagents. Some of the reagents for which there are substitutes are as follows.
1. Ethyl acetate (22) with a higher flash point (4.0\degree C) replaced ether (flash point of \(45\degree C\)) in the formalin-ether concentration technique. Hemo-De (Medical Industries, Los Angeles, Calif., or Fisher Scientific, Los Angeles, Calif.) can also replace ethyl acetate in the concentration procedure (19). This reagent has a flash point of 57.8\degree C and is generally regarded as safe by the Food and Drug Administration.
2. Xylene, as used in the trichrome or iron hematoxylin staining of PVA-fixed fecal smears, is a potential toxic and fire hazard. Again, Hemo-De has successfully replaced xylene in both the carbol-xylene and xylene steps of the trichrome procedure (6, 7). There are other substitutes available as well (Hemo-Sol; Fisher Scientific). Check with your local pathology departments or reagent suppliers for other alternatives.
3. Mercuric chloride, used in PVA fixative and Schaudinn’s fixative, presents both a toxic hazard and a disposal problem. Copper sulfate has been suggested as a substitute for mercuric chloride (6, 9); however, protozoan morphology will not be as clear and precise if this formula is used. Many single vial systems are now available; most fixatives use a zinc base. However, the total formulas for these stool preservatives are proprietary (6).
4. Before any of these substitute reagents are incorporated into the routine procedures, perform comparative studies.
5. Consult section 15 for further cautions.

V. EQUIPMENT

A. Microscope
1. If the stage of the microscope becomes contaminated with fecal material, wipe it clean with a disinfectant (70% isopropyl or ethyl alcohol).
2. Observe the general safety precautions governing the use of electrical equipment; i.e., do not use near water, check for bare wires, turn off power before servicing, etc.

B. Centrifuge
1. Use plastic tubes to reduce the hazards of breakage during centrifugation. If glass centrifuge tubes are used, inspect them, and use only those free of defects. To prevent aerosols, cap tubes prior to centrifugation.
2. Always balance the centrifuge prior to use.
3. Close the centrifuge lid when the centrifuge is in operation. Should noise or vibration develop, stop the centrifuge and check for symmetrical loading of the head.

C. Fume hood
1. If a fume hood is used to store flammable solvents such as ethyl acetate, ether, or xylene, keep the hood in operation to prevent the buildup of dangerous or toxic levels of these reagents. An adequate face velocity of 60 to 100 ft/min is recommended (3).
2. If the hood is used for other purposes, place reagents so that they do not interfere with the proper operation of the hood.

PART 3 Quality Control

I. INTERNAL QUALITY CONTROL

The internal QC component of a QC program generally consists of documenting the proper functioning of reagents and equipment at prescribed intervals and evaluating performance on split samples (day to day, batch to batch) and within-batch reproducibility studies (see section 14).

A. Check the permanent staining procedure with known positive control specimens when the stain is prepared (or a new lot number is purchased) and at least weekly. Record all QC results.
B. If positive specimens are not available, use smears of feces containing epithelial cells or pus cells (6, 10, 17).
C. Check stains for blood parasites for staining quality at the time of preparation, when put into use, and quarterly. If positive blood smears are not available, use a negative smear.
D. Introduce previously identified specimens as blind specimens to evaluate the overall performance of the parasitology laboratory. The use of these samples is of particular value in those laboratories that process few specimens and in those that receive a small number of positive specimens or lack a variety of specimens.

II. EXTERNAL QUALITY CONTROL PROFICIENCY TESTING

A. Every parasitology laboratory should subscribe to a proficiency testing program to provide an unbiased evaluation of its performance.
B. For tests for which there is no commercially available proficiency testing, an alternative performance or assessment system must be established to determine the reliability of testing.

III. REFERENCE MATERIALS

A. Reference materials should be available for comparison with unknown organisms, refresher training, and the training of additional personnel.
B. Ideal reference materials include formalin-preserved specimens of helminth eggs, larvae, and protozoan cysts; stained fecal smears of protozoan oocysts, spores, cysts, and trophozoites; and positive blood smears.
C. Color slides and atlases are recommended, although the level of microscopic focus cannot be changed.
D. Reference books and manuals from a number of publishers are available, and selected ones should be part of the parasitology library.

PART 4 Quality Assurance

I. QUALITY ASSURANCE

QA is the sum of all the activities necessary to produce consistently accurate test results. These include preanalytical, analytical, and postanalytical activities (see section 14).
I. QUALITY ASSURANCE

A. Preanalytical activities
These are all activities up to but not including the actual laboratory manipulation of the specimen that influence the quality of laboratory results.

1. Training of personnel
   a. The person advising the patient or medical staff must be adequately trained in all facets of specimen collection (preparation of the patient, specimen collection times, sample quality and volume, condition of specimen container, use of preservatives, and proper labeling).
   b. The person performing the parasitologic examination must be familiar with appropriate technical procedures to be used for each type of specimen and morphologic recognition and differentiation of parasites.

2. Preparation of the patient
   This involves the use of proper laxatives and/or enemas and the proper method for handling the patient prior to collecting a pinworm specimen, i.e., before bowel movement and bathing.

3. Specimen collection
   a. Consideration must be given to the sequence of collecting multiple specimens, i.e., the time interval between administering antibiotics, X-ray dyes (barium), or nonabsorbable antidiarrheal preparations and the collection of the specimen and the time between subsequent collections.
   b. Blood collections for blood-borne parasites should be optimized for the suspected parasites (16).

4. Specimen quality and volume (see individual protocols)
5. Specimen handling and labeling (see individual protocols)

B. Analytical activities
1. These include the technical or laboratory procedures necessary to produce accurate test results.
   a. A procedure manual should include a detailed description of the procedures necessary to process the specimens and to identify the various parasites and their diagnostic stages.
   b. Description of procedures must be part of a written procedure manual (13) that is reviewed and, when approved by the laboratory director, becomes the legal protocol for the laboratory by which each laboratorian is to be held responsible for performing tests exactly as described.
   c. The procedure manual should be reviewed at least annually and before procedural changes are adopted by the laboratory director.

2. Also included are all aspects of QC and proficiency testing, including a corrective action plan when the appropriate results are not obtained. Preventive maintenance and equipment calibration data are also required.

C. Postanalytical activities
1. These activities include information that is transferred verbally, in writing, or by electronic means from the laboratory to the clinician and that provides the clinician with the meaningful laboratory report necessary to optimally manage the patient. This report includes the extent of laboratory procedures performed (i.e., complete workup or limited to direct smear), presence of abnormalities seen in the specimen, excessive amounts of blood or yeast cells, etc., in addition to the identity of the parasite(s) found.

   2. Include qualifying statements regarding the quality of the specimen such as “inadequately preserved when received in the laboratory” or “contaminated with water or urine.”

II. QUALITY ASSURANCE DOCUMENTATION

All QA monitors must be properly documented. Use of the 10-step protocol developed by the JCAHO is recommended (5).
REFERENCES

9.2.1 Collection of Fresh Specimens

**PREANALYTICAL CONSIDERATIONS**

I. **PRINCIPLE**

One of the most important steps in the diagnosis of intestinal parasites is the proper collection of specimens (1–4). Improperly collected specimens can result in inaccurate results. Fresh specimens are mandatory for the recovery of motile trophozoites. However, unless strict collection and delivery times are adhered to, the specimen may have little value for diagnostic testing.

II. **SPECIMEN**

A. Collect all fecal specimens prior to the administration of antibiotics or antidiarrheal agents. Avoid the use of mineral oil, bismuth, and barium prior to fecal collection, since all of these substances may interfere with the detection or identification of intestinal parasites (1). The examination of purged specimens is less frequently performed and will not be discussed in this procedure. However, the same time limits for fixation and/or examination of diarrheic stools can be used for purged specimens submitted for examination.

B. Collect the fecal specimen in a clean, widemouthed container or on newspaper, and transfer it to a container with a tight-fitting lid.

C. Avoid contamination with urine or water from the toilet.

D. Transport the specimen to the laboratory as soon as possible, or keep it refrigerated until transport is possible. Obviously, dried specimens (diarrheic, semifomed, or formed) are not acceptable for fecal examination.

**Observe standard precautions.**

III. **MATERIALS**

A. **Reagents**

None

B. **Supplies**

1. Collection container(s)
2. Newspaper (optional)

C. **Equipment**

Standard

**ANALYTICAL CONSIDERATIONS**

IV. **QUALITY CONTROL**

A. There is no maximum limit on the amount of stool collected.

B. As a minimum amount, collect several grams (or teaspoon amounts). Smaller amounts can be examined, but the specimen is likely to dry out before examination (unacceptable for testing).

C. Reject any specimen that appears to be dry on the surface or edges.
V.  PROCEDURE

A. Wear gloves when performing this procedure.
B. Sample areas of the feces that appear bloody, purulent, or watery for examination as direct wet smears, fecal concentration, and permanent stained smears.
C. For adequate sampling of a formed fecal specimen, collect and examine material from the sides, ends, and middle by using fecal concentration procedures and permanent stained smears (4).

POSTANALYTICAL CONSIDERATIONS

VI.  PROCEDURE NOTES

A. To ensure the recovery of parasitic organisms that are passed intermittently and in fluctuating numbers, the examination of a minimum of three specimens collected over a 7- to 10-day period is recommended (1–4). Refer to Appendix 9.2.1–1 for other collection algorithms and options.
B. Infections with Entamoeba histolytica/E. dispar or Giardia lamblia may require the examination of up to six specimens before the organism is detected.
C. Liquid specimens should be received and examined or preserved by the laboratory within 30 min of passage, soft or semiformalized specimens within 1 h of passage, and formed specimens on the same day of passage.

VII.  LIMITATIONS OF THE PROCEDURE

A. Protozoan trophozoites will not survive if the stool specimen begins to dry out. Cysts will not form once the specimen has been passed.
B. Unless guidelines for delivery times are monitored and inappropriate specimens are replaced with appropriate ones, laboratory results may be incorrect. Include in the laboratory report a statement indicating that the results may be incorrect.

REFERENCES

### APPENDIX 9.2.1–1

**Fecal specimens for parasites: options for collection and processing**

<table>
<thead>
<tr>
<th>Option</th>
<th>Pros</th>
<th>Cons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rejection of stools from inpatients who have been in-house for &gt;3 days*</td>
<td>Patients may become symptomatic with diarrhea after they have been inpatients for a few days; symptoms are usually attributed not to parasitic infections but generally to other causes.</td>
<td>There is always the chance that the problem is related to a nosocomial parasitic infection (rare), but <em>Cryptosporidium</em> and microsporidia may be possible considerations.</td>
</tr>
<tr>
<td>Examination of a single stool (O&amp;P examination). Data suggest that 40–50% of organisms present will be found with only a single stool exam.</td>
<td>Some feel that most intestinal parasitic infections can be diagnosed from examination of a single stool. If the patient becomes asymptomatic after collection of the first stool, then subsequent specimens may not be necessary.</td>
<td>Diagnosis from a single stool examination depends on experience of the microscopist, proper collection, and the parasite load in the specimen. In a series of 3 stool specimens, frequently not all 3 specimens are positive and/or they may be positive for different organisms.</td>
</tr>
<tr>
<td>Two O&amp;P exams (concentration, permanent stained smear) are acceptable but are not always as good as three specimens (may be a relatively cost-effective approach); any patient remaining symptomatic would require additional testing.</td>
<td>With additional examinations, the yield of protozoa increases (<em>Entamoeba histolytica</em>, 22.7%; <em>Giardia lamblia</em>, 11.3%; <em>Dientamoeba fragilis</em>, 31.1%).</td>
<td>Assumes that the second (or third) stool is collected within the recommended 10-day time frame for a series of stools; protozoa are shed periodically. May be inconvenient for patient.</td>
</tr>
<tr>
<td>Examine a second stool only after the first is negative and the patient is still symptomatic.</td>
<td>If the examinations are negative and the patient’s symptoms subside, then probably no further testing is required.</td>
<td>Patients may exhibit symptoms (off and on), so it may be difficult to rule out parasitic infections with only a single stool and immunoassay. If the patient remains symptomatic, then even if the <em>Giardia</em> immunoassay is negative, other protozoa may be missed (<em>E. histolytica</em>/<em>E. dispar</em>, <em>D. fragilis</em>).</td>
</tr>
<tr>
<td>Examination of a single stool and an immunoassay (EIA, FA, cartridge) (<em>Giardia</em>). This approach is a mixture: one screen is acceptable; one O&amp;P exam is not the best approach (review last option below).</td>
<td>Three specimens are collected over 7–10 days and may save time and expense.</td>
<td>Organisms present in low numbers may be missed due to the dilution factor.</td>
</tr>
<tr>
<td>Pool three specimens for examination; perform one concentrate and one permanent stain.</td>
<td>Three specimens are collected over 7–10 days; would maximize recovery of protozoa in areas of the country where these organisms are most common.</td>
<td>Might miss light helminth infection (eggs, larvae) due to the pooling of the three specimens for the concentration; however, with a permanent stain performed on each of the three specimens, this approach would probably be the next best option in lieu of the standard approach (concentration and permanent stained smear performed on every stool).</td>
</tr>
<tr>
<td>Pool three specimens for examination; perform a single concentrate and three permanent stained smears.</td>
<td>Pooling of the specimens would require only a single vial.</td>
<td>This would complicate patient collection and very likely result in poorly preserved specimens, especially regarding the recommended ratio of stool to preservative and the lack of proper mixing of specimen and fixative.</td>
</tr>
<tr>
<td>Actually collect three stools, but put sample of stool from all three into a single vial (patient given a single vial only).</td>
<td></td>
<td>Laboratories rarely receive information that would allow them to place a patient in a particular risk group: children &lt;5 yrs old, children from day care centers (may or may not be symptomatic), patients with immunodeficiencies, and patients from outbreaks. Performance of immunoassay procedures on every stool is not cost-effective, and the positivity rate will be low unless an outbreak situation is involved.</td>
</tr>
<tr>
<td>Perform immunoassays on selected patients using methods for <em>G. lamblia</em>, <em>Cryptosporidium parvum</em> and/or the <em>E. histolytica/E. dispar</em> group, or <em>E. histolytica</em>.</td>
<td>Would be more cost-effective than performing immunoassay procedures on all specimens; however, information required to group patients is often not received with specimens.</td>
<td></td>
</tr>
</tbody>
</table>

(continued)
Appending 9.2.1–1 (continued)

Fecal specimens for parasites: options for collection and processing* (continued)

<table>
<thead>
<tr>
<th>Option</th>
<th>Pros</th>
<th>Cons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perform immunoassays and O&amp;P examinations on request for <em>G. lamblia</em>, <em>C. parvum</em> and/or <em>E. histolytica</em>/<em>E. dispar</em> group, or <em>E. histolytica</em></td>
<td>Will limit the number of stools on which immunoassay procedures are performed for parasites.</td>
<td>Will require education of the physician clients regarding appropriate times and patients for whom immunoassays should be ordered. Educational initiatives must also include information on the test report indicating the pathogenic parasites that will not be detected using these methods. It is critical to make sure clients know that if patients have become asymptomatic, further testing may not be required. However, if the patient remains symptomatic, then further testing (O&amp;P exams) is required. Remember that a single O&amp;P may not reveal all organisms present. Present plan to physicians for approval: immunoassays or O&amp;P examinations, procedure discussion, report formats, clinical relevance, limitations on each approach.</td>
</tr>
<tr>
<td>A number of variables will determine the approach to immunoassay testing and the O&amp;P examination (geography, organisms recovered, positivity rate, physician requests). Immunoassays and/or O&amp;P examinations should be separately ordered and billed.</td>
<td>Immunoassay results do not have to be confirmed by any other testing (such as O&amp;P examinations or modified acid-fast stains).</td>
<td></td>
</tr>
</tbody>
</table>

* O&P, ova and parasite; FA, fluorescent-antibody assay.

† Two key references addressed this issue and served as guidelines for microbiologists in reviewing clinically relevant recommendations for specimen submission:

‡ It is difficult to know when you may be in an early outbreak situation in which testing of all specimens for either *G. lamblia*, *C. parvum*, or both may be relevant. Extensive efforts are under way to encourage communication among laboratories, water companies, pharmacies, and public health officials regarding the identification of potential or actual outbreaks. If it appears that an outbreak is in the early stages, then performing the immunoassays on request can be changed to screening all stools.

Approaches to stool parasitology: test ordering

<table>
<thead>
<tr>
<th>Patient(s) and/or situation</th>
<th>Test ordered*</th>
<th>Follow-up test ordered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient with diarrhea and AIDS or other cause of immune deficiency; potential waterborne outbreak (municipal water supply)</td>
<td><em>Cryptosporidium</em> or <em>Giardia/Cryptosporidium</em> immunoassay</td>
<td>If immunoassays are negative and symptoms continue, special tests for microsporidia (modified trichrome stain) and other coccidia (modified acid-fast stain) and O&amp;P exam should be performed.</td>
</tr>
<tr>
<td>Patient with diarrhea (person associated with a nursery school or day care center or a camper or backpacker); patient with diarrhea and potential waterborne outbreak (resort setting)</td>
<td><em>Giardia</em> or <em>Giardia/Cryptosporidium</em> immunoassay</td>
<td>If immunoassays are negative and symptoms continue, special tests for microsporidia and other coccidia (see above) and O&amp;P exam should be performed.</td>
</tr>
<tr>
<td>Patient with diarrhea and relevant travel history; patient with diarrhea who is a past or present resident of a developing country</td>
<td>O&amp;P exam, <em>Entamoeba histolytica</em>/<em>E. dispar</em> immunoassay; immunoassay for confirmation of <em>E. histolytica</em>; various tests for <em>Strongyloides</em> may be relevant (eosinophilia)</td>
<td>If exams are negative and symptoms continue, special tests for coccidia and microsporidia should be performed.</td>
</tr>
<tr>
<td>Patient in an area of the United States where parasites other than <em>Giardia</em> are found</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient with unexplained eosinophilia</td>
<td>May want to consider agar plate culture for <em>Strongyloides stercoralis</em></td>
<td>If test is negative and symptoms continue, O&amp;P exam and special tests for microsporidia and other coccidia should be performed.</td>
</tr>
<tr>
<td>Patient with diarrhea (suspected food-borne outbreak)</td>
<td>Test for <em>Cyclospora cayetanensis</em> (modified acid-fast stains)</td>
<td>If test is negative and symptoms continue, special procedures for microsporidia and other coccidia and O&amp;P exam should be performed.</td>
</tr>
</tbody>
</table>

* Depending on the particular immunoassay kit used, various single or multiple organisms may be included. Selection of a particular kit depends on many variables: clinical relevance, cost, ease of performance, training, personnel availability, number of test orders, training of physician clients, sensitivity, specificity, equipment, time to result, etc. Very few laboratories will handle this type of testing exactly the same. Many options are clinically relevant and acceptable for good patient care. It is critical that the laboratory report indicate specifically which organisms could be identified using the kit; a negative report should list the organisms relevant to that particular kit.
9.2.2 Preservation of Specimens

**PREANALYTICAL CONSIDERATIONS**

**I. PRINCIPLE**
Fecal specimens that cannot be processed and examined in the recommended time should be placed in an appropriate preservative or combination of preservatives for examination later (1, 2, 8, 9). Preservatives will prevent the deterioration of any parasites that are present. A number of fixatives for preserving protozoa and helminths are available. Each preservative has specific limitations, and no single solution enables all techniques to be performed with optimal results. The choice of preservative should give the laboratory the capability to perform a concentration technique and prepare a permanent stained smear for every specimen submitted for fecal examination (1, 2, 8, 9).

**II. SPECIMEN**
The fecal specimen should be collected as described previously (see procedures 9.1 and 9.2.1). A portion of the specimen should be placed in the preservative immediately after passage. Excellent directions are available with stool collection vials and/or kits, and this approach eliminates many of the problems encountered with fresh stool collection. See Appendix 9.2.2–1 for a summary table on fecal preservatives and specimen collection.

**III. MATERIALS**
A. Ice cream sticks, applicator sticks, tongue depressors, or other appropriate implement for transferring and mixing the specimen
B. Reagents (see Appendix 9.2.2–1)
C. Equipment
   Standard

**ANALYTICAL CONSIDERATIONS**

**IV. QUALITY CONTROL**
A. Obtain a fresh, anticoagulated blood specimen, and prepare a buffy coat sample.
B. Mix approximately 2 g of soft, fresh fecal specimen (normal stool, containing no parasites) with several drops of the buffy coat cells.
C. Prepare several fecal smears, and fix immediately in Schaudinn’s fixative to be quality controlled.
D. Mix the remaining feces-buffy coat mixture in 10 ml of polyvinyl alcohol (PVA), modified PVA, or sodium acetate-acetic acid-formalin (SAF) to be quality controlled.
E. Allow 30 min for fixation, and then prepare several fecal smears. Allow to dry thoroughly (30 to 60 min) at room temperature.
F. Stain slides by using normal staining procedure.
G. After staining, if WBCs appear well fixed and display typical morphology, assume that any intestinal protozoa placed in the same lot number of preservative would also be well fixed, provided the fecal sample was fresh and fixed within the recommended time limits.
V. PROCEDURE

A. Wear gloves when performing this procedure.
B. Add a portion of fecal material to the preservative vial to give a 3:1 or 5:1 ratio of preservative to fecal material (a grape-sized formed specimen or about 5 ml of liquid specimen).
C. Mix well by stirring with an applicator stick or the “Spork” insert that is attached to the fixative vial lid to give a homogeneous solution.
D. Allow to stand for 30 min at room temperature to allow adequate fixation.
E. If using commercial collection systems, follow the manufacturer’s directions concerning shaking the vials, etc.

POSTANALYTICAL CONSIDERATIONS

VI. PROCEDURE NOTES

A. Most of the commercially available kits have a “fill to” line on the vial label to indicate how much fecal material to add to ensure adequate preservation of the fecal material.
B. Although the two-vial system (one vial of 10% buffered formalin [concentration] and one vial of PVA [permanent stained smear]) has always been the “gold standard,” laboratories are beginning to use other options. Changes in the selection of fixatives are based on the following.
   1. Problems with disposal of mercury-based fixatives
   2. Cost of a two-vial system compared with that of a single collection vial
   3. Selection of specific stains (trichrome, iron-hematoxylin) to use with specific fixatives
   4. Immunoassay procedures (EIA, fluorescent-antibody assay [FA], immunochromatographic cartridge) cannot be performed on specimens preserved in certain fixatives; check with the manufacturer.

VII. LIMITATIONS OF THE PROCEDURE

A. Adequate fixation still depends on the following.
   1. Meeting recommended time limits for lag time between passage of the specimen and fixation
   2. Use of the correct ratio of specimen to fixative
   3. Thorough mixing of the preservative and specimen
B. Unless the appropriate stain is used with each fixative, the final permanent stained smear may be difficult to examine (organisms hard to see and/or identify).

REFERENCES

APPENDIX 9.2.2–1

Include QC information on reagent container and in QC records.

Reagents

Indicate the expiration date on the label and in the work record or on the manufacturer’s label.

A. Schaudinn’s fixative

This preservative is used with fresh stool specimens or samples from the intestinal mucosal surface. Many laboratories that receive specimens from in-house patients (no problem with delivery times) may select this approach. Permanent stained smears are then prepared from fixed material.

1. Mercuric chloride, saturated aqueous solution

   mercuric chloride (HgCl₂) ................. 110 g
   distilled water .......................... 1,000 ml

   Dissolve the mercuric chloride in distilled water by heating (use a hood if available); allow the solution to cool until crystals form. Filter the solution into a glass-stoppered bottle and store until needed for stock solution preparation.

2. Schaudinn’s fixative (stock solution)

   mercuric chloride, saturated aqueous solution .................................. 600 ml
   ethyl alcohol, 95% ........................ 300 ml

   Immediately before use, add 5 ml of glacial acetic acid per 100 ml of stock solution.

3. Advantages
   a. Designed to be used for the fixation of slides prepared from fresh fecal specimens or samples from the intestinal mucosal surfaces
   b. Prepared slides can be stored in the fixative for up to a week without distortion of protozoan organisms.
   c. Easily prepared in the laboratory
   d. Available from a number of commercial suppliers

4. Disadvantages
   a. Not recommended for use in concentration techniques
   b. Has poor adhesive properties with liquid or mucoid specimens
   c. Contains mercury compounds (mercuric chloride), which may cause disposal problems. Additional information can be found in section 15.

B. PVA

PVA (1, 2, 8) is a plastic resin that is normally incorporated into Schaudinn’s fixative. The PVA powder serves as an adhesive for the stool material; i.e., when the stool-PVA mixture is spread onto the glass slide, it adheres because of the PVA component. Fixation is still accomplished by the Schaudinn’s fluid itself. Perhaps the greatest advantage in the use of PVA is the fact that a permanent stained smear can be prepared. PVA fixative solution is highly recommended as a means of preserving cysts and trophozoites for examination at a later time. The use of PVA also permits specimens to be shipped (by regular mail service) from any location in the world to a laboratory for subsequent examination. PVA is particularly useful for liquid specimens and should be used at a ratio of 3 parts PVA to 1 part fecal specimen.

1. PVA fixative

   PVA ........................................ 10.0 g
   ethyl alcohol, 95% ......................... 62.5 ml
   mercuric chloride, saturated aqueous solution .................................. 125.0 ml
   acetic acid, glacial ........................ 10.0 ml
   glycerin .................................... 3.0 ml

   a. Mix the liquid ingredients in a 500-ml beaker.
   b. Add the PVA powder (stirring is not recommended).
   c. Cover the beaker with a large petri dish, heavy waxed paper, or foil, and allow PVA to soak overnight.
   d. Heat the solution slowly to 75°C. When this temperature is reached, remove the beaker and swirl the mixture until a homogeneous, slightly milky solution is obtained (30 s).
APPENDIX 9.2.2–1 (continued)

2. Advantages
   a. Ability to prepare permanent stained smears and perform concentration techniques
   b. Good preservation of protozoan trophozoites and cyst stages
   c. Long shelf life (months to years) in tightly sealed containers at room temperature
   d. Commercially available from a number of sources
   e. Allows shipment of specimens

3. Disadvantages
   a. Some organisms (Trichuris trichiura eggs, Giardia lamblia cysts, Isospora belli oocysts) are not concentrated as well from PVA as from formalin-based fixatives, and morphology of some ova and larvae may be distorted.
   b. Contains mercury compounds (Schaudinn’s fixative), which may cause disposal problems
   c. May turn white and gelatinous when aliquotted into small amounts (begins to dehydrate) or if refrigerated
   d. Difficult to prepare in the laboratory

C. Modified PVA (copper base) (6)

Although there has been a great deal of interest in developing preservatives that do not use mercury compounds, substitute compounds may not provide good preservation of protozoan morphology on the permanent stained smear. Copper sulfate has been tried most frequently, but it does not provide results equal to those seen with mercuric chloride (4, 6).

1. Copper sulfate solution
   \[ \text{CuSO}_4 \cdot 5\text{H}_2\text{O} \] \[ \text{distilled water} \]
   \[ \text{20.0 g} \] \[ \text{1,000 ml} \]

   Add the \( \text{CuSO}_4 \cdot 5\text{H}_2\text{O} \) to 1,000 ml of distilled water heated to 100°C. Mix until dissolved.

2. Modified PVA fixative (stock solution)
   copper sulfate solution \[ \text{600 ml} \]
   ethyl alcohol, 95% \[ \text{300 ml} \]

   Immediately before use, add 5 ml of glacial acetic acid per 100 ml of stock solution.

3. Advantages
   a. Can be used for concentration techniques and stained smears
   b. Contains no mercury compounds (usually prepared with copper sulfate)
   c. Commercially available from a number of suppliers. These products (commercial formulas) apparently contain other “fixation agents” that may produce better overall fixation than the formula presented above.

4. Disadvantages
   a. Does not provide high quality of preservation of protozoan morphology on stained slides (4)
   b. Staining characteristics of protozoan organisms are variable; identification may be difficult, particularly when compared with staining characteristics seen with mercuric chloride-based fixatives.

D. Modified PVA (zinc base) (5, 7)

A number of these fixatives are now available commercially, although they do not provide the same overall quality of fixation seen with mercury-based fixatives and the specific formulas are proprietary. Morphologic differences based on organism fixation can be placed in perspective as follows:

Mercury-based fixatives: How beautiful is the organism?
Zinc-based fixatives: Can you identify the organism?
(In terms of clinical relevance, the last is the critical question.)

Although some of the organisms do not present with “textbook-quality” morphologic features, the majority of the time most organisms can be identified. In general, trophozoites tend to fix well, while cyst forms do not. The most difficult organisms to identify in zinc-preserved specimens are Endolimax nana cysts.
APPENDIX 9.2.2–1 (continued)

1. Advantages
   a. Can be used for concentration techniques and stained smears
   b. Contains no mercury compounds (usually prepared with zinc sulfate)
   c. Commercially available from a number of suppliers. These products (commercial formulas) contain other “fixation agents” that are proprietary.

2. Disadvantages
   a. Does not provide the same quality of preservation for good protozoan morphology on stained slides as seen in mercury-fixed solutions (5)
   b. Staining characteristics of protozoan organisms are variable; identification may be difficult, particularly when compared with staining characteristics seen from mercuric chloride-based fixatives.

E. SAF

SAF (10) lends itself to both the concentration technique and the permanent stained smear and has the advantage of not containing mercuric chloride, as is found in Schaudinn’s fluid and PVA. It is a liquid fixative much like 10% formalin. The sediment is used to prepare the permanent smear, and it is recommended that the stool material be placed on an albumin-coated slide to improve adherence to the glass. SAF is considered a “softer” fixative than mercuric chloride. The morphology of organisms will not be quite as sharp after staining as that of organisms originally fixed in solutions containing mercuric chloride. Staining SAF-fixed material with iron-hematoxylin appears to reveal organism morphology more clearly than staining SAF-fixed material with trichrome.

1. SAF fixative

   sodium acetate .................................1.5 g
   acetic acid, glacial .............................2.0 ml
   formaldehyde, 37 to 40% solution ..........4.0 ml
   distilled water ................................ 92.0 ml

2. Mayer’s albumin

   Equal parts egg white and glycerin. Place 1 drop on a microscope slide, and add 1 drop of SAF-preserved fecal sediment (from the concentration procedure). Allow the smear to dry at room temperature for 30 min prior to staining.

3. Advantages
   a. Can be used for concentration techniques and stained smears
   b. Contains no mercury compounds
   c. Long shelf life
   d. Easily prepared or commercially available from a number of suppliers

4. Disadvantages
   a. Has poor adhesive properties. Albumin-coated slides are recommended for stained smears.
   b. Protozoan morphology with trichrome stain not as clear as with PVA smears.
   c. More difficult for inexperienced workers to use

F. MIF

Merthiolate (thimerosal)-iodine-formalin (MIF) (1) is a good stain preservative for most kinds and stages of parasites found in feces and is useful for field surveys. It is used with all common types of stools and aspirates; protozoa, eggs, and larvae can be diagnosed without further staining in temporary wet mounts. Many laboratories using this fixative examine the material only as a wet preparation (direct smear and/or concentration sediment). MIF is prepared in two stock solutions that are stored separately and mixed immediately before use.

1. MIF fixative
   a. Solution I (stored in a brown bottle)

      distilled water .............................50 ml
      formaldehyde (USP) .....................5 ml
      thimerosal (tincture of merthiolate, 1:1,000) .............................40 ml
      glycerin .................................1 ml
b. Solution II (Lugol’s solution: good for several weeks in a tightly stoppered brown bottle)

- distilled water ........................................ 100 ml
- potassium iodide (KI) crystals ................. 10 g
- iodine crystals (add after KI dissolves) ...... 5 g

Combine 9.4 ml of solution I with 0.6 ml of solution II just before use.

2. Advantages
   a. Combination of preservative and stain (merthiolate), especially useful in field surveys
   b. Protozoan cysts and helminth eggs and larvae can be diagnosed from temporary wet-mount preparations.

3. Disadvantages
   a. Difficult to prepare permanent stained smears
   b. Iodine component unstable; needs to be added immediately prior to use
   c. Concentration techniques may give unsatisfactory results.
   d. Morphology of organisms becomes distorted after prolonged storage.

G. 5 or 10% formalin (1, 2, 8, 9)

Formalin (1) is an all-purpose fixative that is appropriate for helminth eggs and larvae and protozoan cysts. Two concentrations are commonly used: 5% which is recommended for preservation of protozoan cysts, and 10%, which is recommended for helminth eggs and larvae. Most commercial manufacturers provide 10%, which is most likely to kill all helminth eggs. To help maintain organism morphology, formalin can be buffered with sodium phosphate buffers, i.e., neutral formalin.

1. 10% Formalin

   formaldehyde (USP) ........ 100 or 50 ml (for 5%)

   Formaldehyde is normally purchased as a 37 to 40% HCHO solution; however, for dilution, it should be considered to be 100%.

   0.85% NaCl .................. 900 or 950 ml (for 5%)

   Dilute 100 ml of formaldehyde with 900 ml of 0.85% NaCl solution. (Distilled water may be used instead of NaCl solution.)

   If you want to use buffered formalin, the following approach is recommended.

   Na₂HPO₄  ........................................ 6.10 g
   NaH₂PO₄ .................................... 0.15 g

   Mix the two thoroughly, and store the dry mixture in a tightly closed bottle. Prepare 1 liter of 10 or 5% formalin, and add 0.8 g of the buffer salt mixture.

2. Advantages
   a. Good routine preservative for protozoan cysts and helminth eggs and larvae. Materials can be preserved for several years.
   b. Can be used for concentration techniques (sedimentation techniques)
   c. Long shelf life and commercially available
   d. Neutral formalin (buffered with sodium phosphate) helps maintain organism morphology with prolonged storage.

3. Disadvantage
   a. Permanent stained smears cannot be prepared from formalin-preserved fecal specimens.
Preservation of Specimens

APPENDIX 9.2.2–1 (continued)

Preservatives used in diagnostic parasitology (stool specimens) (1, 2, 3, 9)

<table>
<thead>
<tr>
<th>Preservative</th>
<th>Concn</th>
<th>Permanent stained smear</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 or 10% formalin(^a)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>5 or 10% buffered formalin</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>MIF</td>
<td>X</td>
<td>X polychrome IV stain</td>
</tr>
<tr>
<td>SAF</td>
<td>X</td>
<td>X iron hematoxylin</td>
</tr>
<tr>
<td>PVA(^a)</td>
<td>X</td>
<td>X trichrome or iron hematoxylin</td>
</tr>
<tr>
<td>PVA, modified(^b)</td>
<td></td>
<td>X trichrome or iron hematoxylin</td>
</tr>
<tr>
<td>PVA, modified(^c)</td>
<td></td>
<td>X trichrome or iron hematoxylin</td>
</tr>
<tr>
<td>Single-vial systems(^d)</td>
<td></td>
<td>X trichrome or iron hematoxylin</td>
</tr>
<tr>
<td>Schaudinn’s (without PVA)(^e)</td>
<td></td>
<td>X trichrome or iron hematoxylin</td>
</tr>
</tbody>
</table>

\(^a\) Formaldehyde is normally purchased at a 37 to 40% HCHO solution; however, for dilution, it should be considered to be 100%. Example: 10% formalin = 10 ml of formaldehyde plus 90 ml of distilled water or buffered water.

\(^b\) This fixative uses the mercuric chloride base in the Schaudinn’s fluid; this formulation is still considered to be the “gold standard,” against which all other fixatives are evaluated (organism morphology after permanent staining). Additional fixatives prepared with non-mercuric chloride-based compounds are continuing to be developed and tested.

\(^c\) This modification uses a copper sulfate base rather than mercuric chloride.

\(^d\) This modification uses a zinc base rather than mercuric chloride and apparently works well with trichrome stain; trials using iron-hematoxylin are currently under way.

\(^e\) These modifications use a combination of ingredients (including zinc), but are prepared from proprietary formulas.

Considerations when selecting a stool fixative

A. Overall fixation efficacy for trophozoites, cysts, oocysts, microsporidial spores, eggs, and larvae
B. Ability to perform both the concentration and permanent stained smear from the preserved specimen
C. Selection of a one- or two-vial collection system
D. Preparation of reagents in-house or commercial purchase
E. Decision to perform immunoassay procedures (EIA, fluorescent-antibody assay, cartridge formats) from preserved specimen
F. Need to perform special stains from the preserved specimen (modified acid-fast stains for Cryptosporidium, Cyclospora, and Isospora and modified trichrome stains for microsporidia)
9.2.3 Shipment of Specimens

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE
In outpatient situations, it may be necessary for a specimen to be shipped to the laboratory for examination. Only preserved fecal specimens should be shipped, as any delays in examination may result in deterioration of parasitic organisms (1–3). Prior fixation also reduces the risk of infection from any etiologic agents present in the specimen. The U.S. Postal Service regulates the shipment of clinical specimens through the mail. It is the responsibility of the sender to conform to these regulations (1–3).

II. SPECIMENS
A. Preserved fecal specimens in collection vials
B. Fecal smears for staining and examination for parasitic organisms
C. Blood smears for staining and examination for blood parasites (thin blood films should be fixed in methyl alcohol prior to shipment)

III. MATERIALS
A. Reagents
   None
B. Supplies
   1. Primary container (vial, test tube, or bottle) containing the clinical specimen (feces or blood)
   2. Secondary container in which the primary container will be placed (sealed and water tight, durable) with absorbent material to contain leakage
   3. Shock-absorbent material for protecting the contents (when shipping fecal or blood smears or tubes of blood)
   4. Outer shipping container of fiberboard or other sturdy material in which the secondary container will be placed
   5. Instructions for patient specimen collection and handling
   6. Appropriate mailing labels
C. Equipment
   None

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL
A. Familiarity with U.S. postal regulations is mandatory. Post up-to-date regulations.
B. Review relevant postal regulations at routine safety meetings for the staff, and document the review.
V. PROCEDURE

A. Place the primary container of preserved fecal material into the secondary container (metal sleeve or a sealable bag), and seal.
B. Place into the mailing container, and seal.
C. Label appropriately for shipment.
D. Wrap glass slides in shock-absorbent material to protect from breakage, or place them in a sturdy slide container.
E. Slides need not be placed in double containers for shipping.
F. Place padded slides in shipping container and label appropriately.

VI. PROCEDURE NOTES

A. Some commercially available mailing containers consist of a screw-cap inner metal sleeve that will hold a preservative vial that is placed inside a screw-cap cardboard mailing sleeve. Absorbent material can be placed between the two containers to absorb any leakage. This container can be used to mail a single specimen.
B. Padded envelopes for mailing sets of preservative vials are available in several sizes. The absorbent material is shredded newspaper inside the envelope layers.
C. Clinical specimens exceeding 50 ml per parcel must be packaged in a fiberboard box or shipping container of equivalent strength.

VII. LIMITATIONS OF THE PROCEDURE

A. The same limitations apply to all fixatives, regardless of whether they are delivered by mail.
B. Each vial or tube containing liquid should be as full as possible. If it is only partly full, some of the liquid may get splashed onto the walls of the container and dry out during transit. Also, the larger the air space, the more shaking will occur, and this may be detrimental to any delicate organisms in the specimen (even if they are preserved).

REFERENCES

9.3.1 Macroscopic Examination of Fecal Specimens: Age and Physical Description

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE
The age of fresh fecal specimens is an important factor in the diagnosis of parasitic infections (1–4). The date and time of passage must be provided for each specimen submitted to the laboratory. The physical characteristics of a fresh fecal specimen may aid in determining what types of organisms may be present (1–4). Fecal specimens are described as formed, semiformed, soft, loose, or watery. Loose or watery specimens may contain trophozoites, whereas formed or semifomed specimens are more likely to contain cyst stages. Helminth eggs or larvae may be found in any type of specimen but are more difficult to find in liquid specimens because of the dilution factor. One can also see if blood and/or mucus is present, although if present, neither one necessarily indicates a parasitic infection. When the fresh specimen is examined visually in the collection container, adult pinworms (Enterobius vermicularis) and tapeworm proglottids may also be seen.

II. SPECIMENS
(See procedure 9.2.1.)
A. Fresh fecal specimen in waterproof container
B. Preserve or examine liquid or soft stools containing blood or mucus within 30 min of passage.
C. Preserve or examine semifomed specimens within 1 h of passage.
D. Preserve or examine formed specimens within the same day.
E. Fecal specimens that cannot be processed and examined in the recommended time period should be placed in an appropriate preservative or combination of preservatives for examination at a later time. Preservatives will prevent the deterioration of any parasites that are present. A number of fixatives are available (see Appendix 9.2.2–1) for preserving protozoan and helminth organisms. The choice of preservative should give the laboratory the capability to perform a concentration technique and prepare a permanent stained smear on every specimen submitted for fecal examination (2, 4).

ANALYTICAL CONSIDERATIONS

III. PROCEDURE
A. Wear gloves when performing this procedure.
B. Examine the specimen macroscopically to determine consistency.
C. Examine the surface of the fecal specimen for the presence of blood or mucus.
D. Sample areas of blood or mucus for examination for trophic amebae.
E. Examine the surface of the specimen and the area underneath the specimen for possible organisms (adult pinworms or tapeworm proglottids).
F. Check information for date and time of passage.
IV. RESULTS

A. Adult helminths or portions of helminths may be recovered and seen with the naked eye. Examples include *E. vermicularis* adult worms, *Ascaris lumbricoides* adult worms, and tapeworm proglottids.

B. Occasionally, other helminths may be recovered (hookworm, *Strongyloides stercoralis*), but identification requires the use of the microscope.

**POSTANALYTICAL CONSIDERATIONS**

V. REPORTING RESULTS

A. Report the presence of adult helminths or portions of helminths. Morphology and size are usually adequate for identification of pinworm and *Ascaris* adults and tapeworm proglottids. (Identification to the species level will require India ink injection [see procedure 9.5.6].)

**Examples:**
- *Ascaris lumbricoides* adult worm identified.
- *Enterobius vermicularis* adult worm identified.
- *Taenia saginata* gravid proglottid identified.

B. Report the presence of blood on or in the fecal specimen.

**Example:** Fresh blood seen on stool specimen.

VI. PROCEDURE NOTES

A. Trophic amebae or flagellates are found most frequently in liquid or soft specimens and tend to disintegrate rapidly at room temperature.

B. Trophozoites and cyst stages may be found in semiformal specimens.

C. Cyst stages are found most frequently in formed specimens and will not lose characteristic morphology at room temperature for approximately 1 day.

D. Eggs and larvae in fresh fecal specimens do not lose characteristic morphology at room temperature as rapidly as trophozoites or cysts. Some eggs (hookworm) may hatch if the specimen is kept unpreserved at room temperature for more than a day.

E. Refrigeration of the fresh fecal specimen will delay deterioration of the parasitic organisms.

F. Freezing of the fecal specimen is not recommended, as characteristic morphology of the parasitic organisms may be altered.

G. Never incubate fecal specimens.

H. Laboratories that receive the fecal specimen in preservative vials must rely on information that is submitted with the specimen as to the consistency of the specimen.

I. Many commercially available kits contain vials with labels which allow the patient to indicate the original consistency of the specimen.

J. A clean vial (containing no preservative) can be provided to patients for submitting a portion of the fresh specimen for determining consistency.

K. Dark or tarry fecal specimens usually indicate bleeding in the upper gastrointestinal tract.

L. Bright red blood indicates bleeding at a lower level or around the rectum.

M. Barium causes feces to be light tan to white. These specimens should be rejected.

N. Ingested iron and some antidiarrheal compounds may cause the specimen to be dark to black.

O. Yellowish specimens may be noted in cases of fat malabsorption, which is seen commonly in infection with *Giardia lamblia*.

P. Vegetable material is frequently seen in fecal specimens and must be differentiated from helminths. Size and gross morphology are used to differentiate vegetable material from helminth parasites *(see Appendixes 9.10.1–1 and 9.10.3–1 at the end of this section).*
Although there are some benefits (organism motility) associated with the examination of fresh fecal specimens, many laboratories have switched to stool preservative collection kits. Many intestinal parasites tend to disintegrate soon after collection, particularly if there is a time lag between specimen collection and preservation. In order to eliminate time lag problems and ensure adequate organism morphology, stool collection kits are recommended. Also, remember that organisms other than parasites can cause diarrhea; you might want to check whether bacterial cultures have been ordered.

REFERENCES

9.3.2 Calibration of Microscope with an Ocular Micrometer

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE
The identification of protozoa and other parasites depends on several factors, one of which is size. Any laboratory doing diagnostic work in parasitology should have a calibrated microscope available for precise measurements. Measurements are made with a micrometer disk that is placed in the ocular of the microscope; the disk is usually calibrated as a line divided into 50 units (U). Depending on the objective magnification used, the divisions in the disk represent different measurements. The ocular disk division must be compared with a known calibrated scale, usually a stage micrometer with a scale of 0.1- and 0.01-mm divisions (1) (Fig. 9.3.2–1).

II. MATERIALS

A. Supplies
1. Ocular micrometer disk (line divided into 50 U) (any laboratory supply distributor: Fisher, Baxter, Scientific Products, VWR, etc.)
2. Stage micrometer with a scale of 0.1- and 0.01-mm divisions (Fisher, Baxter, Scientific Products, VWR, etc.)
3. Immersion oil
4. Lens paper

B. Equipment
1. Binocular microscope with 10×, 40×, and 100× objectives. Other objective magnifications (50× oil or 60× oil immersion lenses) may also be used.
2. Oculars should be 10×. Some may prefer 5×; however, smaller magnification may make final identifications more difficult.
3. Single 10× ocular to be used to calibrate all laboratory microscopes (to be used when any organism is being measured)

III. QUALITY CONTROL

A. Recalibrate the microscope periodically. If the scope receives heavy use, once a year is recommended, or when any parts are replaced or changed on the microscope.
B. Often the measurement of RBCs (approximately 7.5 μm) is used to check the calibrations of the three magnifications (×100, ×400, ×1,000).
C. Latex or polystyrene beads of a standardized diameter can be used to check the calculations and measurements (Sigma, J. T. Baker, etc.). Beads of 10 and 90 μm are recommended.
D. Record all measurements in QC records.

IV. PROCEDURE

A. Unscrew the eye lens of a 10× ocular, and place the micrometer disk (engraved side down) within the ocular. Use lens paper to handle the disk; keep all surfaces free of dust or lint.
Figure 9.3.2–1 Ocular micrometer, top scale; stage micrometer, bottom scale (from L. S. Garcia, Diagnostic Medical Parasitology, 4th ed., 2001, ASM Press, Washington, D.C.).

IV. PROCEDURE (continued)

B. Place the calibrated micrometer on the stage, and focus on the scale. You should be able to distinguish the difference between the 0.1- and 0.01-mm divisions. Make sure you understand the divisions on the scale before proceeding.

C. Adjust the stage micrometer so that the “0” line on the ocular micrometer is exactly lined up on top of the 0 line on the stage micrometer.

D. When these two 0 lines are lined up, do not move the stage micrometer any farther. Look to the right of the 0 lines for another set of lines superimposed on each other. The second set of lines should be as far to the right of the 0 lines as possible; however, the distance varies with the objectives being used (Fig. 9.3.2–1).

E. Count the number of ocular divisions between the 0 lines and the point where the second set of lines is superimposed. Then, on the stage micrometer, count the number of 0.1-mm divisions between the 0 lines and the second set of superimposed lines.

F. Calculate the portion of a millimeter that is measured by a single small ocular unit.

G. When the high dry and oil immersion objectives are used, the 0 line of the stage micrometer will increase in size, whereas the ocular 0 line will remain the same size. The thin ocular 0 line should be lined up in the center or at one edge of the broad stage micrometer 0 line. Thus, when the second set of superimposed lines is found, the thin ocular line should be lined up in the center or at the corresponding edge of the broad stage micrometer line.

H. Calculate the factors as follows.

**Examples:**

\[
\frac{\text{stage reading (mm)}}{\text{ocular reading}} \times \frac{1,000 \, \mu\text{m}}{1 \, \text{mm}} = \text{ocular units (\mu m)}
\]

- **Low power (10×):**
  \[
  \frac{0.8 \, \text{mm}}{100 \, \text{U}} \times \frac{1,000 \, \mu\text{m}}{1 \, \text{mm}} = 8.0 \, \mu\text{m} \text{ (factor)}
  \]

- **High dry power (40×):**
  \[
  \frac{0.1 \, \text{mm}}{50 \, \text{U}} \times \frac{1,000 \, \mu\text{m}}{1 \, \text{mm}} = 2.0 \, \mu\text{m} \text{ (factor)}
  \]

- **Oil immersion (100×):**
  \[
  \frac{0.05 \, \text{mm}}{62 \, \text{U}} \times \frac{1,000 \, \mu\text{m}}{1 \, \text{mm}} = 0.8 \, \mu\text{m} \text{ (factor)}
  \]
IV. PROCEDURE (continued)  

**Examples:** If a helminth egg measures 15 ocular units by 7 ocular units with the high dry objective, then multiply the measurements by the factor 2.0 μm (for that objective). The egg then measures 30 by 14 μm and is probably *Clonorchis sinensis.*

If a protozoan cyst measures 27 ocular units with the oil immersion objective, then multiply the measurement by the factor 0.8 μm (for that objective). The cyst then measures 21.6 μm.

V. RESULTS  

A. For each objective magnification, a factor will be generated (1 ocular unit = certain number of micrometers).

B. If standardized latex or polystyrene beads or an RBC is measured with various objectives, the size for the object measured should be the same (or very close), regardless of the objective magnification.

VI. REPORTING RESULTS  

A. Post the multiplication factor for each objective either on the base of the microscope or on a nearby wall or bulletin board for easy reference.

B. Once the number of ocular lines per width and length of the organism is measured, then, depending on the objective magnification, the factor (1 ocular unit = certain number of micrometers) can be applied to the number of lines to obtain the width and length of the organism.

C. Comparison of these measurements with reference measurements in various books and manuals should confirm the organism identification.

VII. PROCEDURE NOTES  

A. The final multiplication factors will be only as good as your visual comparison of the ocular 0 and stage micrometer 0 lines.

B. As a rule of thumb, the high dry objective (40×) factor should be approximately 2.5 times more than the factor obtained from the oil immersion objective (100×). The low-power objective (10×) factor should be approximately 10 times that of the oil immersion objective (100×).

VIII. LIMITATIONS OF THE PROCEDURE  

A. After each objective has been calibrated, the oculars containing the disk and/or these objectives cannot be interchanged with corresponding objectives or oculars on another microscope.

B. Each microscope used to measure organisms must be calibrated as a unit. The original oculars and objectives that were used to calibrate the microscope must also be used when an organism is measured.

C. The objective containing the ocular micrometer can be stored until needed. This single ocular can be inserted when measurements are taken. However, this particular ocular containing the ocular micrometer disk must also have been used as the ocular during microscope calibration.

REFERENCE  

Microscopic Examination of Fecal Specimens: Direct Smears

**PREANALYTICAL CONSIDERATIONS**

**I. PRINCIPLE**

The microscopic examination of a direct smear has several purposes: to assess the worm burden of a patient, to provide a quick diagnosis of a heavily infected specimen, to check organism motility, and to diagnose parasites that may be lost in concentration techniques (1–4).

**II. SPECIMEN**

Any fresh stool specimen that has not been refrigerated is acceptable. Since trophozoites within preserved specimens would exhibit no motility on a direct smear, it is not necessary to perform this procedure on specimens submitted in preservatives (10% formalin, sodium acetate-acetic acid-formalin, and various types of polyvinyl alcohol, single-vial preservative collection systems).

*Observe standard precautions.*

**III. MATERIALS**

**A. Reagents** *(see Appendix 9.3.3–1)*

The reagents indicated below are available commercially.

1. 0.85% NaCl
2. D’Antoni’s iodine or Lugol’s iodine
   Aliquot some of the iodine into a brown dropper bottle. The working solution should resemble a strong tea color and should be discarded when it lightens in color (usually within 10 to 14 days).

**B. Supplies**

1. Microscope slides (1 by 3 in. or larger)
2. Coverslips (no. 1, 22 by 22 mm or larger)
3. Pasteur pipettes and bulbs
4. Screw-cap test tubes (16 by 125 mm)
5. Beaker (500 ml)
6. Brown glass-stoppered bottle (250 ml)
7. Two brown glass dropper bottles

**C. Equipment**

1. Binocular microscope with 10×, 40×, and 100× objectives; phase-contrast and/or differential interference contrast optics preferred
2. Oculars should be 10×. Some may prefer 5×; however, smaller magnification may make final identifications more difficult.
3. Magnetic stirrer and stir bar

**ANALYTICAL CONSIDERATIONS**

**IV. QUALITY CONTROL**

A. Check the working iodine solution each time it is used or periodically (once a week).
   1. The iodine should be free of any signs of bacterial and/or fungal contamination.
IV. QUALITY CONTROL
(continued)

2. The color should be that of strong tea (discard if too light).
3. Protozoan cysts should contain yellow-gold cytoplasm, brown glycogen material, and paler refractile nuclei. Human WBCs mixed with negative stool can be used as a QC specimen. The human cells will stain with the same color as that seen in the protozoa.

B. The microscope should be calibrated, and the original optics used for the calibration should be in place on the microscope. Post the calibration factors for all objectives on the microscope for easy access (see procedure 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

C. Record all QC results.

V. PROCEDURE

A. Wear gloves when performing this procedure.
B. Place one drop of 0.85% NaCl on the left side of the slide and 1 drop of iodine (working solution) on the right side of the slide.
C. Take a very small amount of fecal specimen (about the amount picked up on the end of an applicator stick when introduced into the specimen), and thoroughly emulsify the stool in the saline and iodine preparations (use separate sticks for each).
D. Place a coverslip (22 by 22 mm) on each suspension.
E. Systematically scan both suspensions with the 10× objective. The entire coverslip area should be examined.
F. If you see something suspicious, use the 40× objective for more-detailed study. At least one-third of the coverslip should be examined with the 40× objective, even if nothing suspicious has been seen.

VI. RESULTS

Protozoan trophozoites and/or cysts and helminth eggs and larvae may be seen and identified.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. You may or may not be able to identify motile trophozoites to the species level (depending on the clarity of the morphology).
Example (positive report): *Giardia lamblia* trophozoites present.
B. You may or may not be able to identify protozoan cysts to the species level (depending on the clarity of the morphology).
Example (positive report): *Entamoeba coli* cysts present.
C. You may identify helminth eggs and/or larvae.
Examples: *Ascaris lumbricoides* eggs present.
*Strongyloides stercolaris* larvae present.
D. You may see artifacts and/or other structures. Report them.
Examples: Moderate Charcot-Leyden crystals present.
Few RBCs present.

VIII. PROCEDURE NOTES

A. In preserved specimens, formalin replaces the saline and can be used directly; however, you will not be able to see any organism motility (organisms are killed by 10% formalin).
VIII. PROCEDURE NOTES (continued)

B. Some workers prefer to make the saline and iodine mounts on separate slides. There is less chance of getting fluids on the microscope stage if separate slides are used (less total fluid on the slide and under the coverslip).

C. The microscope light should be reduced for low-power observations, since most organisms will be overlooked with bright light. Illumination should be regulated so that some of the cellular elements in the feces show refraction. Most protozoan cysts will refract under these light conditions.

IX. LIMITATIONS OF THE PROCEDURE

A. Results obtained with wet smears should usually be confirmed by permanent stained smears. Some protozoa are very small and difficult to identify to the species level by direct wet smears alone.

B. Confirmation is particularly important in the case of Entamoeba histolytica/E. dispar versus Entamoeba coli.

REFERENCES


APPENDIX 9.3.3–1

Reagents

Include QC information on reagent container and in QC records.

A. 0.85% NaCl

<table>
<thead>
<tr>
<th>Sodium chloride (NaCl)</th>
<th>0.85 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water (H2O)</td>
<td>100.0 ml</td>
</tr>
</tbody>
</table>

1. Dissolve the sodium chloride in distilled water in a flask or bottle by using a magnetic stirrer.
2. Distribute 10 ml of the solution into each of 10 screw-cap tubes.
3. Label as 0.85% NaCl with an expiration date of 1 year.
4. Sterilize by autoclaving at 121°C for 15 min.
5. When cool, store at 4°C.

B. D’Antoni’s iodine

<table>
<thead>
<tr>
<th>Potassium iodide (KI)</th>
<th>1.0 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Powdered iodine crystals</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Distilled water (H2O)</td>
<td>100.0 ml</td>
</tr>
</tbody>
</table>

1. Dissolve the potassium iodide and iodine crystals in distilled water in a flask or bottle by using a magnetic stirrer.
2. Some excess crystals of iodine should remain on the bottom of the bottle.
3. Store in a brown, glass-stoppered bottle at room temperature.
4. The solution is ready for immediate use. Label as D’Antoni’s iodine with an expiration date of 1 year (the stock solution remains good as long as an excess of iodine crystals remains on the bottom of the bottle).
5. Aliquot some of the iodine into a brown dropper bottle. The working solution should resemble strong tea and should be discarded when it lightens in color (usually within 10 to 14 days).
C. Lugol’s iodine

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>potassium iodide</td>
<td>10.0 g</td>
</tr>
<tr>
<td>powdered iodine crystals</td>
<td>5.0 g</td>
</tr>
<tr>
<td>distilled water</td>
<td>100.0 ml</td>
</tr>
</tbody>
</table>

1. Follow the directions given above for D’Antoni’s iodine, including the expiration date of 1 year.
2. Dilute a portion 1:5 with distilled water for routine use (working solution).
3. Place this working solution into a brown dropper bottle. The working solution should resemble strong tea and should be discarded when it lightens in color (usually within 10 to 14 days).

### Diagnostic characteristics of organisms in wet mounts

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Protozoa</th>
<th>Helminths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stool, other specimens from the gastrointestinal tract or urogenital system</td>
<td>Size, shape, stage (trophozoite, precyst, cyst, oocyst), motility (fresh specimens only), refractility, cytoplasm inclusions (chromatoidal bars, glycogen vacuoles, axonemes, axostyles, median bodies, sporozoites)</td>
<td>Egg, larvae, or adult; size; internal structure, as follows: for the egg, embryonated, opercular shoulders, abopercular thickenings or projections, hooklets, polar filaments, and spines; for larvae, head and tail morphology and digestive tract; and for the adult, nematode, cestode, or trematode</td>
</tr>
</tbody>
</table>
9.3.4 Microscopic Examination of Fecal Specimens: Concentration by Formalin-Ethyl Acetate Sedimentation

Fecal concentration has become a routine procedure as a part of the complete ova and parasite examination for parasites and allows the detection of small numbers of organisms that may be missed by using only a direct wet smear. There are two types of concentration procedures: flotation and sedimentation, both of which are designed to separate protozoan organisms and helminth eggs and larvae from fecal debris by centrifugation and/or differences in specific gravity.

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE
By centrifugation, this concentration procedure leads to the recovery of all protozoa, eggs, and larvae present; however, the preparation contains more debris than the flotation procedure. Ethyl acetate is used as an extractor of debris and fat from the feces and leaves the parasites at the bottom of the suspension. Concentration by formalin-ethyl acetate sedimentation is recommended because it is the easiest to perform, allows recovery of the broadest range of organisms, and is least subject to technical error (1–5). It is also important to remember that concentrated fecal sediment is recommended for the modified acid-fast and modified trichrome stains used for the coccidia and microsporidia, respectively.

II. SPECIMEN

A. The specimen must be fresh or formalinized stool (5 or 10% buffered or nonbuffered formalin or sodium acetate-acetic acid-formalin [SAF]) (see procedures 9.1, 9.2.1, and 9.2.2).

B. Polyvinyl alcohol (PVA)-preserved specimens can be used (see item VIII below), as can specimens preserved in the single-vial collection systems (zinc-based, proprietary formulas).

III. MATERIALS

A. Reagents
1. Ethyl acetate
2. Formalin (5 or 10% buffered or nonbuffered or SAF)
3. 0.85% NaCl
4. D’Antoni’s or Lugol’s iodine

B. Supplies
1. Funnel
2. Gauze
3. Centrifuge tubes (15 ml)
4. Applicator sticks
5. Glass slides (1 by 3 in. or larger)

C. Equipment
1. Centrifuge (tabletop or floor model)
2. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
3. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.

Observe standard precautions.

Include QC information on reagent container and in QC records.
Fecal Specimens: Formalin-Ethyl Acetate Sedimentation

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Check the reagents each time they are used. The formalin and saline should appear clear, without any visible contamination.

B. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements done with the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (see procedure 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

C. Concentrate known positive specimens and verify organism recovery at least quarterly and particularly after the centrifuge has been recalibrated.

D. Record all QC results.

V. PROCEDURE

A. Wear gloves when performing this procedure.

B. Transfer a half-teaspoon (about 4 g) of fresh stool into 10 ml of 10% formalin in a shell vial, unwaxed paper cup, or round-bottom tube (container may be modified to suit individual laboratory preferences). Mix the stool and formalin thoroughly. Let the mixture stand a minimum of 30 min for fixation. If the specimen is already in 5 or 10% formalin (or SAF or one of the other available single-vial collection system fixatives), restir the stool-formalin mixture.

C. Depending on the amount and viscosity of the specimen, strain a sufficient quantity through wet gauze into a conical 15-ml centrifuge tube to give the desired amount of sediment (0.5 to 1 ml) in step V.D below. Usually, 8 ml of the stool-formalin mixture prepared in step V.B will be sufficient. If the specimen is received in vials of preservative, then approximately 3 to 4 ml will be sufficient unless there is very little stool in the vial.

D. Add 0.85% NaCl (see item VIII below) almost to the top of the tube, and centrifuge for 10 min at 500 × g. The amount of sediment obtained should be approximately 0.5 to 1 ml.

E. Decant the supernatant fluid, and resuspend the sediment in saline. Add saline almost to the top of the tube, and centrifuge again for 10 min at 500 × g. This second wash may be eliminated if the supernatant fluid after the first wash is light tan or clear.

F. Decant the supernatant fluid, and resuspend the sediment on the bottom of the tube in 10% formalin. Fill the tube half full only. If the amount of sediment left in the bottom of the tube is very small or if the original specimen contained a lot of mucus, do not add ethyl acetate in step V.G; merely add the formalin, spin, decant, and examine the remaining sediment.

G. Add 4 to 5 ml of ethyl acetate. Stopper the tube, and shake it vigorously for at least 30 s, exerting pressure on the stopper throughout. Hold the tube so the stopper is directed away from your face.

H. After a 15- to 30-s wait, carefully remove the stopper.

I. Centrifuge for 10 min at 500 × g. 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 ethyl acetate at the top (Fig. 9.3.4–1).

J. Free the plug of debris by ringing the plug with an applicator stick; decant all of the supernatant fluid. After proper decanting, a drop or two of fluid remaining...
V. PROCEDURE (continued)

on the side of the tube may run down into the sediment. Mix this fluid with the sediment.

K. If the sediment is still somewhat solid, add a drop or two of saline to the sediment, mix, and add a small amount of material to a slide, add a coverslip, and examine.

L. Systematically scan with the 10× objective. The entire coverslip area should be examined.

M. If you see something suspicious, use the 40× objective for more-detailed study. At least one-third of the coverslip should be examined with the 40× objective, even if nothing suspicious has been seen. As in the direct wet smear, iodine can be added to enhance morphological detail.

VI. RESULTS

Protozoan trophozoites and/or cysts and helminth eggs and larvae may be seen and identified. Protozoan trophozoites are less likely to be seen.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. You may or may not be able to identify protozoan cysts to the species level (depending on the clarity of the morphology).

Example (positive report): *Giardia lamblia* cysts present.

B. You may identify helminth eggs and/or larvae.

Example: *Trichuris trichiura* eggs present.

C. You may also see artifacts and/or other structures. Report them.

Examples: Few Charcot-Leyden crystals present.

Moderate PMNs present.

VIII. PROCEDURE NOTES

A. Tap water may be substituted for 0.85% NaCl throughout this procedure, although the addition of water to fresh stool will cause *Blastocystis hominis* cyst forms to rupture. In addition to the original 10% formalin fixation, some workers prefer to use 10% formalin for all the rinses throughout the procedure.

B. Ethyl acetate is widely recommended as a substitute for ether. It can be used the same way in the procedure and is much safer. Hemo-De can also be used (6).

1. After the plug of debris is rimmed and excess fluid is decanted, while the tube is still upside down, swab the sides of the tube with a cotton-tipped applicator stick to remove excess ethyl acetate. This is particularly important if you are working with plastic centrifuge tubes. If the sediment is too dry after the tube has been swabbed, add several drops of saline before preparing your wet smear for examination.

2. If you have excess ethyl acetate in the smear of the sediment prepared for examination, bubbles will obscure the material you are trying to see.

C. If specimens are received in SAF, then begin at step V.B.

D. If specimens are received in PVA or one of the other available single vial collection system fixatives, modify the first two steps of the procedure (steps V.A and B) as follows:

1. Immediately after stirring the stool-fixative mixture with applicator sticks, pour approximately one-half of the mixture into a tube (container optional) and add 0.85% saline almost to the top of the tube.

2. Filter the stool-fixative-saline mixture through wet gauze into a 15-ml centrifuge tube. Follow the standard procedure from here to completion, beginning with step V.C.
Fecal Specimens: Formalin-Ethyl Acetate Sedimentation

VIII. PROCEDURE NOTES (continued)

E. Too much or too little sediment will result in an ineffective concentration.
F. Let the centrifuge reach the recommended speed before you begin to monitor centrifugation time. If the centrifugation time at the proper speed is reduced, some organisms (Cryptosporidium oocysts or microsporidial spores) may not be recovered in the sediment.

IX. LIMITATIONS OF THE PROCEDURE

A. Results obtained with wet smears should usually be confirmed by permanent stained smears. Some protozoa are very small and difficult to identify to the species level by direct wet smears alone.
B. Confirmation is particularly important in the case of Entamoeba histolytica/E. dispar versus Entamoeba coli.
C. Certain organisms, such as G. lamblia, hookworm eggs, and occasionally Trichuris eggs, may not concentrate as well from PVA-preserved specimens as they do from those preserved in formalin. However, if there are enough G. lamblia organisms present to concentrate from formalin, then PVA should contain enough for detection on the permanent stained smear. In clinically important infections, the number of helmint eggs present would ensure detection regardless of the type of preservative used. Also, the morphology of Strongyloides stercoralis larvae is not as clear from PVA as from specimens fixed in formalin.
D. For unknown reasons, Isospora belli oocysts concentrated from PVA-preserved specimens are routinely missed in the concentrate sediment.
E. At the centrifugation speed and time recommended in this procedure, there is anecdotal evidence to strongly indicate that Cryptosporidium oocysts and microsporidial spores should be recovered if present in the specimen. The current recommendation is centrifugation at 500 × g for a minimum of 10 min for the recovery of coccidia and microsporidia.

REFERENCES


APPENDIX 9.3.4–1

Automated Workstation for the Microscopic Analysis of Fecal Concentrates

The FE-2 (DiaSys Corporation, Waterbury, Conn.) is a countertop workstation that automates the microscopic analysis of fecal concentrates (Fig. 9.3.4–A1). The system automates the aspiration, resuspension, staining or dilution (based on user preference), transfer, presentation, and disposal of fecal concentrates. When the sample button is pressed, within 5 s two samples of fecal concentrate are automatically and simultaneously aspirated from the concentrate tube and transported to the glass dual-flow cells of the Optical Slide Assembly (Fig. 9.3.4–A2). Based on user preference, the FE-2 will also simultaneously stain or dilute one of the two samples to be examined. After the microscopic examination of the fecal suspension within the glass viewing chambers, the flow chambers can be purged and cleaned so that they are ready for the next specimen. The dual-flow-cell Optical Slide Assembly is designed to fit within the stage clips of any standard, upright microscope. The Optical Slide Assembly accommodates bright-field, phase-contrast, polarized-light, and other common forms of microscopy. The system can be moved from one microscope to another or can be set up as a semipermanent station for fecal concentrate microscopy. Removal to another microscope just involves removing the Optical Slide Assembly from the microscope stage.
APPENDIX 9.3.4–1 (continued)

Figure 9.3.4–A1 Countertop workstation that automates the microscopic analysis of fecal concentrates (DiaSys Corp.) (from L. S. García, Diagnostic Medical Parasitology, 4th ed., 2001, ASM Press, Washington, D.C.).

Figure 9.3.4–A2 Dual-flow-cell Optical Slide Assembly (DiaSys Corp.) that fits into the stage clips of any standard upright microscope (from L. S. García, Diagnostic Medical Parasitology, 4th ed., 2001, ASM Press, Washington, D.C.).
Microscopic Examination of Fecal Specimens: Concentration by Zinc Sulfate Flotation

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE
The flotation procedure permits the separation of protozoan cysts and certain helminth eggs from excess debris through the use of a liquid with a high specific gravity. The parasitic elements are recovered in the surface film, and the debris remains in the bottom of the tube. This technique yields a cleaner preparation than the sedimentation procedure; however, some helminth eggs (perculated eggs and/or very dense eggs such as unfertilized Ascaris eggs) do not concentrate well with the flotation method (1–5). The specific gravity of the zinc sulfate may be increased, although this usually causes more distortion in the organisms present and is not recommended for routine clinical use. To ensure detection of all possible organisms, examine both the surface film and the sediment. For most laboratories, this is not a practical approach.

II. SPECIMEN
Observe standard precautions.

The specimen must be fresh or formalinized stool (5 or 10% buffered or nonbuffered formalin or sodium acetate-acetic acid-formalin [SAF]).

III. MATERIALS

A. Reagents (see Appendix 9.3.5–1)
1. Formalin (5 or 10% buffered or nonbuffered) or SAF
2. 0.85% NaCl
3. Zinc sulfate (33% aqueous solution)

B. Supplies
1. Funnel
2. Gauze
3. Centrifuge tubes (15 ml)
4. Applicator sticks
5. Glass slides (1 by 3 in. or larger)
6. Coverslips (22 by 22 mm; no. 1 or larger)
7. Disposable glass or plastic pipettes
8. Wire loop (bacteriology)
9. Graduated cylinder

IV. QUALITY CONTROL

A. Check the reagents each time they are used. The formalin, saline, and zinc sulfate should appear clear, without any visible contamination.

B. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the micro-
IV. QUALITY CONTROL (continued)

Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (see procedure 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

C. Concentrate known positive specimens, and verify organism recovery at least quarterly and particularly after the centrifuge has been recalibrated.

D. Record all QC results.

V. PROCEDURE

A. Wear gloves when performing this procedure.

B. Transfer a half-teaspoon (about 4 g) of fresh stool into 10 ml of 10% formalin in a shell vial, unwaxed paper cup, or round-bottom tube (container may be modified to suit individual laboratory preferences). Mix the stool and formalin thoroughly. Let the mixture stand a minimum of 30 min for fixation. If the specimen is already in 5 or 10% formalin (or SAF), restir the stool-formalin mixture.

C. Depending on the size and density of the specimen, strain a sufficient quantity through wet gauze into a conical 15-ml centrifuge tube to give the desired amount of sediment (0.5 to 1 ml) in step V.D below. Usually, 8 ml of the stool-formalin mixture prepared in step V.B will be sufficient. If the specimen is received in vials of preservative (10% formalin or SAF), then approximately 3 to 4 ml will be sufficient unless the specimen has very little stool in the vial.

D. Add 0.85% NaCl (see item VIII below) almost to the top of the tube, and centrifuge for 10 min at 500 × g. The amount of sediment obtained should be approximately 0.5 to 1 ml. Too much or too little sediment will result in an ineffective concentration.

E. Decant the supernatant fluid, resuspend the sediment in 0.85% NaCl almost to the top of the tube, and centrifuge for 10 min at 500 × g. This second wash may be eliminated if the supernatant fluid after the first wash is light tan or clear.

F. Decant the supernatant fluid, and resuspend the sediment on the bottom of the tube in 1 to 2 ml of zinc sulfate. Fill the tube to within 2 to 3 mm of the rim with additional zinc sulfate.

G. Centrifuge for 1 min at 500 × g. Allow the centrifuge to come to a stop without interference or vibration. Two layers should result: a small amount of sediment in the bottom of the tube and a layer of zinc sulfate. The protozoan cysts and some helminth eggs will be in the surface film; some operculated and/or heavy eggs will be in the sediment (Fig. 9.3.5–1).

H. Without removing the tube from the centrifuge, remove 1 or 2 drops of the surface film with a Pasteur pipette or a freshly flamed (and allowed to cool) wire loop and place them on a slide. Do not use the loop as a “dipper”; simply touch the surface (bend the loop portion of the wire 90° so the loop is parallel with the surface of the fluid). Make sure the pipette tip or wire loop is not below the surface film.

I. Add a coverslip to the preparation. Iodine may be added to the preparation.

J. Systematically scan with the 10× objective. The entire coverslip area should be examined.

K. If you see something suspicious, use the 40× objective for more-detailed study. At least one-third of the coverslip should be examined with the 40× objective, even if nothing suspicious has been seen. As in the direct wet smear, iodine can be added to enhance morphological detail.
VI. RESULTS

Protozoan trophozoites and/or cysts and helminth eggs and larvae may be seen and identified.

POSTANALYTICAL CONSIDERATIONS

A. You may or may not be able to identify protozoan cysts to the species level (depending on the clarity of the morphology).
   Example: *Giardia lamblia* cysts present.

B. You may identify helminth eggs and/or larvae.
   Example: *Trichuris trichiura* eggs present.

C. You may see and report artifacts and/or other structures.
   Examples: Few Charcot-Leyden crystals present.
   Moderate PMNs present.

VII. REPORTING RESULTS

A. Tap water (see step V.D below) may be substituted for 0.85% NaCl throughout this procedure; some workers prefer to use 10% formalin for all the rinses throughout the procedure.

B. If fresh stool is used (nonformalin preservatives), then the zinc sulfate should be prepared with a specific gravity of 1.18.

C. If specimens are received in SAF, then begin at step V.B.

D. If fresh specimens are received, the standardized procedure requires the stool to be rinsed in distilled water prior to the addition of zinc sulfate in step V.F. However, the addition of fresh stool to distilled water will destroy any Blastocystis hominis organisms present and is not a recommended approach.

E. Some workers prefer to remove the tubes from the centrifuge prior to sampling the surface film. This is acceptable; however, there is more chance that the surface film will be disturbed prior to sampling.

F. Some workers prefer to add a small amount of zinc sulfate to the tube so that the fluid forms a slightly convex meniscus. A coverslip is then placed on top of the tube so that the undersurface touches the meniscus. Leave undisturbed for 5 min. Carefully remove the coverslip, and place it on a slide for examination.

G. When using the hydrometer (with the solution at room temperature), mix the solution well. Float the hydrometer in the solution, giving it a slight twist to see that it is completely free from the sides of the container. Read the bottom meniscus, and correct for temperature if necessary. Most hydrometers are calibrated at 20°C. A difference of 3°C between the solution temperature (room temperature) and the hydrometer calibration temperature requires a correction of 0.001, to be added if above 20°C and subtracted if below 20°C.

H. The longer centrifugation times for the wash steps (V.D and E) are necessary for the possible recovery of *Cryptosporidium* oocysts or microsporidial spores.

VIII. PROCEDURE NOTES

A. Some workers prefer to remove the tubes from the centrifuge prior to sampling the surface film. This is acceptable; however, there is more chance that the surface film will be disturbed prior to sampling.

IX. LIMITATIONS OF THE PROCEDURE

A. Results obtained with wet smears should usually be confirmed by permanent stained smears. Some protozoa are very small and difficult to identify to the species level by direct wet smears alone.

B. Confirmation is particularly important in the case of *Entamoeba histolytica*/*E. dispar* versus *Entamoeba coli*.

C. Protozoan cysts and thin-shelled helminth eggs are subject to collapse and distortion when left in contact with high-specific-gravity zinc sulfate for more than a few minutes. Remove the surface film for examination within 5 min of the time the centrifuge comes to a stop. The longer the organisms are in contact with the zinc sulfate, the more distortion you will see on microscopic examination of the surface film.
IX. LIMITATIONS OF THE PROCEDURE (continued)

D. If zinc sulfate is the only concentration method used, examine both the surface film and the sediment to ensure detection of all possible organisms.

REFERENCES


APPENDIX 9.3.5–1

Reagents

Indicate the expiration date on the label and in the work record or on the manufacturer’s label.

A. Formalin (5 or 10% buffered or nonbuffered) or SAF (see procedure 9.2.2)
B. 0.85% NaCl
C. Zinc sulfate (33% aqueous solution)

\[
\begin{align*}
\text{zinc sulfate} & \quad \text{330 g} \\
\text{distilled water} & \quad \text{670 ml}
\end{align*}
\]

1. Dissolve the zinc sulfate in distilled water in an appropriate flask or beaker with a magnetic stirrer.
2. Adjust the specific gravity to 1.20 by adding more zinc sulfate or distilled water. Use a specific gravity of 1.18 when using fresh stool (nonformalinized).
3. Store in a glass-stoppered bottle with an expiration date of 24 months.

NOTE: There are no specific guidelines for how often the specific gravity of the zinc sulfate solution should be checked. However, due to the possibility of evaporation, the specific gravity of the solution should probably be checked twice each year. If the zinc sulfate flotation concentration method is used routinely, the specific gravity of the solution will be checked after preparation and then twice each year. Remember to store the solution in a tightly stoppered bottle.
9.3.6 Microscopic Examination of Fecal Specimens: Permanent Stained Smear (Trichrome)

It is generally recognized that stained fecal films are the single most productive means of stool examination for intestinal protozoa. The permanent stained smear facilitates detection and identification of cysts and trophozoites and affords a permanent record of the protozoa encountered. Small protozoa missed by direct smear and concentration techniques are often seen on the stained smear. It also allows laboratories to refer the slide to a specialist for help when they have encountered an organism with an unusual morphology or have difficulty with the identification. For these reasons, the permanent stained smear is recommended for use with every stool specimen submitted for a routine parasite examination (1, 2, 4–6).

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The trichrome technique of Wheatley (8) for fecal specimens is a modification of Gomori’s original staining procedure for tissue (3). It is a rapid, simple procedure that produces uniformly well-stained smears of intestinal protozoa, human cells, yeast cells, and artifact material.

II. SPECIMEN

Observe standard precautions.

A. The specimen usually consists either of unconcentrated fresh stool smeared on a microscope slide and immediately fixed in Schaudinn’s fixative or of polyvinyl alcohol (PVA)-preserved stool smeared on a slide and allowed to air dry (see procedure 9.2.2).

B. Stool preserved in sodium acetate-acetic acid-formalin (SAF) or any of the single-vial fixatives for parasitology can also be used (see procedure 9.2.2).

III. MATERIALS

A. Reagents (see Appendix 9.3.6–1)

- Trichrome stain

B. Supplies

1. Disposable glass or plastic pipettes
2. Glass slides (1 by 3 in., or larger if you prefer)
3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)
4. 12 covered Coplin jars or staining dishes (with slide rack)

C. Equipment

1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
3. Fume hood to contain staining setup (optional)
IV. QUALITY CONTROL

A. For QC of Schaudinn’s, SAF, or PVA fixative, see procedure 9.2.2.
B. Stool samples used for QC can be either fixed stool specimens known to contain protozoa or PVA-preserved negative stools to which buffy coat cells have been added. Use a QC smear prepared from a positive PVA specimen or PVA containing buffy coat cells when a new stain is prepared or at least once each month. Cultured protozoa can also be used.
C. Include a QC slide when you use a new lot number of reagents, when you add new reagents after cleaning the dishes, and at least monthly.
D. If the xylene becomes cloudy or has an accumulation of water in the bottom of the staining dish, use fresh 100% ethanol and xylene.
E. Cover all staining dishes to prevent evaporation of reagents.
F. Depending on the volume of slides stained, change staining solutions on an as-needed basis.
G. When the smear is thoroughly fixed and the stain is performed correctly, the cytoplasm of protozoan trophozoites will be blue-green, with sometimes a tinge of purple. Cysts tend to be slightly more purple. Nuclei and inclusions (chromatoid bodies, RBCs, bacteria, and Charcot-Leyden crystals) are red, sometimes tinged with purple. The background material usually stains green, providing a nice color contrast with the protozoa. This contrast is more distinct than that obtained with the hematoxylin stain.
H. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (see procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.
I. Known positive microscope slides, Kodachrome 2-by-2 projection slides, and photographs (reference books) should be available at the workstation.
J. Record all QC results, including a description of QC specimens tested.

V. PROCEDURE

A. Wear gloves when performing this procedure.
B. Slide preparation
   1. Fresh fecal specimens
      a. When the specimen arrives, prepare two slides with applicator sticks and immediately (without drying) place them in Schaudinn’s fixative (see procedure 9.2.2). Allow the specimen to fix for a minimum of 30 min; overnight fixation is acceptable. The stool smeared on the slide should be thin enough that newsprint can be read through the smear. Proceed with the trichrome staining procedure (see item V.C.1 below).
      b. If the fresh specimen is liquid, place 3 or 4 drops of PVA (see procedure 9.2.2) on the slide, mix several drops of fecal material with the PVA, spread the mixture, and allow it to dry for several hours in a 37°C incubator or overnight at room temperature (25°C). Proceed with the trichrome staining procedure by placing the slides in iodine-alcohol (see item V.B.2 below).
V.  PROCEDURE (continued)

2. PVA-preserved fecal specimens (mercuric chloride base) (see procedure 9.2.2)
   a. Allow the stool specimens that are preserved in PVA to fix for at least 30 min. Thoroughly mix the contents of the PVA bottle with two applicator sticks.
   b. Pour some of the PVA-stool mixture onto a paper towel, and allow it to stand for 3 min to absorb the PVA. Do not eliminate this step.
   c. With an applicator stick, apply some of the stool material from the paper towel to two slides, and allow them to dry for several hours in a 37°C incubator or overnight at room temperature (25°C).
   d. Place the dry slides into iodine-alcohol (see item V.C.2 below).
   e. If the stool was not thoroughly mixed with PVA by the patient, apply some stool material to two slides and immediately immerse in Schaudinn’s fixative for a minimum of 30 min; then proceed with the trichrome method (see item V.C.1 below).

3. Modified PVA-preserved fecal specimens (copper or zinc base, single-vial systems)
   a. Allow the stool specimens that are preserved in PVA or another fixative to fix for at least 30 min. Thoroughly mix the contents of the fixative vial with two applicator sticks.
   b. Pour some of the fixative-stool mixture onto a paper towel, and allow it to stand for 3 min to absorb the PVA. Do not eliminate this step if the fixative contains PVA.
   c. With an applicator stick, apply some of the stool material from the paper towel to two slides, and allow them to dry for several hours in a 37°C incubator or overnight at room temperature (25°C).
   d. Begin the trichrome staining process at step V.C.4 or V.C.5.

4. SAF-preserved fecal specimens (see procedure 9.2.2)
   a. Thoroughly mix the SAF-stool mixture, and strain through gauze into a 15-ml centrifuge tube.
   b. After centrifugation (10 min at 500 × g), decant the supernatant fluid. The final sediment should be about 0.5 to 1.0 ml. If necessary, adjust by repeating step V.B.1 or by suspending the sediment in saline (0.85% NaCl) and removing part of the suspension.
   c. Prepare a smear from the sediment for later staining.
   d. After drying, place the smear in 70% alcohol (see item V.C.3 below) (iodine-alcohol step can be eliminated).

C. Staining smears

1. Remove slide from Schaudinn’s fixative, and place slide in 70% ethanol for 5 min.
2. Place slide in 70% ethanol plus iodine for 1 min for fresh specimens or 5 to 10 min for PVA-preserved air-dried smears. All slides exposed to mercuric-chloride-based fixatives must be placed in the iodine dish to remove the mercury. The subsequent rinses in ethanol remove the iodine. At the point the slide is placed into trichrome stain, both the mercury and iodine have been removed from the fecal smear.
3. Place slide in 70% ethanol for 5 min.*
4. Place in 70% ethanol again for 3 min.* Fecal smears prepared from SAF-preserved stool material do not require the iodine step (V.C.2 above) and can be placed in this alcohol dish before trichrome staining.
5. Place in trichrome stain for 10 min. Fecal smears prepared from modified PVA-fixed material (copper or zinc base) do not require the iodine step (V.C.2 above) or subsequent alcohol rinses (V.C.3 and 4 above) but can be placed directly into the trichrome stain (this step). One alcohol rinse may
V. PROCEDURE (continued)

be used (see item V.C.4) before this trichrome step; some labs prefer this approach.

6. Place in 90% ethanol plus acetic acid for 1 to 3 s. Immediately drain the rack (see item VIII below), and proceed to the next step. Do not allow slides to remain in this solution.

7. Dip several times in 100% ethanol. Use this step as a rinse.

8. Place in two changes of 100% ethanol for 3 min each.*

9. Place in xylene for 5 to 10 min.*

10. Place in xylene again for 5 to 10 min.*

11. Mount with coverslip (no. 1 thickness) by using mounting medium (e.g., Permount).

12. Allow the smear to dry overnight or for 1 h at 37°C.

13. Examine the smear microscopically with the 100× objective. Examine at least 200 to 300 oil immersion fields.

*Slides may be held for up to 24 h in these solutions without harming the quality of the smear or the stainability of organisms.

VI. RESULTS

A. Protozoan trophozoites and cysts will be readily seen.

B. Helminth eggs and larvae may not be easily identified; therefore, examine wet mounts of concentrates.

C. Yeast and human cells can be identified. Human cells include macrophages, PMNs, and RBCs. Yeasts include single and budding cells and pseudohyphae.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the organism and stage (do not use abbreviations).

Example: Entamoeba histolytica/E. dispar trophozoites

B. Quantitate the number of Blastocystis hominis seen (rare, few, moderate, many).

Do not quantitate other protozoa.

C. Note and quantitate the presence of human cells.

Example: Moderate WBCs, many RBCs, few macrophages, rare Charcot-Leyden crystals

D. Report and quantitate yeast cells.

Example: Moderate budding yeast cells and few pseudohyphae

E. Save positive slides for future reference. Record information prior to storage (name, patient number, organisms present).

F. Quantitation of parasites, cells, yeast cells, and artifacts

Few = ≤2 per 10 oil immersion fields (×1,000)

Moderate = 3 to 9 per 10 oil immersion fields (×1,000)

Many = ≥10 per 10 oil immersion fields (×1,000)

VIII. PROCEDURE NOTES

A. Fixation of specimens is important. Improperly fixed specimens will result in protozoan forms that are nonstaining or predominantly red.

B. Spread the PVA-stool mixture to the edges of the glass slide; this will cause the film to adhere to the slide during staining. It is also important to thoroughly dry the slides to prevent the material from washing off during staining.

C. Always drain slides between solutions. Touch the end of the slide to a paper towel for 2 s to remove excess fluid before proceeding to the next step.

D. Incomplete removal of mercuric chloride (Schaudinn’s fixative and PVA) may cause the smear to contain highly refractive granules that may prevent finding or identifying any organisms present (1). Since the 70% ethanol–iodine solution
VIII. PROCEDURE NOTES  
(continued)

removes the mercury complex, it should be changed at least weekly to maintain the strong-tea color.

E. To restore weakened trichrome stain, remove cap and allow the ethanol to evaporate (ethanol carried over on staining rack from previous dish). After a few hours, add fresh stain to restore lost volume. Older, more concentrated stain produces more intense colors and may require slightly longer destaining times (an extra dip).

F. Smears that are predominantly green may be due to the inadequate removal of iodine by the 70% ethanol (see items VIII.D and E above). Lengthening the time of these steps or changing the 70% ethanol more frequently will help.

G. In the final stages of dehydration (steps V.C.8, 9, and 10), keep the 100% ethanol and the xylene as free from water as possible. Coplin jars must have tight-fitting caps to prevent both evaporation of reagents and absorption of moisture. If the xylene becomes cloudy after addition of slides from the 100% ethanol, return the slides to fresh 100% ethanol, and replace the xylene.

H. If the smears peel or flake off, the specimen might have been inadequately dried on the slide (in the case of PVA-fixed specimens) or the slides might have been greasy. Slides do not have to be cleaned with alcohol prior to use.

I. If the stain appears unsatisfactory and it is not possible to obtain another slide to stain, restain the unsatisfactory slide. Place the slide in xylene to remove the coverslip, and reverse the dehydration steps by adding 50% ethanol as the last step. Destain the slide in 10% acetic acid for several hours, and then wash it thoroughly first in water, then in 50% ethanol, and then in 70% ethanol. Place the slide in the trichrome stain for 8 min, and complete the staining procedure (7).

IX. LIMITATIONS OF THE PROCEDURE

A. The permanent stained smear is not recommended for staining helminth eggs or larvae. However, occasionally they may be recognized and identified.

B. Examine the smear under the oil immersion lens (100×) for the identification of protozoa, human cells, Charcot-Leyden crystals, yeast cells, and artifact material.

C. This high-magnification examination is recommended for protozoa.

D. Screening the smear under the low-magnification lens (10×) might reveal eggs or larvae, but this is not recommended as a routine approach.

E. Helminth eggs and larvae and *Isospora belli* oocysts are best seen in wet preparations.

F. *Cryptosporidium parvum* and *Cyclospora cayetanensis* are generally not seen on a trichrome-stained smear (modified acid-fast stains or immunoassays are recommended).

G. Microsporidial spores will not be seen on a trichrome-stained smear (modified trichrome stains are recommended).

REFERENCES

APPENDIX 9.3.6–2

As an alternative to using mounting fluid on every slide, the following method can be used.

This approach saves time (drying the slides after they are mounted) and eliminates the need for routine use of mounting fluids.

A. Remove the stained slides from the last dehydrating dish (step V.C.10 above).

B. Allow the slide to air dry (minimum of 30 min, especially if using xylene substitutes).

C. Place a drop of immersion oil directly onto the dry stool smear.

D. Allow the oil to “sink in” for a minimum of ~15 min.

E. Place a no. 1 coverslip onto the oil-covered stool smear.

F. Add 1 drop of immersion oil onto the coverslip and proceed to examine the smear using the 100× oil immersion objective. A 50× or 60× oil immersion objective can be used for screening.

G. Do not use this approach unless you add the coverslip before examination of the smear. The dry stool material may be quite hard; the objective lens could accidentally be scratched if the stool smear is not covered before reading.

REFERENCES (continued)


APPENDIX 9.3.6–1

Reagents

- Indicate the expiration date on the label and in the work record or on the manufacturer’s label.

A. Trichrome stain

- chromotrope 2R ................................0.6 g
- light green SF ..................................0.3 g
- phosphotungstic acid ..........................0.7 g
- acetic acid (glacial) .......................... 1.0 ml
- distilled water .................................100 ml

Prepare the stain by adding 1.0 ml of acetic acid to the dry components. Allow the mixture to stand (ripen) for 15 to 30 min at room temperature. Add 100 ml of distilled water. Properly prepared stain will be purple. Store in a glass or plastic bottle at room temperature. The shelf life is 24 months.

B. 70% Ethanol

C. 70% Ethanol plus iodine

Prepare a stock solution by adding iodine crystals to 70% alcohol until you obtain a dark solution (1 to 2 g/100 ml). To use, dilute the stock solution with 70% alcohol until a dark reddish brown (strong-tea color) is obtained. As long as the color is acceptable, new working solution does not have to be replaced. Replacement time will depend on the number of smears stained and the size of the container (one to several weeks).

D. 90% Ethanol, acidified

- 90% ethanol ......................................99.5 ml
- acetic acid (glacial) ...........................0.5 ml

Prepare by combining.

E. 100% Ethanol

F. Xylene (or xylene substitute)
### 9.3.7 Microscopic Examination of Fecal Specimens: Iron Hematoxylin Stain (Modified Spencer-Monroe Method)

#### Preanalytical Considerations

**I. Principle**

The iron hematoxylin stain is one of a number of stains used to make a permanent stained slide for detecting and quantitating parasitic organisms. Iron hematoxylin was the stain used for most of the original morphological descriptions of intestinal protozoa found in humans (1–3). Under oil immersion power (×1,000), one can examine the diagnostic features used to identify the protozoan parasite.

**II. Specimen**

*Observe standard precautions.*

A. The specimen usually consists either of unconcentrated fresh stool smeared on a microscope slide and immediately fixed in Schaudinn’s fixative or of polyvinyl alcohol (PVA)-preserved stool smeared on a slide and allowed to air dry (see procedure 9.2.2).

B. Stool preserved in sodium acetate-acetic acid-formalin (SAF) can also be used (see procedure 9.2.2).

**III. Materials**

<table>
<thead>
<tr>
<th>A. Reagents</th>
<th>(see Appendix 9.3.7–1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Iron hematoxylin stain</td>
<td></td>
</tr>
<tr>
<td>2. D’Antoni’s iodine solution</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. Supplies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Glass slides (1 by 3 in.)</td>
</tr>
<tr>
<td>2. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)</td>
</tr>
<tr>
<td>3. Coplin jars or other suitable staining containers</td>
</tr>
<tr>
<td>4. Hematoxylin, crystals or powder</td>
</tr>
<tr>
<td>5. Ferrous ammonium sulfate [Fe(NH₄)₂(SO₄)₂·6H₂O]</td>
</tr>
<tr>
<td>6. Ferric ammonium sulfate [FeNH₄(SO₄)₂·12H₂O]</td>
</tr>
<tr>
<td>7. Hydrochloric acid, concentrated (HCl)</td>
</tr>
<tr>
<td>8. Potassium iodide (KI)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C. Equipment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)</td>
</tr>
<tr>
<td>2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.</td>
</tr>
<tr>
<td>3. Fume hood to contain staining setup (optional)</td>
</tr>
</tbody>
</table>

| 9. Iodine crystals, powdered (I₂) |
| 10. Permount or other suitable mounting medium |
IV. QUALITY CONTROL

A. For QC of Schaudinn’s SAF or PVA fixatives, see procedure 9.2.2.
B. Stool samples used for QC can be either fixed stool specimens known to contain protozoa or PVA-preserved negative stools to which buffy coat cells have been added. A QC smear prepared from a positive PVA specimen or PVA containing buffy coat cells should be used when a new stain is prepared or at least once each month. Cultured protozoa can also be used (1).
C. Include a QC slide when you use a new lot number of reagents, when you add new reagents after cleaning the dishes, and at least monthly.
D. If the xylene becomes cloudy or has an accumulation of water in the bottom of the staining dish, use fresh 100% ethanol and xylene.
E. Cover all staining dishes to prevent evaporation of reagents.
F. Depending on the volume of slides stained, change staining solutions on an as-needed basis.
G. Background material will stain blue-gray. Cells and organisms will stain various intensities of blue-gray. Inclusions, chromatoidal bodies, and nuclear structures will stain darker than the surrounding cytoplasm.
H. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (see procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.
I. Known positive microscope slides, Kodachrome 2-by-2 projection slides, and photographs (reference books) should be available at the work station.
J. Record all QC results, including a description of QC specimens tested.

V. PROCEDURE

A. Wear gloves when performing this procedure.
B. Slide preparation
   1. Fresh fecal specimens
      a. When the specimen arrives, prepare two slides with applicator sticks and immediately (without drying) place them in Schaudinn’s fixative (see procedure 9.1). Allow the slides to fix for a minimum of 30 min; overnight fixation is acceptable. The amount of stool smeared on the slide should be thin enough that newsprint can be read through the smear.
      b. If the fresh specimen is liquid, place 3 or 4 drops of PVA (see procedure 9.1) on the slide, mix several drops of fecal material with the PVA, spread the mixture, and allow it to dry for several hours in a 37°C incubator or overnight at room temperature (25°C).
      c. Proceed with the hematoxylin staining procedure by placing the dry slides in iodine-alcohol (see item V.B.2 below).
   2. PVA-preserved fecal specimens (see procedure 9.1)
      a. Allow the stool specimens that are preserved in PVA to fix at least 30 min. Thoroughly mix the contents of the PVA bottle with two applicator sticks.
      b. Pour some of the PVA-stool mixture onto a paper towel, and allow it to stand for 3 min to absorb the PVA. Do not eliminate this step.
Iron Hematoxylin Stain of Fecal Specimens

V. PROCEDURE (continued)

c. With an applicator stick, apply some of the stool material from the paper towel to two slides, and allow them to dry for several hours in a 37°C incubator or overnight at room temperature.
d. Place the dry slides in iodine-alcohol (see item V.C.2 below).
e. If the stool was not thoroughly mixed with PVA by the patient, apply some stool material to two slides, and immediately immerse in Schaudinn’s fixative for a minimum of 30 min; then proceed with the hematoxylin method.

3. SAF-preserved fecal specimens (see procedure 9.1)
a. Thoroughly mix the SAF-stool mixture, and strain through gauze into a 15-ml centrifuge tube.
b. After centrifugation (10 min at 500 \times g), decant the supernatant fluid. The final sediment should be about 0.5 to 1.0 ml. If necessary, adjust by repeating step 1 or by suspending the sediment in saline (0.85% NaCl) and removing part of the suspension.
c. Prepare a smear from the sediment for later staining.
d. After drying, place the smear into 70% alcohol (see item V.C.3 below) (iodine-alcohol step can be eliminated).

C. Staining smears
1. Place slide in 70% ethanol for 5 min.
2. Place slide in iodine–70% ethanol (70% alcohol to which is added enough D’Antoni’s iodine to obtain a strong tea color) solution for 2 to 5 min.
3. Place in 70% ethanol for 5 min. Begin procedure for SAF-fixed slides at this point.*
4. Wash slide in running tap water (constant stream of water into the container) for 10 min.
5. Place slide in iron hematoxylin working solution for 4 to 5 min.
6. Wash slide in running tap water (constant stream of water into the container) for 10 min.
7. Place slide in 70% ethanol for 5 min.*
8. Place slide in 95% ethanol for 5 min.*
9. Place slide in two changes of 100% ethanol for 5 min each.*
10. Place slide in two changes of xylene for 5 min each.*
11. Add Permount to the stained area of the slide, and cover with a coverslip.
12. Examine the smear microscopically with the 100× objective. Examine at least 200 to 300 oil immersion fields.

*Slides may be held for up to 24 h in these solutions without harming the quality of the smear or the stainability of organisms.

VI. RESULTS

A. Protozoan trophozoites and cysts will be readily seen.
B. Helminth eggs and larvae may not be easily identified; therefore, examine wet-mount concentrates.
C. Yeast and human cells can be identified. Human cells include macrophages, PMNs, and RBCs. Yeast cells include single and budding cells and pseudohyphae.
POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the complete scientific name (genus and species) of the organism and the stage seen.
   Example: *Entamoeba histolytica*/*E. dispar* trophozoites

B. Quantitate the number of *Blastocystis hominis* seen (rare, few, moderate, many).
   Do not quantitate other protozoa.

C. Note and quantitate the presence of human cells.
   Examples: Moderate WBCs, many RBCs, few macrophages, rare Charcot-Leyden crystals

D. Report and quantitate yeast cells.
   Example: Moderate budding yeast cells and few pseudohyphae

E. Save positive slides for future reference. Record information prior to storage (name, patient number, organisms present).

F. Quantitation of parasites, cells, yeast cells, and artifacts
   Few = \leq 1 per 10 oil immersion fields (×1,000)
   Moderate = 3 to 9 per 10 oil immersion fields (×1,000)
   Many = \geq 10 per 10 oil immersion fields (×1,000)

VIII. PROCEDURE NOTES

A. Once the staining process has begun, do not allow the slides to dry until they have been placed in xylene.

B. Always drain slides between solutions. Touch the end of the slide to a paper towel for 2 s to remove excess fluid before proceeding to the next step.

C. Incomplete removal of mercuric chloride (Schaudinn’s fixative and PVA) may cause the smear to contain highly refractive granules that may prevent finding or identifying any organisms present (3). Since the 70% ethanol–iodine solution removes the mercury complex, it should be changed at least weekly to maintain the port wine or strong tea color.

D. When large numbers of slides are stained, the working hematoxylin solution may be diluted and affect the quality of the stain. If dilution occurs, discard the working solution, and prepare a fresh working solution.

E. The shelf life of the stock hematoxylin solutions may be extended by keeping the solutions in the refrigerator at 4°C. Because of crystal formation in the working solutions, it may be necessary to filter them before preparing a new working solution.

F. In the final stages of dehydration (see items V.C.9 and 10 above), keep the 100% ethanol and the xylenes as free from water as possible. Coplin jars must have tight-fitting caps to prevent both evaporation of reagents and absorption of moisture. If the xylene becomes cloudy after addition of slides from the 100% ethanol, return the slides to fresh 100% ethanol, and replace the xylene.

G. If the smears peel or flake off, the specimen might have been inadequately dried on the slide (in the case of PVA-fixed specimens) or the slides might have been greasy. Slides do not have to be cleaned with alcohol prior to use.

H. Other iron hematoxylin formulations may call for 10 g of stain per 100 ml of 95 or 100% ethanol. These are also acceptable.

IX. LIMITATIONS OF THE PROCEDURE

A. The permanent stained smear is not recommended for staining helminth eggs or larvae. However, occasionally they may be recognized and identified.

B. The smear should be examined with the oil immersion lens (100×) for the identification of protozoa, human cells, Charcot-Leyden crystals, yeast cells, and artifact material.

C. This high-magnification examination is recommended for protozoa.
Iron Hematoxylin Stain of Fecal Specimens

IX. LIMITATIONS OF THE PROCEDURE (continued)

D. Screening the smear under low magnification (10×) might reveal eggs or larvae, but this is not recommended as a routine approach.

E. Helminth eggs and larvae and *Isospora belli* oocysts are best seen in wet preparations.

F. *Cryptosporidium parvum* will not be seen on an iron hematoxylin-stained smear (acid-fast stains or immunoassays are recommended).

G. Microsporidial spores will not be seen on an iron hematoxylin-stained smear (modified trichrome stains are recommended).

REFERENCES


APPENDIX 9.3.7–1

Reagents

- Include QC information on reagent container and in QC records.

A. Iron hematoxylin stain

1. Solution 1
   - hematoxylin (crystal or powder) ..........10 g
   - ethanol (absolute) .............................1,000 ml

   Place solution in a stoppered clear flask or bottle, and allow to ripen in a lighted room for at least 1 week at room temperature.

2. Solution 2
   - ferrous ammonium sulfate
     \[ \text{[Fe(NH}_4\text{)}_2\text{(SO}_4\text{)}_2 \cdot 6\text{H}_2\text{O}] \] ..........................10 g
   - ferric ammonium sulfate
     \[ \text{[FeNH}_4\text{(SO}_4\text{)}_2 \cdot 12\text{H}_2\text{O}] \] ..........................10 g
   - hydrochloric acid (HCl) (concentrated) ....10 ml
   - distilled water ..........................1,000 ml

3. Working solution
   - Mix equal volumes of solutions 1 and 2. The working solution should be made fresh every week.

B. D’Antoni’s iodine solution

- potassium iodide (KI) ..........................1 g
- distilled water ..........................100 ml

Add 1.5 g of powdered iodine crystals to the KI solution to saturate the solution. Undissolved iodine crystals should be present. Store the solution in the dark in a brown bottle (stock solution). Working solutions should be made fresh every 2 weeks and can be made by filtering the stock solution to remove undissolved crystals.

C. Ethanol: 70, 95, and 100%

D. Xylene (or xylene substitute)
APPENDIX 9.3.7–2  Modified Iron Hematoxylin Stain (Incorporating Carbol Fuchsin Step)

The following combination staining method for SAF-preserved fecal specimens was developed to allow the microscopist to screen for acid-fast organisms in addition to other intestinal parasites. For those laboratories using iron hematoxylin stains in combination with SAF-fixed material and modified acid-fast stains for *C. parvum*, *Cyclospora cayetanensis*, and *I. belli*, this modification represents an improved approach to current staining methods. This combination stain provides savings in both time and personnel use. Any fecal specimen submitted in SAF fixative can be used. Fresh fecal specimens after fixation in SAF for 30 min can also be used. This combination stain approach is not recommended for specimens preserved in Schaudinn’s fixative or PVA.

I. REAGENTS

A. Mayer’s albumin

Add an equal quantity of glycerin to a fresh egg white. Mix gently and thoroughly. Store at 4°C and indicate an expiration date of 3 months. Mayer’s albumin from commercial suppliers can normally be stored at 25°C for 1 year [e.g., product 756; E. M. Diagnostic Systems Inc., 480 Democrat Rd., Gibbstown, NJ 08027; (800) 443-3637].

B. Stock solution of hematoxylin stain

- hematoxylin powder .........................10 g
- ethanol (95 or 100%) .....................1,000 ml

1. Mix well until dissolved.
2. Store in a clear glass bottle in a light area. Allow to ripen for 14 days before use.
3. Store at room temperature with an expiration date of 1 year.

C. Mordant

- ferrous ammonium sulfate
  
  \[
  [\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2\cdot6\text{H}_2\text{O}] \quad \ldots \quad 10 \text{ g}
  \]

- ferric ammonium sulfate

  \[
  [\text{FeNH}_4(\text{SO}_4)_2\cdot12\text{H}_2\text{O}] \quad \ldots \quad 10 \text{ g}
  \]

- hydrochloric acid (concentrated) ...........10 ml
- distilled water to .........................1,000 ml

D. Working solution of hematoxylin stain

1. Mix equal quantities of stock solution of stain and mordant.
2. Allow mixture to cool thoroughly before use (prepare at least 2 h prior to use).
   The working solution should be made fresh every week.

E. Picric acid

Mix equal quantities of distilled water and an aqueous saturated solution of picric acid to make a 50% saturated solution.

F. Acid-alcohol decolorizer

- hydrochloric acid (concentrated) ...........30 ml
- alcohol to .................................1,000 ml

G. 70% alcohol and ammonia

- 70% alcohol ..............................50 ml
- ammonia .................................0.5 to 1.0 ml

Add enough ammonia to bring the pH to approximately 8.0.

H. Carbol fuchsin

1. To make basic fuchsin (solution A), dissolve 0.3 g of basic fuchsin in 10 ml of 95% ethanol.
2. To make phenol (solution B), dissolve 5 g of phenol crystals in 100 ml of distilled water. (Gentle heat may be needed.)
3. Mix solution A with solution B.
4. Store at room temperature. Solution is stable for 1 year.
APPENDIX 9.3.7–2 (continued)

II. PROCEDURE
A. Prepare slide.
   1. Place 1 drop of Mayer’s albumin on a labeled slide.
   2. Mix the sediment from the SAF concentration well with an applicator stick.
   3. Add approximately 1 drop of the fecal concentrate to the albumin and spread the mixture over the slide.
B. Allow slide to air dry at room temperature (smear will appear opaque when dry).
C. Place slide in 70% alcohol for 5 min.
D. Wash in container (not running water) of tap water for 2 min.
E. Place slide in Kinyoun’s stain for 5 min.
F. Wash slide in running tap water (constant stream of water into container) for 1 min.
G. Place slide in acid-alcohol decolorizer for 4 min.
H. Wash slide in running tap water (constant stream of water into container) for 1 min.
I. Place slide in iron hematoxylin working solution for 8 min.
J. Wash slide in distilled water (in container) for 1 min.
K. Place slide in picric acid solution for 3 to 5 min.
L. Wash slide in running tap water (constant stream of water into container) for 10 min.
M. Place slide in 70% alcohol plus ammonia for 3 min.
N. Place slide in 95% alcohol for 5 min.
O. Place slide in 100% alcohol for 5 min.
P. Place slide in two changes of xylene for 5 min.

III. PROCEDURE NOTES
A. The first 70% alcohol step acts with the Mayer’s albumin to “glue” the specimen to the glass slide. The specimen may wash off if insufficient albumin is used or if the slides are not completely dry prior to staining.
B. The working hematoxylin stain should be checked each day of use by adding a drop of stain to alkaline tap water. If a blue color does not develop, prepare fresh working stain solution.
C. The picric acid differentiates the hematoxylin stain by removing more stain from fecal debris than from the protozoa and removing more stain from the organism cytoplasm than from the nucleus. When properly stained, the background should be various shades of gray-blue and protozoa should be easily seen with medium blue cytoplasm and dark blue-black nuclei.

Supplemental Reading
9.3.8 Calcofluor White for Detection of Microsporidial Spores and Acanthamoeba Cysts
[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

The diagnosis of intestinal microsporidiosis (Enterocytozoon bieneusi, Encephalitozoon intestinalis) has depended on the use of invasive procedures and subsequent examination of biopsy specimens, often by electron microscopy methods. However, the need for a practical method for the routine clinical laboratory has stimulated some work in the development of additional methods. Slides prepared from fresh or formalin-fixed stool specimens can be stained using calcofluor white (CFW) (optical brightening agent) and can be examined using fluorescence microscopy. This staining method is based on the fact that routine stain penetration of the microsporidial spore is very difficult; thus, the use of CFW enhances the ability of the spores to be seen. The spore coat will stain with CFW, but the stain is nonspecific and may also stain other structures within the specimen (yeast, etc.).

I. PRINCIPLE

Acanthamoeba, a free-living ameba found in soil, sewage, and fresh, salt, or brackish water, can cause painful keratitis that may lead to eye removal or loss of eye function and has also been implicated in granulomatous amebic encephalitis (1) (see also procedure 9.9.2). A key to successful treatment of Acanthamoeba-caused infection is the rapid detection of the organisms in patient samples. Cultures may require several days, delaying diagnosis and treatment.

CFW can be used for direct detection of Acanthamoeba cysts from clinical specimens (4). The active ingredient is the disodium salt of 4,4’-bis-(4-anilino-bis-diethylamino-5-triazin-2-y lamino)-2,2’-stilbene disulfonic acid, which is a nonspecific fluorescent dye binding to the polysaccharide polymers of amebic cysts and microsporidial spores (3). It is very simple to use and provides a rapid and reliable means of demonstrating these organisms.

II. SPECIMEN

With the exception of stool, collect all specimens aseptically, and hold them at room temperature (24 to 28°C). Do not freeze or refrigerate specimens. Use sterile containers and solutions where indicated; any remaining specimen may be used to inoculate culture media.

A. Stool

1. The specimen can be fresh stool or stool that has been preserved in 5 or 10% formalin, sodium acetate-acetic acid-formalin (SAF), or some of the newer single-vial system fixatives.
2. Any specimen other than tissue thought to contain microsporidia could be stained by these methods.
3. Polyvinyl alcohol-preserved fecal material is not recommended.

B. CSF

1. Centrifuge at 250 × g for 10 min.
2. Remove and place into another sterile tube all except 0.5 ml of the supernatant fluid.
3. Suspend the sediment with remaining supernatant fluid for examination.
II. SPECIMEN (continued)

C. Tissue
Triturate a small portion in sterile water or saline.
1. Brain
2. Corneal scrapings or biopsy specimen
3. Lung
4. Skin lesion

D. Swab of conjunctiva or corneal ulcer
1. Place swab in 2 ml of sterile water or saline in a tube.
2. Vigorously shake the cotton-tipped portion of the swab in the liquid to suspend specimen.
3. Remove swab from tube, and return to original holder.
4. The sterile water can be examined directly or concentrated by centrifugation if a larger volume is used.

E. Contact lens paraphernalia
1. Contact lens solutions
   Solutions must be from opened containers already used by patient. If volume of solution is greater than 2 ml, then centrifuge prior to examination (250 × g for 10 min).
2. Contact lenses
   Submit in 2 ml of sterile water or saline. Examine a small portion of lens and the fluid containing the lens.

F. Water samples
1. Collect at least 100 ml of water in a sterile container.
2. Concentrate specimen by filtration or centrifugation prior to examination (2).

G. Slide prepared by physician
Submit at least two slides.
1. Place specimen in the center of the slide, covering no more than a dime-sized area.
2. Circle the material with a wax pencil or magic marker to denote location of the specimen.
3. Air dry slides thoroughly.
4. Place the slide in a slide holder or envelope for staining in the laboratory.

III. MATERIALS

A. Reagents (see Appendix 9.3.8–1)
B. Supplies
1. Microscope slides (1 by 3 in.), one ring (15 mm), frosted
2. Microscope slides (1 by 3 in.), two rings (12 mm), frosted
3. Coverslip (24 by 40 mm), no. 1 thickness
4. Sterile polystyrene, round-bottom tubes (with cap), 12 by 75 mm
5. Sterile transfer pipettes
6. Sterile wooden applicator sticks
7. Sterile swabs
8. Disposable latex gloves
9. Biohazard container for disposal of contaminated supplies and patient specimens.
10. Positive fecal specimen (microsporidial spores)
11. Acanthamoeba castellanii ATCC 30010 stock culture
12. Escherichia coli ATCC 25922 stock culture
13. Wax pencil or magic marker

C. Equipment
1. Epifluorescence microscope equipped with an exciter filter that transmits the 250- to 400-nm group of intense mercury spectral emission lines (Zeiss UGI or G365). View through a barrier filter (Zeiss 41 or LP420), which removes UV while transmitting visible blue light and longer wavelengths (1, 3).
2. Biological safety cabinet
IV. QUALITY CONTROL

A. A QC slide must be run with each batch of specimens stained with CFW.
B. Unfortunately, the only way to perform acceptable QC procedures for this method is to use actual microsporidial spores as the control organisms (Medical Chemical Corporation; http://www.med-chem.com). Obtaining these positive controls may be somewhat difficult. It is particularly important to use the actual organisms because the spores are difficult to stain and the size is very small (1 to 2.5 μm). Prepare control slides for microsporidia as follows:
1. Using a 10-μl aliquot of concentrated (formalin-ethyl acetate sedimentation concentration; centrifugation at 500 × g for 10 min), preserved liquid stool (5 or 10% formalin or SAF), prepare the smear by spreading the material over an area of 45 by 25 mm.
2. Allow the smear to air dry.
C. Prepare control slides for amebae as follows.
1. Make a suspension of A. castellanii from stock culture with sterile water or Page ameba saline.
2. Make a suspension of E. coli from stock culture with sterile water or Page ameba saline.
3. Add 1 drop of the A. castellanii suspension to one of the rings of a two-ring slide and 1 drop of the E. coli suspension to the other ring.
4. Allow the smear to air dry.
5. Fix the smear in absolute methanol for 3 to 5 min.
6. Store at room temperature. The smears are stable for 1 year.
D. Positive control
1. Acanthamoeba cysts are doubled walled (10 to 25 μm), and outer wall is wrinkled. The cysts will fluoresce.
2. The spores will be ovoid and refractile, and the spore wall will fluoresce. Occasionally, the polar tube can be seen either as a stripe or as a diagonal line across the spore; however, the internal spore contents will normally not be visible.
E. Negative control
1. E. coli will not fluoresce.
2. Most of the bacteria and other debris will not fluoresce. However, there will still be some yeast and debris that may also fluoresce.
F. Perform all scheduled maintenance on all equipment.
G. Record all QC results, including a description of QC specimens tested.
H. Known positive microscope slides, projection slides (2 by 2 in.), photographs, and reference books should be available at the workstation.

V. PROCEDURE

A. Slide preparation of clinical specimens
1. Using a sterile swab, stick, or pipette, thinly spread the specimen evenly over the area circumscribed by the ring.
   ☰ NOTE: Do not apply excessive specimen on the slide, because the smear may be too thick to visualize any organisms present.
2. Allow the smear to air dry.
3. Fix the smear in absolute methanol for 3 to 5 min.
4. Air dry.
B. Stain procedure
1. Add 3 or 4 drops of CFW and 3 or 4 drops of Evan’s blue into a tube (12 by 75 mm) and mix well.
2. Add several drops of this mixture to the specimen and allow to stand for 5 min.
V.  PROCEDURE  (continued)

3. Turn slide on its side and allow excess stain to run off.
4. Add coverslip, blot excess stain from slide, and examine immediately.

VI. RESULTS

A. Microsporidial spores will fluoresce. The outer wall (1 to 3 μm) will fluoresce, with the interior being clear, or perhaps the horizontal or diagonal stripe will fluoresce.

B. *Acanthamoeba* cysts will fluoresce. Cysts are double walled (10 to 25 μm), and the outer cyst wall is wrinkled (hexacanth cyst). Although more rare than *Acanthamoeba*, the cysts of *Balamuthia mandrillaris* are usually spherical, appear to have two walls (outer irregular wall and inner round wall), and measure 6 to 30 μm in diameter. *Naegleria* cysts can be confirmed from culture plates but are not seen in clinical specimens. They tend to measure from 7 to 15 μm and have a thick double wall.

C. Yeast cells, pseudohyphae, hyphae, and other fungal elements will stain with CFW (2).

D. *Pneumocystis carinii* can be detected with CFW (3).

E. Bacteria will not fluoresce.

F. Epithelial cells and blood cells will stain red by Evan’s blue counterstain.

G. Cotton fibers will fluoresce strongly and can be distinguished as artifacts.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Positive
   Report as microsporidial spores or *Acanthamoeba* cysts seen. Notify physician immediately.

B. Negative
   No organisms seen.

VIII. PROCEDURE NOTES

A. Collagen, elastin, and keratin also fluoresce.

B. Other microorganisms (e.g., yeast cells, fungi) will fluoresce.

C. Although bacteria will not fluoresce, microsporidial spores (approximately the same size as some small yeasts and bacteria [1 to 2 μm]) will fluoresce. These organisms have been implicated as a cause of eye disease and have been found in other body tissues. The use of modified trichrome stains would be helpful in differentiating microsporidial spores from other organisms.

D. One drop of 10% KOH can be added to the CFW reagent, and a wet mount can be made of the specimens which require clearing or teasing (e.g., skin scrapings, hair, or viscous specimens).

E. Various concentrations of CFW and Evan’s blue are commercially available. Some work better than others.

F. Confirm findings (positive or negative) with culture.

G. Depending on what filter combination is used, the cysts will fluoresce either blue-white or apple green.

H. If *Naegleria* cysts are present, they may be seen using calcofluor.

REFERENCES

SUPPLEMENTAL READING


APPENDIX 9.3.8–1

Reagents

A. Commercially available solution of CFW with an Evan’s blue counterstain (such as Fungi-Fluor kit, catalog no. 17442; Polysciences, Inc., Warrington, Pa.) or solution prepared as follows:

1. **0.1% CFW**
   
   CFW M2R, purified .......................... 0.1 g
   distilled water .............................. 99.9 ml
   
   Mix, Filter, and store in dark container. The mixture is stable at room temperature for 1 year.

2. **0.5% Evan’s blue**
   
   Evan’s blue .................................. 0.5 g
   distilled water .............................. 99.5 ml
   
   Mix. The mixture is stable at room temperature for 1 year.

B. **Page’s ameba saline (1X)**

   NaCl .............................................. 6 mg
   MgSO₄•7H₂O .................................... 0.2 mg
   CaCl₂•2H₂O .................................... 0.2 mg
   Na₂HPO₄ ........................................ 7.1 mg
   KH₂PO₄ .......................................... 6.8 mg
   distilled water .............................. 500 ml
   
   Autoclave at 121°C for 15 min. Store refrigerated in a glass bottle. The mixture is stable for 3 months.

C. **Absolute methanol**

D. **Sterile distilled water**

Include QC information on reagent container and in QC records.
9.4.1 Special Stains for Coccidia: Modified Kinyoun’s Acid-Fast Stain (Cold)

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE
Cryptosporidium and Isospora species have been recognized as causes of severe diarrhea in immunocompromised hosts, but they can also cause diarrhea in immunocompetent hosts. Oocysts in clinical specimens may be difficult to detect without special staining. Cyclospora cayetanensis has also been reported to be acid fast. Modified acid-fast stains are recommended for demonstrating these organisms. Unlike the Ziehl-Neelsen modified acid-fast stain, the modified Kinyoun acid-fast stain does not require heating the reagents used for staining and uses a mild decolorizer (1–3).

II. SPECIMEN
Concentrated sediment of fresh or formalin-preserved stool may be used. Other types of clinical specimens such as duodenal fluid, bile, or pulmonary (induced sputum, bronchial washings, biopsy specimens) may also be stained after centrifugation.

III. MATERIALS

A. Reagents (see Appendix 9.4.1–1)
   1. Absolute methanol
   2. 50% Ethanol
   3. Kinyoun carbol fuchsin
   4. 1% Sulfuric acid
   5. Methylene blue

B. Supplies
   1. Disposable glass or plastic pipettes
   2. Glass slides (1 by 3 in., or larger if you prefer)
   3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)

C. Equipment
   1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
   2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
   3. Tabletop centrifuge
   4. Staining rack

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL
A. A control slide of Cryptosporidium parvum from a 10% formalin-preserved specimen is included with each staining batch run. If the cryptosporidia stain well, any Isospora belli oocysts present will also take up the stain, as will C. cayetanensis.
IV. QUALITY CONTROL (continued)

B. Cryptosporidia stain pink-red. Oocysts are 4 to 6 μm in diameter, and four sporozoites may be present internally. The background should stain uniformly blue.
C. Check the specimen (macroscopically) for adherence to the slide.
D. Record all QC results.

V. PROCEDURE

A. Smear 1 or 2 drops of specimen on the slide, and allow it to air dry. Do not make the smears too thick (you should be able to see through the wet material before it dries). Prepare two smears.
B. Fix with absolute methanol for 1 min. Allow to air dry.
C. Flood slide with Kinyoun’s carbol fuchsin, and stain for 5 min.
D. Rinse slide briefly (3 to 5 s) with 50% ethanol.
E. Rinse thoroughly with water.
F. Decolorize with 1% sulfuric acid for 2 min or until no more color runs from the slide.
G. Rinse slide with water. Drain.
H. Counterstain with methylene blue for 1 min.
I. Rinse slide with water. Air dry.
J. Examine using low-power or high dry power objectives. To see internal morphology, use oil immersion objective (100×).

VI. RESULTS

A. With this cold Kinyoun acid-fast method, C. cayetanensis and the oocysts of Cryptosporidium and Isospora will stain pink to red to deep purple. Some of the four sporozoites may be visible in the Cryptosporidium oocysts. Some of the Isospora immature oocysts (entire oocyst) will stain, while in oocysts that are mature, the two sporocysts within the oocyst wall will usually stain pink to purple and there will be a clear area between the stained sporocysts and the oocyst wall. The background will stain blue. If Cyclospora oocysts are present (uncommon), they tend to be approximately 8 to 10 μm, they resemble C. parvum but are larger, and they have no definite internal morphology; the acid-fast staining will tend to be more variable than that seen with Cryptosporidium or Isospora spp. Modified acid-fast stains stain the Cyclospora oocysts from light pink to deep red, and some of the oocysts will contain granules or have a bubbly appearance, often being described as looking like “wrinkled cellophane.” Even with the 1% acid decolorizer, some oocysts of Cyclospora may appear clear or very pale. If the patient has a heavy infection with microsporidia (immunocompromised patient), small (1- to 2-μm) spores may be seen but may not be recognized as anything other than bacteria or small yeast cells.
B. There is usually a range of color intensity in the organisms present.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the organism and stage (oocyst or C. cayetanensis). Do not use abbreviations.
   Examples: Cryptosporidium parvum oocysts or Isospora belli oocysts or Cyclospora cayetanensis oocysts
B. Call the physician when these organisms are identified.
VIII. PROCEDURE NOTES

A. Routine stool examination stains are not recommended; however, the sedimentation concentration is acceptable \((500 \times g \text{ for } 10 \text{ min})\) for the recovery and identification of Cryptosporidium and Cyclospora spp. Routine concentration (formalin-ethyl acetate) can be used to recover Isospora oocysts, but routine permanent stains are not reliable for this purpose.

B. Polyvinyl alcohol-preserved specimens are not acceptable for staining with the modified acid-fast stain. However, specimens preserved in SAF are perfectly acceptable.

C. Avoid the use of wet gauze filtration (an old, standardized method of filtering stool prior to centrifugation) with too many layers of gauze that may trap organisms and prevent them from flowing into the fluid to be concentrated. It is recommended that no more than two layers of gauze be used; another option is to use the commercially available concentrators that use no gauze but instead use plastic or metal screens.

D. Other organisms, such as acid-fast bacteria and some Nocardi a spp., stain positive.

E. It is very important that smears not be too thick. Thick smears may not adequately destain.

F. Concentration of the specimen is essential for demonstrating organisms \((500 \times g \text{ for } 10 \text{ min})\). The number of organisms seen in the specimens may vary from numerous to very few.

G. Because of their mucoid consistency, some specimens require treatment with 10% KOH. Add 10 drops of 10% KOH to the sediment, and vortex until homogeneous. Rinse with 10% formalin, and centrifuge \((500 \times g \text{ for } 10 \text{ min})\). Without decanting the supernatant, take 1 drop of the sediment and smear it thinly on a slide.

H. Commercial concentrators and reagents are available (see Appendix 9.10.6–1 at the end of this section).

I. Weak concentrations of sulfuric acid (1.0 to 3.0%) are normally used. Stronger concentrations will remove too much stain.

J. There is some debate about whether organisms lose their abilities to take up the acid-fast stain after long-term storage in 10% formalin. Use of the hot modified acid-fast method might eliminate this problem (1).

K. Centrifuge specimens in capped tubes, and wear gloves during all phases of specimen processing.

L. Currently, no commercial immunoassays are available for C. cayetanensis. However, several reagents are in the research phase.

IX. LIMITATIONS OF THE PROCEDURE

A. Light infections (low number of oocysts) may be missed. Immunoassay methods for C. parvum are more sensitive.

B. Multiple specimens must be examined, since the numbers of oocysts in the stool will vary from day to day. A series of three specimens submitted on alternate days is recommended.

REFERENCES


APPENDIX 9.4.1–1

Include QC information on reagent container and in QC records.

Reagents

Indicate the expiration date on the label and in the work record or on the manufacturer’s label.

A. 50% Ethanol
   1. Add 50 ml of absolute ethanol to 50 ml of distilled water.
   2. Store at room temperature. Stable for 1 year.

B. Kinyoun carbol fuchsin
   1. Dissolve 4 g of basic fuchsin in 20 ml of 95% ethanol (solution A).
   2. Dissolve 8 g of phenol crystals in 100 ml of distilled water (solution B).
   3. Mix solutions A and B together.
   4. Store at room temperature. Stable for 1 year.

C. 1% Sulfuric acid
   1. Add 1 ml of concentrated sulfuric acid to 99 ml of distilled water.
   2. Store at room temperature. Stable for 1 year.

D. Methylene blue
   1. Dissolve 0.3 g of methylene blue in 100 ml of 95% ethanol.
   2. Store at room temperature. Stable for 1 year.

APPENDIX 9.4.1–2

(Top) C. parvum oocysts; sporozoites are visible within some oocysts. (Bottom) I. belli immature oocyst; note that the entire oocyst stains with modified acid-fast stain.
9.4.2 Special Stains for Coccidia: Modified Ziehl-Neelsen Acid-Fast Stain (Hot)
[Updated March 2007]

Preanalytical Considerations

I. Principle
Cryptosporidium and Isospora species have been recognized as causes of severe diarrhea in immunocompromised hosts, but they can also cause diarrhea in immunocompetent hosts. Oocysts in clinical specimens may be difficult to detect without special staining. Cyclospora cayetanensis has also been reported to be acid fast. Modified acid-fast stains are recommended for demonstrating these organisms. Application of heat to carbol fuchsin assists in the staining, and the use of a mild decolorizer allows the organisms to retain their pink-red color (1–5).

II. Specimens
Concentrated sediment of fresh or formalin-preserved stool may be used. Other types of clinical specimens such as duodenal fluid, bile, or pulmonary (induced sputum, bronchial washings, biopsy specimens) may also be stained, after centrifugation.

Observe standard precautions.

III. Materials
A. Reagents (see Appendix 9.4.2–1)
1. Carbol fuchsin
2. 5% Sulfuric acid
3. Methylene blue
B. Supplies
1. Disposable glass or plastic pipettes
2. Glass slides (1 by 3 in., or larger if you prefer)
3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)
C. Equipment
1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
3. Tabletop centrifuge
4. Staining rack
5. 70°C heating block
6. Alcohol lamp or Bunsen burner

Analytical Considerations

IV. Quality Control
A. A control slide of Cryptosporidium parvum from a 10% formalin-preserved specimen is included with each staining batch run. If the cryptosporidia stain well, any Isospora belli oocysts present will also take up the stain, as will C. cayetanensis.
B. Cryptosporidia stain pink-red. Oocysts are 4 to 6 μm in diameter, and four sporozoites may be present internally. The background should stain uniformly blue.
C. Check the specimen (macroscopically) for adherence to the slide.
D. Record all QC results, including a description of QC specimens tested.
V. PROCEDURE

A. Smear 1 or 2 drops of specimen on the slide, and allow it to air dry. Do not make the smears too thick (you should be able to see through the wet material before it dries). Prepare two smears.

B. Dry on a heating block (70°C) for 5 min, or air dry.

C. Place slide on staining rack, and flood with carbol fuchsin.

D. With alcohol lamp or Bunsen burner, gently heat slide to steaming by passing flame under slide. Discontinue heating once the stain begins to steam. Do not boil.

E. Allow to stain 5 min. If slide dries, add more stain without additional heating.

F. Rinse thoroughly with water. Drain.

G. Decolorize with 5% sulfuric acid for 30 s. (Thicker smears may require longer to destain.)

H. Rinse with water. Drain.

I. Flood slide with methylene blue for 1 min.

J. Rinse with water, drain, and air dry.

K. Examine with low-power or high dry power objectives. To see internal morphology, use oil immersion objective (100×).

VI. RESULTS

A. With this modified acid-fast method, the oocysts of C. cayetanensis, Cryptosporidium, and Isospora will stain pink to red to deep purple. Some of the four sporozoites may be visible in the Cryptosporidium oocysts. Some of the Isospora immature oocysts (entire oocyst) will stain, while in oocysts that are mature, the two sporocysts within the oocyst wall will stain pink to purple and there will be a clear area between the stained sporocysts and the oocyst wall. The background will stain blue. If Cyclospora oocysts are present (uncommon), they tend to be approximately 8 to 10 μm, they resemble C. parvum but are larger, and they have no definite internal morphology; the acid-fast staining will tend to be more variable than that seen with Cryptosporidium or Isospora spp. Modified acid-fast stains stain the Cyclospora oocysts from light pink to deep red, and some of the oocysts will contain granules or have a bubbly appearance, often being described as looking like “wrinkled cellophane.” With the 5% acid decolorizer, some oocysts of Cyclospora may appear clear or very pale. If the patient has a heavy infection with microsporidia (immunocompromised patient), small (1- to 2-μm) spores may be seen but may not be recognized as anything other than bacteria or small yeast cells.

B. There is usually a range of color intensity in the organisms present.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the organism and stage (oocyst or C. cayetanensis). Do not use abbreviations.

Examples: Cryptosporidium parvum oocysts or Isospora belli oocysts or Cyclospora cayetanensis oocysts

B. Call the physician when these organisms are identified.

VIII. PROCEDURE NOTES

A. Routine stool examination stains are not recommended for the recovery and identification of Cryptosporidium and Cyclospora spp.; however, the sedimentation concentration is acceptable (500 × g for 10 min). Routine concentration (formalin-ethyl acetate) can be used to recover Isospora oocysts, but routine permanent stains are not reliable for this purpose.
VI. PROCEDURE NOTES
(continued)

B. Polyvinyl alcohol-preserved specimens are not acceptable for staining with the modified acid-fast stain. However, specimens preserved in sodium acetate-acetic acid-formalin are perfectly acceptable.
C. Avoid the use of wet gauze filtration (an old, standardized method of filtering stool prior to centrifugation) with too many layers of gauze that may trap organisms and prevent them from flowing into the fluid to be concentrated. It is recommended that no more than two layers of gauze be used; another option is to use the commercially available concentrators that use no gauze but instead use plastic or metal screens.
D. Other organisms, such as acid-fast bacteria and some Nocardia spp., stain positive.
E. It is very important that smears not be too thick. Thick smears may not adequately destain.
F. Concentration of specimen is essential for demonstrating organisms. The number of organisms seen in the specimen may vary from numerous to very few.
G. Because of their mucoid consistency, some specimens require treatment with 10% KOH. Add 10 drops of 10% KOH to the sediment, and vortex until homogeneous. Rinse with 10% formalin, and centrifuge (500 × g for 10 min). Without decanting the supernatant, take 1 drop of the sediment and smear it thinly on a slide.
H. Commercial concentrators and reagents are available (see Appendix 9.10.6–1 at the end of this section).
I. Do not boil stain. Gently heat until steam rises from the slide. Do not allow the stain to dry on the slide.
J. Various concentrations of sulfuric acid (0.25 to 10%) may be used, but destaining time will vary according to the concentration used. Generally, a 1 or 5% solution is used.
K. There is some debate about whether organisms lose their abilities to take up the acid-fast stain after long-term storage in 10% formalin. Use of this hot modified acid-fast method might eliminate the problem (1).
L. Centrifuge specimens in capped tubes, and wear gloves during all phases of specimen processing.
M. Currently, no commercial immunoassays are available for C. cayetanensis. However, several reagents are in the research phase.

IX. LIMITATIONS OF THE PROCEDURE

A. Light infections (low number of oocysts) may be missed. Immunoassay methods for C. parvum are more sensitive.
B. Multiple specimens must be examined, since the numbers of oocysts in the stool will vary from day to day. A series of three specimens submitted on alternate days is recommended.

REFERENCES

APPENDIX 9.4.2–1

Reagents

- Indicate the expiration date on the label and in the work record or on the manufacturer’s label.

A. Carbol fuchsin
1. Basic fuchsin (solution A)
   - Dissolve 0.3 g of basic fuchsin in 10 ml of 95% ethanol.
2. Phenol (solution B)
   - Dissolve 5 g of phenol crystals in 100 ml of distilled water. (Gentle heat may be needed.)
3. Mix solution A with solution B.
4. Store at room temperature. Stable for 1 year.

B. 5% Sulfuric acid
1. Add 5 ml of concentrated sulfuric acid to 95 ml of distilled water.
2. Store at room temperature. Stable for 1 year.

C. Methylene blue
1. Dissolve 0.3 g of methylene blue chloride in 100 ml of distilled water.
2. Store at room temperature. Stable for 1 year.

APPENDIX 9.4.2–2

(Left) C. cayetanensis oocysts (note variation in staining). (Right) The large, clear object is a C. cayetanensis oocyst (8 to 10 μm); the darker object to the right is a C. parvum oocyst (4 to 6 μm). Note that Cyclospora tends to look like wrinkled cellophane when it does not stain well.
9.4.3 Special Stains for Microsporidia: Modified Trichrome-Weber Green

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The diagnosis of intestinal microsporidiosis (Enterocytozoon bieneusi, Encephalitozoon intestinalis) has depended on the use of invasive procedures and subsequent examination of biopsy specimens, often by electron microscopy methods. However, the need for a practical method for the routine clinical laboratory has stimulated some work in the development of additional methods. Slides prepared from fresh or formalin-fixed stool specimens can be stained by a new chromotrope-based technique and can be examined with light microscopy. This staining method is based on the fact that stain penetration of the microsporidial spore is very difficult; thus, the dye content in the chromotrope 2R is higher than that routinely used to prepare Wheatley’s modification of Gomori’s trichrome method, and the staining time is much longer (90 min) (1–3). At least several of these stains are available commercially from a number of suppliers.

II. SPECIMEN

Observe standard precautions.

The specimen can be fresh stool or stool that has been preserved in 5 or 10% formalin, sodium acetate-acetic acid-formalin (SAF), or some of the newer single-vial system fixatives. Actually, any specimen other than tissue thought to contain microsporidia could be stained by these methods. Polyvinyl alcohol-preserved fecal material is not recommended.

III. MATERIALS

A. Reagents (see Appendix 9.4.3–1)

B. Supplies

1. Disposable glass or plastic pipettes
2. Glass slides (1 by 3 in., or larger if you prefer)
3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)
4. Glass or plastic centrifuge tubes (15 ml)
5. Coplin jars or other suitable staining containers
6. Chromotrope 2R
7. Aniline blue
8. Phosphotungstic acid
9. Acetic acid (glacial)
10. Distilled water

C. Equipment

1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
3. Tabletop centrifuge
IV. QUALITY CONTROL

A. Unfortunately, the only way to perform acceptable QC procedures for this method is to use actual microsporidial spores as the control organisms. Obtaining these positive controls may be somewhat difficult. It is particularly important to use the actual organisms because the spores are difficult to stain and the size is very small (1 to 2.5 μm).

B. A QC slide should be included with each run of stained slides, particularly if the staining setup is used infrequently.

C. All staining dishes should be covered to prevent evaporation of reagents (screw-cap Coplin jars or glass lids).

D. Depending on the volume of slides stained, staining solutions will have to be changed on an as-needed basis.

E. When the smear is thoroughly fixed and the stain is performed correctly, the spores will be ovoid and refractile, with the spore wall being bright pinkish red. Occasionally, the polar tube can be seen either as a stripe or as a diagonal line across the spore. The majority of the bacteria and other debris will tend to stain green. However, there will still be some bacteria and debris that will stain red.

F. The specimen is also checked for adherence to the slide (macroscopically).

G. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted on the body of the microscope). Although recalibration every 12 months may not be necessary, this will vary from laboratory to laboratory, depending on equipment care and use. Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

H. Known positive microscope slides, Kodachrome 2-by-2 projection slides, and photographs (reference books) should be available at the workstation.

I. Record all QC results; the laboratory should also have an action plan for “out-of-control” results.

V. PROCEDURE

A. Using a 10-μl aliquot of concentrated (formalin-ethyl acetate sedimentation concentration; centrifugation at 500 × g for 10 min), preserved liquid stool (5 or 10% formalin or SAF), prepare the smear by spreading the material over an area of 45 by 25 mm.

B. Allow the smear to air dry.

C. Place the smear in absolute methanol for 5 min.

D. Allow the smear to air dry.

E. Place in trichrome stain for 90 min.

F. Rinse in acid-alcohol for no more than 10 s.

G. Dip slides several times in 95% alcohol. Use this step as a rinse.

H. Place in 95% alcohol for 5 min.

I. Place in 100% alcohol for 10 min.

J. Place in xylene substitute for 10 min.

K. Mount with coverslip (no. 1 thickness), using mounting medium (this step is optional).

L. Examine smears under oil immersion (1,000×) and read at least 100 fields; the examination time will probably be at least 10 min per slide.
VI. RESULTS

A. Microsporidial spores might be seen. The spore wall should stain pinkish to red, with the interior of the spore being clear or perhaps showing a horizontal or diagonal stripe that represents the polar tube. The background will appear green (Weber stain).

B. Other bacteria, some yeast cells, and some debris will stain pink to red; the shapes and sizes of the various components may be helpful in differentiating the spores from other structures.

C. The results from this staining procedure should be reported only if the positive control smears are acceptable. The production of immunoassay reagents should provide a more specific and sensitive approach to the identification of the microsporidia in fecal specimens.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

Report the organism and stage. Do not use abbreviations.

Examples (stool specimens): Microsporidial spores present. Enterocytozoon bieneusi or Encephalitozoon (Septata) intestinalis present (if from fecal specimen); the two organisms cannot be differentiated on the basis of size or morphology.

Example (from urine): Encephalitozoon (Septata) intestinalis present (identification to the species level highly likely); generally this organism is involved in disseminated cases from the gastrointestinal tract to kidneys and will be found in urine.

VIII. PROCEDURE NOTES

A. It is mandatory that positive control smears be stained and examined each time patient specimens are stained and examined.

B. Because of the difficulty in getting stain penetration through the spore wall, prepare thin smears and do not reduce the staining time in trichrome. Also, make sure that the slides are not left too long in the decolorizing agent (acid-alcohol). If the control organisms are too light, leave them in the trichrome longer and shorten the time to two dips in the acid-alcohol solution. Also, remember that the 95% alcohol rinse after the acid-alcohol should be performed quickly to prevent additional destaining from the acid alcohol reagent.

C. When you purchase the chromotrope 2R, obtain the highest dye content available. Two sources are Harleco (Gibbstown, N.J.) and Sigma Chemical Co. (St. Louis, Mo.) (dye content among the highest [85%]). Fast green and aniline blue can be obtained from Allied Chemical and Dye (New York, N.Y.).

D. In the final stages of dehydration, the 100% ethanol and the xylenes (or xylene substitutes) should be kept as free from water as possible. Coplin jars must have tight-fitting caps to prevent both evaporation of reagents and absorption of moisture. If the xylene becomes cloudy after addition of slides from 100% alcohol, return the slides to 100% alcohol and replace the xylene with fresh stock.

IX. LIMITATIONS OF THE PROCEDURE

A. Although this staining method will stain the microsporidia, the range of stain intensity and the small size of the spores will cause some difficulty in identifying these organisms. Since this procedure will result in many other organisms or objects staining in stool specimens, differentiation of the microsporidia from surrounding material will still be very difficult. There also tends to be some slight size variation among the spores.
B. If the patient has severe watery diarrhea, there will be less artifact material in
the stool to confuse with the microsporidial spores. However, if the stool is
semiformed or formed, the amount of artifact material will be much greater;
thus, the spores will be much harder to detect and identify. Also, remember that
the number of spores will vary according to the stool consistency (the more
diarrhetic, the more spores that will be present).

C. Those who developed some of these procedures feel that concentration proce-
dures result in an actual loss of microsporidial spores; thus, there is a strong
recommendation to use unconcentrated, formalinized stool. However, there are
no data indicating what centrifugation speeds, etc., were used in the study.

D. In the UCLA Clinical Microbiology Laboratory, data (unpublished) have been
generated to indicate that centrifugation at 500 × g for 10 min increases dra-
matically the number of microsporidial spores available for staining (from the
concentrate sediment). This is the same protocol used in that laboratory for
centrifugation of all stool specimens, regardless of the suspected organism.

E. Avoid the use of wet gauze filtration (an old, standardized method of filtering
stool prior to centrifugation) with too many layers of gauze that may trap or-
ganisms and prevent them from flowing into the fluid to be concentrated. It is
recommended that no more than two layers of gauze be used. Another option
is to use the commercially available concentration systems that use metal or
plastic screens for filtration.

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tinal Tract. Approved guideline M28-A.
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croscopical detection of microsporidia spores
in stool and duodenal aspirates. N. Engl. J.

APPENDIX 9.4.3–1

Reagents

A. Trichrome stain (modified for microsporidia) (Weber green)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>chromotrope 2R</td>
<td>6.0 g*</td>
</tr>
<tr>
<td>fast green</td>
<td>0.15 g</td>
</tr>
<tr>
<td>phosphotungstic acid</td>
<td>0.7 g</td>
</tr>
<tr>
<td>acetic acid (glacial)</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>distilled water</td>
<td>100.0 ml</td>
</tr>
</tbody>
</table>

*10 times the normal trichrome stain formula

1. Prepare the stain by adding 3.0 ml of acetic acid to the dry ingredients. Allow the
mixture to stand (ripen) for 30 min at room temperature.
2. Add 100 ml of distilled water. Properly prepared stain will be dark purple.
3. Store in a glass or plastic bottle at room temperature. The shelf life is at least 24
months.

B. Acid-alcohol

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>90% ethyl alcohol</td>
<td>995.5 ml</td>
</tr>
<tr>
<td>Acetic acid (glacial)</td>
<td>4.5 ml</td>
</tr>
</tbody>
</table>

Prepare by combining the two solutions.
9.4.4 Special Stains for Microsporidia: Modified Trichrome-Ryan Blue

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE
The diagnosis of intestinal microsporidiosis (Enterocytozoon bieneusi, Encephalitozoon intestinalis) has depended on the use of invasive procedures and subsequent examination of biopsy specimens, often by electron microscopy methods. However, the need for a practical method for the routine clinical laboratory has stimulated some work in the development of additional methods. Slides prepared from fresh or formalin-fixed stool specimens can be stained by a new chromotrope-based technique and can be examined with light microscopy. This staining method is based on the fact that stain penetration of the microsporidial spore is very difficult; thus, the dye content in the chromotrope 2R is higher than that routinely used to prepare Wheatley’s modification of Gomori’s trichrome method, and the staining time is much longer (90 min) (1–3).

A number of variations to the modified trichrome (Weber green) were tried in an attempt to improve the contrast between the color of the spores and the background staining. Optimal staining was achieved by modifying the composition of the trichrome solution. This stain is also available commercially from a number of suppliers.

II. SPECIMEN
The specimen can be fresh stool or stool that has been preserved in 5 or 10% formalin, sodium acetate-acetic acid-formalin (SAF), or some of the newer single-vial system fixatives. Actually, any specimen other than tissue thought to contain microsporidia could be stained by these methods. PVA-preserved fecal material is not recommended.

Observe standard precautions.

III. MATERIALS

A. Reagents (see Appendix 9.4.4–1)

B. Supplies
1. Disposable glass or plastic pipettes
2. Glass slides (1 by 3 in., or larger if you prefer)
3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)
4. Glass or plastic centrifuge tubes (15 ml)
5. Coplin jars or other suitable staining containers
6. Chromotrope 2R
7. Aniline blue
8. Phosphotungstic acid
9. Acetic acid (glacial)
10. Distilled water

C. Equipment
1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
3. Tabletop centrifuge
IV. QUALITY CONTROL

A. Unfortunately, the only way to perform acceptable QC procedures for this method is to use actual microsporidial spores as the control organisms. Obtaining these positive controls may be somewhat difficult. It is particularly important to use the actual organisms because the spores are difficult to stain and the size is very small (1 to 2.5 \( \mu \)m).

B. A QC slide should be included with each run of stained slides, particularly if the staining setup is used infrequently.

C. All staining dishes should be covered to prevent evaporation of reagents (screw-cap Coplin jars or glass lids).

D. Depending on the volume of slides stained, staining solutions will have to be changed on an as-needed basis.

E. When the smear is thoroughly fixed and the stain is performed correctly, the spores will be ovoid and refractile, with the spore wall being bright pinkish red. Occasionally, the polar tube can be seen either as a stripe or as a diagonal line across the spore. The majority of the bacteria and other debris will tend to stain blue. However, there will still be some bacteria and debris that will stain red.

F. The specimen is also checked for adherence to the slide (macroscopically).

G. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted on the body of the microscope). Although recalibration every 12 months may not be necessary, this will vary from laboratory to laboratory, depending on equipment care and use. Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

H. Known positive microscope slides, Kodachrome 2-by-2 projection slides, and photographs (reference books) should be available at the workstation.

I. Record all QC results; the laboratory should also have an action plan for “out-of-control” results.

V. PROCEDURE

A. Using a 10-\( \mu \)l aliquot of concentrated (formalin-ethyl acetate sedimentation concentration; centrifugation at \( 500 \times g \) for 10 min), preserved liquid stool (5 or 10% formalin or SAP), prepare the smear by spreading the material over an area of 45 by 25 mm.

B. Allow the smear to air dry.

C. Place the smear in absolute methanol for 5 or 10 min.

D. Allow the smear to air dry.

E. Place in trichrome stain for 90 min.

F. Rinse in acid-alcohol for no more than 10 s.

G. Dip slides several times in 95% alcohol. Use this step as a rinse (no more than 10 s).

H. Place in 95% alcohol for 5 min.

I. Place in 95% alcohol for 5 min.

J. Place in 100% alcohol for 10 min.

K. Place in xylene substitute for 10 min.

L. Mount with coverslip (no. 1 thickness), using mounting medium (this step is optional).
V. PROCEDURE (continued)

M. Examine smears under oil immersion (1,000×) and read at least 100 fields; the examination time will probably be at least 10 min per slide.

VI. RESULTS

A. Microsporidial spores might be seen. The spore wall should stain pinkish to red, with the interior of the spore being clear or perhaps showing a horizontal or diagonal stripe that represents the polar tube. The background will appear blue (Ryan stain).

B. Other bacteria, some yeast cells, and some debris will stain pink to red; the shapes and sizes of the various components may be helpful in differentiating the spores from other structures.

C. The results from this staining procedure should be reported only if the positive control smears are acceptable. The production of immunoassay reagents should provide a more specific and sensitive approach to the identification of the microsporidia in fecal specimens.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

Report the organism and stage. Do not use abbreviations.

Examples (stool specimens): Microsporidial spores present. *Enterocytozoon bieneusi* or *Encephalitozoon (Septata) intestinalis* present (if from fecal specimen); the two organisms cannot be differentiated on the basis of size or morphology.

Example (from urine): *Encephalitozoon (Septata) intestinalis* present (identification to species highly likely); generally this organism is involved in disseminated cases from the gastrointestinal tract to kidneys and will be found in urine.

VIII. PROCEDURE NOTES

A. It is mandatory that positive control smears be stained and examined each time patient specimens are stained and examined.

B. Because of the difficulty in getting stain penetration through the spore wall, prepare thin smears and do not reduce the staining time in trichrome. Also, make sure that the slides are not left too long in the decolorizing agent (acid-alcohol). If the control organisms are too light, leave them in the trichrome longer and shorten the time to two dips in the acid-alcohol solution. Also, remember that the 95% alcohol rinse after the acid-alcohol should be performed quickly to prevent additional destaining from the acid-alcohol reagent.

C. When you purchase the chromotrope 2R, obtain the highest dye content available. Two sources are Harleco (Gibbstown, N.J.) and Sigma Chemical Co. (St. Louis, Mo.) (dye content among the highest [85%]). Fast green and aniline blue can be obtained from Allied Chemical and Dye (New York, N.Y.).

D. In the final stages of dehydration, the 100% ethanol and the xylenes (or xylene substitutes) should be kept as free from water as possible. Coplin jars must have tight-fitting caps to prevent both evaporation of reagents and absorption of moisture. If the xylene becomes cloudy after addition of slides from 100% alcohol, return the slides to 100% alcohol and replace the xylene with fresh stock.
IX. LIMITATIONS OF THE PROCEDURE

A. Although this staining method will stain the microsporidia, the range of stain intensity and the small size of the spores will cause some difficulty in identifying these organisms. Since this procedure will result in many other organisms or objects staining in stool specimens, differentiation of the microsporidia from surrounding material will still be very difficult. There also tends to be some slight size variation among the spores.

B. If the patient has severe watery diarrhea, there will be less artifact material in the stool to confuse with the microsporidial spores. However, if the stool is semiformal or formal, the amount of artifact material will be much greater; thus, the spores will be much harder to detect and identify. Also, remember that the number of spores will vary according to the stool consistency (the more diarrhetic, the more spores that will be present).

C. Those who developed some of these procedures feel that concentration procedures result in an actual loss of microsporidial spores; thus, there is a strong recommendation to use unconcentrated, formalinized stool. However, there are no data indicating what centrifugation speeds, etc., were used in the study.

D. In the UCLA Clinical Microbiology Laboratory, data (unpublished) have been generated to indicate that centrifugation at 500 × g for 10 min increases dramatically the number of microsporidial spores available for staining (from the concentrate sediment). This is the same protocol used in that laboratory for centrifugation of all stool specimens, regardless of the suspected organism.

E. Avoid the use of wet gauze filtration (an old, standardized method of filtering stool prior to centrifugation) with too many layers of gauze that may trap organisms and prevent them from flowing into the fluid to be concentrated. It is recommended that no more than two layers of gauze be used. Another option is to use the commercially available concentration systems that use metal or plastic screens for filtration.

REFERENCES


APPENDIX 9.4.4–1

Reagents

A. Trichrome stain (modified for microsporidia) (Ryan blue)

\[
\begin{align*}
\text{chromotrope } 2R & \quad 6.0 \text{ g}^* \\
\text{aniline blue} & \quad 0.5 \text{ g} \\
\text{phosphotungstic acid} & \quad 0.25 \text{ g} \\
\text{acetic acid (glacial)} & \quad 3.0 \text{ ml} \\
\text{distilled water} & \quad 100.0 \text{ ml}
\end{align*}
\]

^10 times the normal trichrome stain formula

1. Prepare the stain by adding 3.0 ml of acetic acid to the dry ingredients. Allow the mixture to stand (ripen) for 30 min at room temperature.

2. Add 100 ml of distilled water and adjust the pH to 2.5 with 1.0 M HCl. Properly prepared stain will be dark purple. The staining solution should be protected from light.

3. Store in a glass or plastic bottle at room temperature. The shelf life is at least 24 months.
APPENDIX 9.4.4–1 (continued)

B. Acid-alcohol

90% ethyl alcohol .................995.5 ml
acetic acid (glacial) .................4.5 ml

Prepare by combining the two solutions.

APPENDIX 9.4.4–2

Modified Trichrome Stain for the Microsporidia (1) (Kokoskin Hot Method)

Changes in temperature from room temperature to 50°C and the staining time from 90 to 10 min have been recommended as improvements for the modified trichrome staining methods. The procedure is as follows.

1. Using a 10-μl aliquot of unconcentrated, preserved liquid stool (5 or 10% formalin or SAF), prepare the smear by spreading the material over an area of 45 by 25 mm.
2. Allow the smear to air dry.
3. Place the smear in absolute methanol for 5 min.
4. Allow the smear to air dry.
5. Place in trichrome stain for 10 min at a temperature of 50°C.
6. Rinse in acid-alcohol for no more than 10 s.
7. Dip slides several times in 95% alcohol. Use this step as a rinse (no more than 10 s).
8. Place in 95% alcohol for 5 min.
9. Place in 100% alcohol for 10 min.
10. Place in xylene substitute for 10 min.
11. Mount with coverslip (no. 1 thickness), using mounting medium.
12. Examine smears under oil immersion (1,000×) and read at least 100 fields; the examination time will probably be at least 10 min per slide.

Reference

9.4.5 Special Stains for Microsporidia: Acid-Fast Modified Trichrome Stain for Coccidia and the Microsporidia

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE
The detection of coccidia and the microsporidia from stool specimens has depended on two separate stains. However, a method is now available that will stain both organisms, an important improvement since dual infections have been demonstrated in AIDS patients. This acid-fast modified trichrome stain yields results comparable to those obtained by the Kinyou and modified trichrome methods and considerably reduces the time necessary for microscopic examination. Also, it appears that modified trichrome stains and staining with fluorochromes are equally useful in the diagnosis of microsporidiosis; however, a combination of both methods may be more sensitive in cases where the number of spores is very low (1, 2).

II. SPECIMEN
Observe standard precautions.

The specimen can be fresh stool or stool that has been preserved in 5 or 10% formalin, SAF, or some of the newer single-vial system fixatives. Actually, any specimen other than tissue thought to contain microsporidia could be stained by these methods. PVA-preserved fecal material is not recommended.

III. MATERIALS

A. Reagents (see Appendix 9.4.5–1)
B. Supplies
   1. Disposable glass or plastic pipettes
   2. Glass slides (1 by 3 in., or larger if you prefer)
   3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)
   4. Glass or plastic centrifuge tubes (15 ml)
   5. Coplin jars or other suitable staining containers
   6. Chromotrope 2R
   7. Aniline blue
   8. Phosphotungstic acid
   9. Carbol fuchsin
   10. Phenol
   11. Acetic acid (glacial)
   12. Distilled water
C. Equipment
   1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
   2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult
   3. Tabletop centrifuge

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL
A. Unfortunately, the only way to perform acceptable QC procedures for this method is to use actual microsporidial spores and coccidian oocysts as the control organisms. Obtaining these positive controls may be somewhat difficult. It is particularly important to use the actual organisms because the spores are difficult to stain and the size is very small (1 to 1.5 μm).
IV. QUALITY CONTROL
(continued)

B. A QC slide should be included with each run of stained slides, particularly if the staining setup is used infrequently.
C. All staining dishes should be covered to prevent evaporation of reagents (screw-cap Coplin jars or glass lids).
D. Depending on the volume of slides stained, staining solutions will have to be changed on an as-needed basis.
E. When the smear is thoroughly fixed and the stain is performed correctly, the spores will be ovoid and refractile, with the spore wall being bright pinkish red. Occasionally, the polar tube can be seen either as a stripe or as a diagonal line across the spore. The majority of the bacteria and other debris will tend to stain blue. However, there will still be some bacteria and debris that will stain red. The coccidia (Cryptosporidium, Cyclospora, Isospora) will stain as with any modified acid-fast stain: from pink to violet.
F. The specimen is also checked for adherence to the slide (macroscopically).
G. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted on the body of the microscope). Although recalibration every 12 months may not be necessary, this will vary from laboratory to laboratory, depending on equipment care and use. Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.
H. Known positive microscope slides, Kodachrome 2-by-2 projection slides, and photographs (reference books) should be available at the workstation.
I. Record all QC results; the laboratory should also have an action plan for “out-of-control” results.

V. PROCEDURE

A. Using a 10-μl aliquot of concentrated (formalin-ethyl acetate sedimentation concentration; centrifugation at 500 × g for 10 min), preserved liquid stool (5 or 10% formalin or SAF), prepare the smear by spreading the material over an area of 45 by 25 mm.
B. Allow the smear to air dry.
C. Place the smear in absolute methanol for 5 or 10 min.
D. Allow the smear to air dry.
E. Place in carbol fuchsin solution for 10 min (no heat required).
F. Briefly rinse with tap water.
G. Decolorize with 0.5% acid-alcohol.
H. Briefly rinse with tap water.
I. Place in modified trichrome stain for 30 min at 37°C.
J. Rinse in acid-alcohol for no more than 10 s.
K. Dip slides several times in 95% alcohol. Use this step as a rinse (no more than 10 s).
L. Place in 95% alcohol for 30 s.
M. Allow slides to air dry.
N. Examine smears under oil immersion (1,000×) and read at least 100 fields; the examination time will probably be at least 10 min per slide.
VI. RESULTS

A. Microsporidial spores might be seen. The spore wall should stain pink, with the interior of the spore being clear or perhaps showing a horizontal or diagonal stripe that represents the polar tube. A vacuole may also be visible in some spores. The Cryptosporidium oocysts will stain bright pink or violet. The background will appear blue.

B. Other bacteria, some yeast cells, and some debris will stain pink to red; the shapes and sizes of the various components may be helpful in differentiating the spores from other structures.

C. The results from this staining procedure should be reported only if the positive control smears are acceptable. The production of immunoassay reagents should provide a more specific and sensitive approach to the identification of the microsporidia in fecal specimens.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

Report the organism and stage. Do not use abbreviations.

Examples (stool specimens):
1. Microsporidial spores present. Enterocytozoon bieneusi or Encephalitozoon (Septata) intestinalis present (if from fecal specimen); the two organisms cannot be differentiated on the basis of size or morphology.
2. Cryptosporidium species seen.

Example (from urine): Encephalitozoon (Septata) intestinalis present (identification to species level highly likely); generally this organism is involved in disseminated cases from the gastrointestinal tract to kidneys and will be found in urine.

VIII. PROCEDURE NOTES

A. It is mandatory that positive control smears be stained and examined each time patient specimens are stained and examined.

B. Because of the difficulty in getting stain penetration through the spore wall, prepare thin smears and do not reduce the staining time in trichrome. Also, make sure that the slides are not left too long in the decolorizing agent (acid-alcohol). If the control organisms are too light, leave them in the trichrome longer and shorten the time to two dips in the acid-alcohol solution. Also, remember that the 95% alcohol rinse after the acid-alcohol should be performed quickly to prevent additional destaining from the acid-alcohol reagent.

C. When you purchase the chromotrope 2R, obtain the highest dye content available. Two sources are Harleco (Gibbstown, N.J.) and Sigma Chemical Co. (St. Louis, Mo.) (dye content among the highest [85%]). Fast green and aniline blue can be obtained from Allied Chemical and Dye (New York, N.Y.).

D. In the final stages of dehydration, the 100% ethanol and the xylenes (or xylene substitutes) should be kept as free from water as possible. Coplin jars must have tight-fitting caps to prevent both evaporation of reagents and absorption of moisture. If the xylene becomes cloudy after addition of slides from 100% alcohol, return the slides to 100% alcohol and replace the xylene with fresh stock.

IX. LIMITATIONS OF THE PROCEDURE

A. Although this staining method will stain the microsporidia, the range of stain intensity and the small size of the spores will cause some difficulty in identifying these organisms. Since this procedure will result in many other organisms or objects staining in stool specimens, differentiation of the microsporidia from
surrounding material will still be very difficult. There also tends to be some slight size variation among the spores.

**B.** If the patient has severe watery diarrhea, there will be less artifact material in the stool to confuse with the microsporidial spores. However, if the stool is semiformed or formed, the amount of artifact material will be much greater; thus, the spores will be much harder to detect and identify. Also, remember that the number of spores will vary according to the stool consistency (the more diarrhetic, the more spores that will be present).

**C.** Those who developed some of these procedures feel that concentration procedures result in an actual loss of microsporidial spores; thus, there is a strong recommendation to use unconcentrated, formalinized stool. However, there are no data indicating what centrifugation speeds, etc., were used in the study.

**D.** In the UCLA Clinical Microbiology Laboratory, data (unpublished) have been generated to indicate that centrifugation at $500 \times g$ for 10 min increases dramatically the number of microsporidial spores available for staining (from the concentrate sediment). This is the same protocol used in that laboratory for centrifugation of all stool specimens, regardless of the suspected organism.

**E.** Avoid the use of wet gauze filtration (an old, standardized method of filtering stool prior to centrifugation) with too many layers of gauze that may trap organisms and allow them to flow into the fluid to be concentrated. It is recommended that no more than two layers of gauze be used. Another option is to use the commercially available concentration systems that use metal or plastic screens for filtration.

**REFERENCES**


**SUPPLEMENTAL READING**

APPENDIX 9.4.5–1

Include QC information on reagent container and in QC records.

**Reagents**

**A. Trichrome stain (modified for microsporidia)**

- chromotrope 2R ................................6.0 g*
- aniline blue ...................................0.5 g
- phosphotungstic acid ........................0.7 g
- acetic acid (glacial) ..........................3.0 ml
- distilled water ...............................100.0 ml

*10 times the normal trichrome stain formula

1. Prepare the stain by adding 3.0 ml of acetic acid to the dry ingredients. Allow the mixture to stand (ripen) for 30 min at room temperature.
2. Add 100 ml of distilled water and adjust the pH to 2.5 with 1.0 M HCl. Properly prepared stain will be dark purple. The staining solution should be protected from light.
3. Store in a glass or plastic bottle at room temperature. The shelf life is at least 24 months.

**B. Carbol fuchsin solution**

- Phenol solution
  - phenol ........................................ 25.0 g
  - distilled water ..............................500.0 ml

- Saturated alcoholic fuchsin solution
  - basic fuchsin ................................2.0 g
  - 96% ethanol ................................. 25.0 ml

Add the mixture of phenol and water to 25.0 ml of the saturated alcoholic fuchsin solution.

**C. Acid-alcohol**

- 90% ethyl alcohol .............................995.5 ml
- acetic acid (glacial) ..........................4.5 ml

Prepare by combining the two solutions.
Special Stain for *Cyclospora* Oocysts: Modified Safranin Technique with Microwave Heating

*Procedure added March 2007*

**PREANALYTICAL CONSIDERATIONS**

**I. PRINCIPLE**
*Cyclospora* is an emerging parasite causing intestinal disease both in immunocompetent persons with or without travel history and in immunocompromised patients. Oocysts stain modified acid-fast variable, and the variability may lead to misidentification of this parasite. An alternative to the modified acid-fast stain is the modified safranin stain with microwave heating, which may be easier to interpret and perform. This safranin-based stain uniformly stains the *Cyclospora* oocysts, unlike the variable staining with the modified acid-fast stain.

**II. SPECIMEN**
A. Concentrated sediment of fresh, 5 or 10% formalin or sodium acetate-acetic acid-formalin preserved stool may be used.
B. Polyvinyl alcohol-preserved fecal material is not recommended.
C. Specimens in potassium dichromate are not acceptable.

**III. MATERIALS**
A. Reagents
1. 1% Safranin
2. 1% Methylene blue
3. Cytoseal 60
B. Supplies
1. Disposable glass or plastic pipettes
2. Glass microscope slides, 1 by 3 in.
3. Coverslips, 22 by 22 mm, no. 1
4. Staining jars
C. Equipment
1. Binocular microscope with 10×, 40×, and 100× objectives. Occlus should be 10×.
2. 60°C slide warmer
3. Microwave oven

**ANALYTICAL CONSIDERATIONS**

**IV. QUALITY CONTROL**
A. The only way to perform acceptable QC procedures for this method is to use actual *Cyclospora* oocysts as the control organism. Obtaining these positive controls may be somewhat difficult.
B. A QC slide should be run with each batch of stained slides, particularly if the stain is used infrequently.
C. Depending on the volume of slides stained, staining solutions will have to be changed on an as-needed basis or every 10 uses.
D. When the smear is thoroughly fixed and the stain is performed correctly, the oocysts will appear uniformly reddish orange.
V. PROCEDURE

A. Using a 10-μL aliquot of concentrated stool, prepare the smear by spreading the material thinly across the slide.
B. Allow the smear to dry on a 60°C slide warmer.
C. Cool slide to room temperature before staining.
D. Place the slide in a Coplin jar containing acidic alcohol (3% [vol/vol] HCl in methanol) and let stand for 5 min.
E. Wash off excess acidic alcohol with cold tap water.
F. Place the slide(s) into the Coplin jar containing the safranin solution in acidified water (pH 6.5) and microwave on full power (650 W) for 1 min. (Place staining jar in another container to catch overflow of stain because of boiling.)
G. Wash off excess stain with cold tap water.
H. Place the slide(s) in a Coplin jar containing 1% methylene blue for 1 min.
I. Rinse gently with cold tap water.
J. Air dry.
K. Coverslip the slide using Cytoseal 60.
L. Examine the smear with low-power or high dry power objectives. To see additional morphology, use the oil immersion objective (100×).

VI. RESULTS

Cyclospora oocysts will stain reddish-orange. The crinkled or wrinkled appearance of the oocyst wall will be evident. The oocysts will measure 8 to 10 μm.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the organism and the stage. Do not use abbreviations.
   Example: Cyclospora cayetanensis oocysts
B. Notify the physician when this organism is identified.

VIII. PROCEDURE NOTES

A. It is mandatory that positive control smears be stained and examined each time patient specimens are stained and examined.
B. For best results, safranin stain should be changed after 10 microwave heatings.
C. The Cyclospora oocyst wall in the safranin-stained smears will exhibit weak to moderate autofluorescence.
D. The Cyclospora oocyst wall may be seen more clearly using Nomarski differential interference contrast microscopy.

SUPPLEMENTAL READING

9.5.1 “Culture” of Larval-Stage Nematodes: Baermann Technique

*Strongyloides stercoralis* larvae are usually the only larvae found in stool specimens. Depending on bowel transit time and the condition of the patient, rhabditiform and, rarely, filariform larvae may be present. If there is delay in examination of the stool, then embryonated ova and larvae of hookworm may be present. Culture of feces for larvae is useful to (i) reveal the presence of larvae when they are too scanty to be detected by concentration methods, (ii) distinguish whether the infection is due to *Strongyloides* or hookworm on the basis of rhabditiform larval morphology by allowing hookworm eggs to hatch and release first-stage larvae, and (iii) allow development of larvae into the filariform stage for further differentiation. Additionally, such techniques are useful for obtaining a large number of infective-stage larvae for research purposes. Three culture techniques and one enhanced-recovery method are described in this section.

Occasionally, it is necessary to examine stool specimens for scolices and proglottids of cestodes and for adult nematodes and trematodes to confirm the diagnosis and/or to identify a species. A method for the recovery of these stages is also described in procedure 9.5.6.

### PREANALYTICAL CONSIDERATIONS

#### I. PRINCIPLE

The Baermann technique uses a special apparatus and relies on the principle that active larvae will migrate out of a fecal specimen that has been placed on a wire mesh covered with several layers of gauze (1, 2). Larvae migrate through the gauze into the water and settle to the bottom of the funnel, where they can be collected and examined. Modifications to simplify the procedure have been reported elsewhere (5). Besides being useful for diagnosis from stool specimens directly or after enhancement by culture, this technique can be used by epidemiologists to examine soil specimens for larvae.

#### II. SPECIMEN

The specimen must be fresh stool that has not been refrigerated.

#### III. MATERIALS

| Include QC information on reagent container and in QC records. |

- **A. Reagent**
  - Indicate the expiration date on the label and in the work record or on the manufacturer’s label.
  - Bleach (full strength)
- **B. Supplies**
  1. Glass funnel (6 in. across at the mouth)
  2. Rubber tubing to fit end of funnel
  3. Clamp
  4. Wire gauze screen or nylon filter
  5. Centrifuge tubes (15-ml capacity)
  6. Glass slides (1 by 3 in. or larger)
  7. Disposable glass or plastic pipettes
  8. Wooden applicator sticks (non-sterile)
  9. Gauze
  10. Ring stand and ring for holding funnel
  11. Glass beaker (500 ml)
- **C. Equipment**
  1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
  2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
  3. Centrifuge, tabletop
ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL
A. To ensure reliable results, follow routine procedures for optimal collection and handling of specimens for parasitologic examination.
B. If available, examine known positive and negative samples of stools (from laboratory animals) to make sure that the procedure is precise.
C. Review larval diagrams (any parasitology text) for confirmation of larval identification.
D. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (see procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.
E. Record all QC results.

V. PROCEDURE
A. Wear gloves when performing this procedure.
B. If possible, use a fresh fecal specimen that has been obtained after administration of a mild saline cathartic, not a stool softener. Soft stool is recommended, but any fresh fecal specimen is acceptable.
C. Set up a clamp supporting a 6-in.-mouth glass funnel. Attach rubber tubing and a pinch clamp to the bottom of the funnel. Place a collection beaker underneath (see Fig. 9.5.1–1).
D. Place wire gauze or a nylon filter over the top of the funnel (or resting within the funnel), and then place a pad consisting of two layers of gauze over that.
E. Close the pinch clamp at the bottom of the tubing, and fill the funnel with tap water until it just soaks the gauze padding.
F. Spread a large amount of fecal material on the gauze padding so the specimen is in contact with water. If the fecal material is very firm, emulsify in water.
G. Allow the apparatus to stand for 2 or more hours, draw off 10 ml of fluid into the beaker by releasing the pinch clamp, centrifuge for 2 min at 500 × g, and examine the sediment under the microscope (100× and 400×) for the presence of motile larvae. Make sure that the end of the tubing is well inside the beaker before slowly releasing the pinch clamp. Infective larvae may be present; wear gloves when performing this procedure.
H. If there are no larvae seen, allow the apparatus to stand at room temperature for 8 to 12 h and examine additional fluid (after centrifugation). Discard after 12 h.

VI. RESULTS
A. Larval nematodes (hookworm, Strongyloides spp., or Trichostrongylus spp.) may be recovered.
B. Both infective and noninfective Strongyloides larvae may be recovered, particularly in a heavy infection.
Larval-Stage Nematodes: Baermann Technique

9.5.1.3

POSTANALYTICAL CONSIDERATIONS

A. Report your findings as “No larvae detected” if no larvae could be detected at the end of incubation.

B. Report larvae detected by fecal culture.

Example: *Strongyloides stercoralis* larvae detected by fecal culture.

VIII. PROCEDURE NOTES

A. It is often difficult to observe details in rapidly moving larvae. If desired, use slight heating or a drop of iodine or formalin to kill the larvae.

B. Preserved fecal specimens or specimens obtained after a barium meal are not suitable for processing by this method.

C. *Wear gloves when you perform this procedure.*

D. When you release the pinch clamp, do it slowly to prevent splashing.

E. For the same reason, hold the end of the tubing toward the bottom of the beaker.

F. Infective larvae may be found any time after the fourth day and occasionally after the first day in heavy infections. *Caution must be exercised in handling the fluid, gauze pad, and beaker to prevent accidental infection. Always remember to wear gloves.*
IX. LIMITATIONS OF THE PROCEDURE

A. This technique allows both parasitic and free-living forms of nematodes to develop. If specimens have been contaminated with soil or water containing these forms, it may be necessary to distinguish parasitic from free-living forms. This distinction is possible, since parasitic forms are more resistant to slight acidity than are free-living forms. Proceed as follows (2–4). Add 0.3 ml of concentrated hydrochloric acid per 10 ml of water containing the larvae (adjust the volume to achieve a 1:30 dilution of acid). Free-living nematodes are killed, while parasitic species live for about 24 h.

B. Specimens that have been refrigerated are not suitable for culture. Larvae of certain species are susceptible to cold.

REFERENCES


SUPPLEMENTAL READING


“Culture” of Larval-Stage Nematodes: Harada-Mori Technique

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE
This filter paper test tube culture technique was initially introduced by Harada and Mori in 1955 (2) and was later modified by others (3, 5). The technique employs a filter paper to which fecal material is added and a test tube into which the filter paper is inserted. Moisture is provided by adding water to the tube. The water continuously soaks the filter paper by capillary action. Incubation under suitable conditions favors hatching of ova and/or development of larvae. Fecal specimens to be cultured should not be refrigerated, since some parasites (especially Necator americanus) are susceptible to cold and may fail to develop after refrigeration.

The specimen must be fresh stool that has not been refrigerated.

II. SPECIMEN
Observe standard precautions.

III. MATERIALS

A. Reagents
None
B. Supplies
1. Disposable glass or plastic pipettes
2. Glass slides (1 by 3 in. or larger)
3. Coverslips (22 by 22 mm; no. 1 or larger)
4. Filter paper (40 or 42 is fine; weight is not critical)
5. Wooden applicator sticks (nonsterile)
6. 15-ml-capacity conical tube
7. Forceps

C. Equipment
1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. To ensure reliable results, follow routine procedures for optimal collection and handling of specimens for parasitologic examination.
B. If available, examine known positive and negative samples of stools (from laboratory animals) to make sure that the procedure is precise.
C. Review larval diagrams (any parasitology text) for confirmation of larval identification.
D. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (see procedures 9.1 and 9.3.2). Although there is not universal agreement, the
IV. QUALITY CONTROL (continued)

The microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

E. Record all QC results.

V. PROCEDURE

A. Wear gloves when performing this procedure.
B. Cut a narrow (3/8 by 5 in.) strip of filter paper, and taper it slightly at one end. Smear 0.5 to 1 g of feces in the center of the strip.
C. Add 3 to 4 ml of distilled water to a 15-ml conical centrifuge tube.
D. Insert the filter paper strip into the tube so that the tapered end is near the bottom of the tube. The water level should be slightly (0.5 in.) below the fecal spot. It is not necessary to cap the tube. However, a cork stopper or a cotton plug may be used (see Fig. 9.5.2–1).
E. Allow the tube to stand upright in a rack at 25 to 28°C. Add distilled water to maintain the original level (usually evaporation takes place over the first 2 days, but then the culture becomes stabilized).
F. Keep the tube for 10 days, and check daily by withdrawing a small amount of fluid from the bottom of the tube. Prepare a smear on a glass slide, cover with a coverslip, and examine with the 10× objective.
G. Examine the larvae for motility and typical morphological features to reveal whether hookworm, Strongyloides, or Trichostrongylus larvae are present.

VI. RESULTS

A. Larval nematodes (hookworm, Strongyloides spp., or Trichostrongylus spp.) may be recovered.
B. If Strongyloides organisms are present, free-living stages and larvae may be found after several days in culture.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report your findings as “No larvae detected” if no larvae could be detected at the end of incubation.
B. Report larvae detected by fecal culture.
   Example: Strongyloides stercoralis larvae detected by fecal culture.

VIII. PROCEDURE NOTES

A. It is often difficult to observe details in rapidly moving larvae. If desired, use slight heating or a drop of iodine or formalin to kill the larvae.
B. Infective larvae may be found anytime after day 4 or even on day 1 in a heavy infection. Since infective larvae may migrate upward as well as downward on the filter paper strip, be careful when handling the fluid and the paper strip itself to prevent infection. Handle the filter paper with forceps.
C. It is important to maintain the original water level to keep optimum humidity.
D. Preserved fecal specimens or specimens obtained after a barium meal are not suitable for processing by this method.
E. Wear gloves when you perform this procedure.
IX. LIMITATIONS OF THE PROCEDURE

A. This technique allows both parasitic and free-living forms of nematodes to develop. If specimens have been contaminated with soil or water containing these forms, it may be necessary to distinguish parasitic from free-living forms. This distinction is possible since parasitic forms are more resistant to slight acidity than are free-living forms. Proceed as follows (4, 6). Add 0.3 ml of concentrated hydrochloric acid per 10 ml of water containing the larvae (adjust the volume to achieve a 1:30 dilution of acid). Free-living nematodes are killed, while parasitic species live for about 24 h.

B. Specimens that have been refrigerated are not suitable for culture. Larvae of certain species are susceptible to cold.

C. This method requires too much time to be clinically useful, but it can be used for field or survey studies where rapid results are not that important. Due to the time factor, this method, as well as the petri dish-filter paper slant method (procedure 9.5.3), may be replaced by the agar plate method (procedure 9.5.4), which is not only more rapid but also more sensitive.

REFERENCES


SUPPLEMENTAL READING


9.5.3 “Culture” of Larval-Stage Nematodes: Petri Dish-Filter Paper Slant

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE
This alternative culture technique for recovery of nematode larvae was originally described by Little (1, 2). As in previously described techniques (procedure 9.5.2), sufficient moisture is provided by continuous soaking of filter paper in water. Stool material is placed on filter paper that has been cut to fit the dimensions of a standard (1 by 3 in.) microscope slide. The filter paper is then placed on a slanted glass slide in a glass or plastic petri dish containing water. This technique has the added advantage of allowing the microbiologist to look for nematode larvae and free-living stages of *Strongyloides stercoralis* in the fecal mass or the surrounding water by direct examination of the preparation with a dissecting microscope without having to sample the preparation.

II. SPECIMEN

The specimen must be fresh stool that has not been refrigerated.

Observe standard precautions.

III. MATERIALS

A. Reagents
None

B. Supplies
1. Disposable glass or plastic pipettes
2. Glass slides (1 by 3 in., or larger if you prefer)
3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)
4. Filter paper (40 or 42 is fine; weight is not critical)
5. Wooden applicator sticks (nonsterile)
6. Glass or plastic petri dish (100 by 15 mm)
7. Piece of glass rod or glass tubing cut to fit a petri dish (100 by 15 mm)

C. Equipment
1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
3. Dissecting microscope with at least two different magnifications

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. To ensure reliable results, follow routine procedures for optimal collection and handling of specimens for parasitologic examination.

B. If available, examine known positive and negative samples of stools (from laboratory animals) to make sure that the procedure is precise.
IV. QUALITY CONTROL (continued)

C. Review larval diagrams (any parasitology text) for confirmation of larval identification.

D. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (see procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

E. Record all QC results.

V. PROCEDURE

A. Wear gloves when performing this procedure.

B. Cut a filter paper strip (1 by 3 in.), and smear a film of 1 to 2 g of fecal material in the center of the strip.

C. Place the strip on a glass slide (1 by 3 in.). Place the slide at an incline (about 10°) at one end of the petri dish by resting the slide on a piece of glass rod or glass tubing.

D. Add water to the petri dish so that the bottom one-fourth of the slide is immersed in water. Cover the dish, and keep at 25 to 28°C. As needed, add water to maintain the original level (see Fig. 9.5.3–1).

E. Keep the dish for 10 days. Examine daily either by using the dissecting microscope or by withdrawing a small amount of fluid and placing it on a microscope slide. Add a coverslip, and examine microscopically with the 10× and 40× objectives.

F. Examine any larvae recovered for typical morphological features.

VI. RESULTS

A. Larval nematodes (hookworm, *Strongyloides* spp., or *Trichostrongylus* spp.)

B. If *Strongyloides* organisms are present, free-living stages and larvae may be found after several days of culture.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report your findings as “No larvae detected” if no larvae could be detected at the end of incubation.

B. Report larvae detected by fecal culture.

   Example: *Strongyloides stercoralis* larvae detected by fecal culture.

Figure 9.5.3–1 Diagram of petri dish-filter paper slant (from reference 1).
VIII. PROCEDURE NOTES

A. It is often difficult to observe details in rapidly moving larvae. If desired, use slight heating or a drop of iodine or formalin to kill the larvae.
B. Infective larvae may be found anytime after day 4. Since infective larvae may migrate upward as well as downward on the filter paper strip, be careful when handling the fluid and the paper strip itself to prevent infection.
C. Wear gloves when handling the cultures.
D. It is important to maintain the original water level to keep optimum humidity.
E. Preserved fecal specimens or specimens obtained after a barium meal are not suitable for processing by this method.

IX. LIMITATIONS OF THE PROCEDURE

A. This technique allows both parasitic and free-living forms of nematodes to develop. If specimens have been contaminated with soil or water containing these forms, it may be necessary to distinguish parasitic from free-living forms. The method for doing so depends on the fact that parasitic forms are more resistant to slight acidity than are free-living forms. Proceed as follows (3, 4). Add 0.3 ml of concentrated hydrochloric acid per 10 ml of water containing the larvae (adjust the volume to achieve a 1:30 dilution of acid). Free-living nematodes are killed, while parasitic species live for about 24 h.
B. Specimens that have been refrigerated are not suitable for culture. Larvae of certain species (Necator americanus) are susceptible to cold.
C. This method requires too much time to be clinically useful, but can be used for field or survey studies where rapid results are not that important. Due to the time factor, this method, as well as the Hara-Mori filter paper slant method (procedure 9.5.2), may be replaced by the agar plate method (procedure 9.5.4), which is not only more rapid but also more sensitive.

REFERENCES


SUPPLEMENTAL READING

“Culture” of Larval-Stage Nematodes: Agar Plate Culture for *Strongyloides stercoralis*

### PREANALYTICAL CONSIDERATIONS

I. **PRINCIPLE**

Agar plate cultures are recommended for the recovery of *Strongyloides stercoralis* larvae and tend to be more sensitive than some of the other diagnostic methods (1, 3, 4, 9). Stool is placed onto agar plates, and the plates are sealed to prevent accidental infections and held for 2 days at room temperature. As the larvae crawl over the agar, they carry bacteria with them, thus creating visible tracks over the agar. The plates are examined under the microscope for confirmation of larvae, the surface of the agar is then washed with 10% formalin, and final confirmation of larval identification is made via wet examination of the sediment from the formalin washings (Fig. 9.5.4–1).

In a study looking at the prevalence of *S. stercoralis* in three areas of Brazil, the diagnostic efficacy of the agar plate culture method (included in this procedure) was as high as 93.9%, compared to only 28.5 and 26.5% for the Harada-Mori filter paper culture and fecal concentration methods, when fecal specimens were processed using all three methods (8). Among the 49 positive samples, about 60% were confirmed as positive using only the agar plate method. These results indicate that the agar plate approach is probably a much more sensitive diagnostic method, and it is recommended for the diagnosis of strongyloidiasis.

It is important to remember that more than half of *S. stercoralis*-infected individuals tend to have low-level infections (10). The agar plate method continues to be documented as a more sensitive method than the usual direct smear or fecal concentration methods (6, 7). Daily search for furrows on agar plates for up to six consecutive days results in increased sensitivity for diagnosis of both *S. stercoralis* and hookworm infections. Also, a careful search for *S. stercoralis* should be made in all patients with comparable clinical findings before deciding on a diagnosis of idiopathic eosinophilic colitis, because consequent steroid treatment may have a fatal outcome by inducing widespread dissemination of the parasite (2).

Human T-cell leukemia virus 1 (HTLV-1) infection is endemic in a number of Latin American countries. HTLV-1-associated myelopathy/tropical spastic paraparesis and adult T-cell leukemia lymphoma are emerging diseases in the region. *S. stercoralis* hyperinfection syndrome and therapeutic failure in apparently healthy patients with nondisseminated strongyloidiasis may be markers of HTLV-1 infection (5).

II. **SPECIMEN**

Observe standard precautions.

III. **MATERIALS**

A. **Reagents** (see Appendix 9.5.4–1)

B. **Supplies**

1. Disposable glass or plastic pipettes
2. Glass slides (1 by 3 in., or larger if you prefer)
3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)
4. Plastic petri dish (100 by 15 mm)
5. Wooden applicator sticks (nonsterile)
6. Glass or plastic centrifuge tube (15 ml)
7. Plastic bags (large enough to hold several petri plates)
8. Metal forceps (no shorter than 3 to 4 in.)
9. Cellulose tape

C. **Equipment**

1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
3. Dissecting microscope with at least two different magnifications
Figure 9.5.4–1 Agar culture method for *S. stercoralis*. (1) Agar plates are prepared; (2) agar is dried for 4 to 5 days on the bench top; (3) plates are stored in plastic bags; (4) fresh stool is submitted to the laboratory; (5) approximately 2 g of stool is placed onto an agar plate; (6) the plate is sealed with tape; (7) the culture plate is incubated at 26 to 33°C for 2 days; (8) the plate is examined microscopically for the presence of tracks (bacteria carried over agar by migrating larvae); (9) 10% formalin is placed onto agar through a hole made in the plastic with hot forceps; and (10) material from the agar plate is centrifuged and (11) examined as a wet preparation for rhabditiform or filariform larvae (high dry power; magnification, ×400). (Illustration by Sharon Belkin; from reference 3).

**ANALYTICAL CONSIDERATIONS**

IV. QUALITY CONTROL

A. To ensure reliable results, follow routine procedures for optimal collection and handling of specimens for parasitologic examination.

B. Examine agar plates to ensure that there is no cracking and the agar pour is sufficient to prevent drying. Also, make sure there is no excess water on the surfaces of the plates.

C. Review larval diagrams and descriptions (any parasitology text) for confirmation of larval identification.

D. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted on the body of the microscope). Although there is not universal agreement, the microscope should
IV. QUALITY CONTROL (continued)

probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

E. Record all QC results (condition of agar plates).

V. PROCEDURE

A. Place approximately 2 g of fresh stool in the center of the agar plate (area of approximately 1 in. in diameter).
B. Replace the lid and seal the plate with cellulose tape or shrink seal.
C. Maintain the agar plate (right side up) at room temperature for 2 days.
D. After 2 days, examine the sealed plates through the plastic lid under the microscope for microscopic colonies that develop as random tracks on the agar and evidence of larvae at the ends of the tracks away from the stool.

**NOTE:** It has been documented that daily search for furrows on agar plates for up to six consecutive days results in increased sensitivity for diagnosis of both *S. stercoralis* and hookworm infections (7). When trying to rule out strongyloidiasis in immunocompromised patients or in those who may receive immunosuppressives, it is recommended that two plates be set up, one that can be examined after 2 days and one that can be examined after the full 6 days.

E. With the end of hot forceps (heat the end of the forceps until able to melt plastic), make a hole in the top of the plastic petri dish.
F. Gently add 10 ml of 10% formalin through the hole onto the agar surface, swirl to cover the surface, and rinse the agar plate. Allow to stand for 30 min.
G. Remove the tape and lid of the agar plate. Pour the 10% formalin through a funnel into a centrifuge tube. Do not try and pour the formalin off directly into the centrifuge tube; the size of the tube opening is too small, and formalin will be spilled onto the counter.

H. Centrifuge formalin rinse fluid for 5 min at 500 \( \times g \).
I. Prepare wet-smear preparation from sediment, and examine using the 10\( \times \) objective (low power) for the presence of larvae. If larvae are found, confirm identification using the 40\( \times \) objective (high dry power).

VI. RESULTS

A. Larval nematodes (hookworm, *Strongyloides* spp., or *Trichostrongylus* spp.)
B. If *Strongyloides* organisms are present, free-living stages and larvae may be found after several days on the agar plates.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report your findings as “No larvae detected” if no larvae could be detected at the end of incubation and rinse procedure.
B. Report larvae detected by agar plate culture.

**Example:** *Strongyloides stercoralis* larvae detected by fecal culture.

VIII. PROCEDURE NOTES

A. It is often difficult to observe details in rapidly moving larvae. If desired, use slight heating or a drop of iodine or formalin to kill the larvae.
B. Infective larvae may be found anytime after the first or second day or even on the first day in a heavy infection. *Since infective larvae may be present on the agar, caution must be exercised in handling the plates once the cellulose tape is removed. Wear gloves when handling the cultures.*
C. It is important to maintain the plates upright at room temperature. Do not incubate or refrigerate at any time; this also applies to the fresh stool specimen.
D. Fresh stool is required for this procedure; preserved fecal specimens or specimens obtained after a barium meal are not suitable for processing by this method.

IX. LIMITATIONS OF THE PROCEDURE

A. This technique is successful if any larvae present are viable. If the fresh stool specimen is too old, larvae may not survive and a negative result will be reported.

B. Specimens that have been refrigerated or preserved are not suitable for culture. Larvae of certain species (Necator americanus) are susceptible to cold environments.

REFERENCES


APPENDIX 9.5.4–1

Agar: 1.5% agar
0.5% meat extract
1.0% peptone
0.5% NaCl

NOTE: Positive tracking on agar plates has been seen with a number of different types of agar. However, the most appropriate agar formula is that seen above.
9.5.5 Determination of Egg Viability: Schistosomal Egg Hatching

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE
The diagnosis of schistosomal infections can be aided by this method. When eggs of Schistosoma mansoni or Schistosoma japonicum are scarce, their presence may be detected by allowing them to hatch. Upon dilution of a specimen with spring (nonchlorinated) water, eggs hatch within a few hours, after which the preparation is examined for free-swimming miracidia (1–4). Miracidia are phototropic; that is, they have a tendency to swim toward light. This procedure takes advantage of the organisms’ phototropism. This is a very sensitive test for indirectly demonstrating small numbers of viable eggs in fecal specimens. This technique also allows one to determine the viability of the eggs. This is not a concentration technique; therefore, unless tests for egg viability are desired, there is seldom any point in using this technique to look for schistosome eggs in urine, in which they are readily concentrated by centrifugation. McMullen and Beaver (3) recommended the use of a side-arm flask, but an Erlenmeyer flask may be an acceptable substitute.

II. SPECIMENS
A. Fresh stool that has not been refrigerated
B. Urine (24 h) collected with no preservatives
C. Urine (random) collected with no preservatives

III. MATERIALS
A. Reagents
1. 0.85% NaCl
2. Spring water (nonchlorinated)
B. Supplies
1. Gauze
2. Funnel
3. Centrifuge tubes (50 ml)
4. 250-ml beaker
5. 500-ml sidearm flask or Erlenmeyer flask
6. Magnifying lens (hand lens)
7. Aluminum foil or brown paper

C. Equipment
1. Lamp (desk, gooseneck, or comparable)
2. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
3. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.

IV. ANALYTICAL CONSIDERATIONS
A. Make sure that water used in this procedure is chlorine free. Chlorine will affect the viability of miracidia.
B. Check saline solution for the presence of any free-living organisms (flagellates or ciliates).
IV. QUALITY CONTROL
(continued)

C. If available, known positive and negative samples should be examined to make sure that the procedure is acceptable. Since this is not usually practical, review drawings and size measurements of schistosomal eggs and/or miracidia (any parasitology text).

D. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (see procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

E. Record all QC results.

V. PROCEDURE

A. Homogenize a stool specimen (40 to 50 g) in 50 to 100 ml of 0.85% NaCl.

B. Strain specimen through two layers of gauze placed on a funnel. Collect material in a centrifuge tube.

C. Allow the suspension to settle for 1 h. Pour off and discard the supernatant fluid, and repeat this process (steps V.B and V.C) at least twice.

D. Decant and discard the saline solution, suspend the sediment in a small quantity of chlorine-free (spring) water, and pour the suspension into a 500-ml sidearm flask or an Erlenmeyer flask (Fig. 9.5.5–1).

E. Add chlorine-free water to the flask so that the fluid level rises to 2 to 3 cm in the sidearm flask or to the top 2 cm of the Erlenmeyer flask. Cover the flask with aluminum foil or black paper, leaving the side arm of the flask exposed to light. If an Erlenmeyer flask is used, cover to 1 cm below the level of fluid in the neck of the flask.

F. Allow the flask to stand at room temperature for several hours or overnight in subdued light.

G. When ready for examination, place a bright light at the side of the flask opposite the surface of exposed water. Do not place the light against the glass to avoid generation of excess heat. As the eggs hatch, the liberated miracidia will swim to the upper layers and collect in the side arm (or neck region of an Erlenmeyer flask) (Fig. 9.5.5–2).

H. Examine the illuminated area with a magnifying lens (hand lens) to look for minute white organisms swimming rapidly in a straight line (placing a piece of dark cardboard behind the flask will help you see the white miracidia against the dark background).
VI. RESULTS

A. Living miracidia may be seen.
B. Failure to see living miracidia does not rule out schistosomiasis.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report as “Miracidia of schistosomes detected, indicating presence of viable eggs.”
B. Report as “No miracidia of schistosomes detected; presence of eggs is not ruled out by this procedure.”

VIII. PROCEDURE NOTES

A. Both urine and stool specimens must be collected without preservatives and should not be refrigerated prior to processing.
B. Hatching will not occur until the saline is removed and nonchlorinated water is added.
C. If a stool concentration is performed, use saline throughout the procedure to prevent premature hatching.
D. Make sure the light is not too close to the side arm or top layer of water in the Erlenmeyer flask. Excess heat will kill the miracidia.

IX. LIMITATIONS OF THE PROCEDURE

A. Absence of swimming miracidia does not rule out the presence of eggs. Non-viable eggs or eggs that did not hatch will not be detected by this method. Use microscopic examination of direct or concentrated specimens to demonstrate the presence or absence of eggs.
B. Egg viability can be determined by placing some stool or urine sediment (same material as that used for the hatching flask) on a microscope slide.
   1. Examine under 100× to locate eggs.
   2. Examine individual eggs under 400×, and look for moving cilia on the flame cells (primitive excretory system) (Fig. 9.5.5–2).
   3. Presence of moving cilia is proof of egg viability.
C. Coprozoic, free-living ciliates may be present in soil-contaminated water. Therefore, it may be necessary to perform the following steps to differentiate those forms from the parasitic miracidia (4).
   1. Transfer a few drops of the suspension containing the organisms to a slide (3 by 2 in.), and examine under the microscope.
   2. Add a drop of weak iodine solution (pale-tea color) or dilute methylene blue (pale blue).
   3. Parasitic miracidia will stop moving, but free-living forms will continue to move.

REFERENCES

### 9.5.6 Recovery of Scolices and Proglottids of Cestodes

#### PREANALYTICAL CONSIDERATIONS

**I. PRINCIPLE**
Occasionally, stool specimens have to be examined for the presence of scolices and gravid proglottids of cestodes for proper species identification. This procedure requires mixing a small amount of feces with water and straining the mixture through a wire screen to look for scolices and proglottids (1). The same procedure may be used to look for small adult nematodes and trematodes (2). Appearance of scolices after therapy is an indication of successful treatment. If scolices have not been passed, they may be attached to the mucosa. The parasite is capable of producing more segments from the neck region of the scolex, and the infection continues.

**II. SPECIMEN**
- A. Fresh feces obtained prior to or after therapy
- B. For posttherapy specimen, the patient must receive a saline purge immediately after taking niclosamide or praziquantel.
- C. Stool specimen (24 h) collected in 10% formalin

**III. MATERIALS**
- **A. Reagent**
  - Indicate the expiration date on the label and in the work record or on the manufacturer’s label.
  - 0.85% NaCl
- **B. Supplies**
  - 1. Small-mesh (30/50 mesh) sieve or screen
  - 2. Glass slides (1 by 3 in. or larger)
  - 3. Wooden applicator sticks (nonsterile)
  - 4. 500-ml beaker
  - 5. Magnifying lens (hand lens)
  - 6. Glass dish
  - 7. Rubber bands (small)
  - 8. Shallow pan
- **C. Equipment**
  - 1. Wood’s lamp (optional)
  - 2. Stereoscope (dissecting microscope)

**IV. QUALITY CONTROL**
- A. To ensure reliable results, follow routine procedures for optimal collection and handling of specimens for parasitologic examination.
- B. Review diagrams and sizes (any parasitology text) of proglottids and scolices of tapeworms.
V. PROCEDURE

A. Mix a 24-h stool specimen (fresh or preserved in 10% formalin) with water, and thoroughly break up the specimen to make a watery suspension.
B. Slowly strain small portions of the suspension (or the purged stool) through a double layer of screen wire or a sieve (one coarse-mesh screen placed over a fine-mesh screen).
C. Wash off the sediment remaining from each portion by running a slow current of water over it.
D. Examine the cleansed debris with a hand lens to look for scolices and proglottids (*Taenia* scolex is 0.5 to 1 cm long and 1 to 2 mm wide).
E. Repeat steps V.C and V.D for each portion of the suspension strained.
F. Collect the strained sediment in a glass dish, and place over a black surface to increase the contrast of organisms against the background.
G. Observe with a magnifying hand lens, and pick out pieces of worms with an applicator stick.
H. Rinse gravid proglottids and/or scolices with tap water, and place between two microscope slides separated at the edges by thin pieces of cardboard.
I. Fasten the preparation by placing rubber bands at each end of the slides so that the segments become somewhat flattened.
J. Use the low power of a dissecting microscope for determining the number of uterine branches and genital pores in the segments and the presence or absence of a rostellum of hooks on the scolex.
K. India ink injection of proglottid
   1. Using a syringe (1 ml or less) and a 25-gauge needle, inject India ink into the central uterine stem of the proglottid, thus filling the uterine branches with ink, or inject ink into the uterine pore.
   2. Rinse the proglottid in water or saline.
   3. Blot the proglottid dry on paper towels, and press it between two slides.
   4. Use the low power of a dissecting microscope for determining the number of uterine branches.

VI. RESULTS

A. Tapeworm proglottids may be recovered (either singly or with several attached together).
B. A scolex may or may not be seen.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report as “A search for adult worms reveals presence/absence of . . . [finding].”
   **Example:** *Taenia saginata* scolex present.
B. Indicate species of gravid proglottid.
   **Example:** *Taenia solium* proglottid

VIII. PROCEDURE NOTES

A. Remember that *T. solium* eggs are infective (cysticercosis), as are the eggs of *Hymenolepis nana*.
B. *Wear gloves when performing this procedure.*
C. Specimens preserved in 70% alcohol are recommended.
D. After the identification has been made, leave the proglottid between the two slides (place a rubber band around the slides), dehydrate through several changes of ethyl alcohol (50, 70, 90, and 100%), clear in two changes of xylene, and mount with Permount for a permanent record. After xylene treatment, the proglottid will be stuck to one of the two slides. Do not try to remove it (will crack; very brittle), but merely put the Permount onto the proglottid and add the cover slip.
E. The proglottid of *T. solium* must be gravid, containing the fully developed uterine branches. If the proglottid is not fully developed (gravid), the branches may not be visible; when the uterine branches cannot be seen and/or counted, the proglottid cannot be accurately identified to the species level.

F. If the patient has received niclosamide or praziquantel, a purged specimen is required, and it should be immediately preserved in 10% formalin.

G. Wood’s lamp may be used to search for scoleces if the patient has been given quinacrine dyes. The worms, having absorbed the dye, will fluoresce at a wavelength of 360 nm. Also, after the use of quinacrine, tapeworm proglottids will appear yellow.

A. Niclosamide or praziquantel therapy leads to dissolution of the tapeworm. Therefore, the scolex and other parts may be difficult to recover unless the patient receives a saline purge soon after taking the medication.

B. It may often be difficult to identify proglottids without staining. This may be achieved by staining with India ink (see item V.K above).

C. If the proglottid is not gravid or has started to disintegrate, it will be difficult to see the uterine branches, even after India ink injection.

REFERENCES


9.5.7 Qualitative Fecal Fat

[Procedure added March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE
The microscopic examination of stool with the addition of Sudan III is a very simple and quick technique and is the most widely used technique to screen for fat.

II. SPECIMEN
A. Fresh, unpreserved fecal material is needed. If there is a time delay prior to testing, then refrigerate the specimen.
B. Specimens greater than 48 h old or dried out should be discarded and another specimen should be collected.

III. MATERIALS
A. Reagents
   1. 95% Ethanol
   2. Glacial acetic acid (36%, vol/vol)
   3. Sudan III stain
B. Supplies
   1. Disposable test tube, 15-ml size
   2. Applicator sticks
   3. Disposable plastic pipettes
   4. Matches
   5. Biohazard discard container
C. Equipment
   1. Bright-field microscope, with 10× and 40× objectives
   2. Alcohol burning lamp or Bunsen burner
   3. Refrigerator
   4. Biological safety cabinet

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL
A. Run QC with each batch of patient tests as in the procedure below.
B. Positive control—mayonnaise: red-stained fat globules should be observed microscopically.
C. Negative control—water: no fat globules observed.
D. If QC is unacceptable, then the test must be repeated and documented on the corrective action sheet.
V. PROCEDURE
A. Mix a small amount of stool with an equal amount of 95% ethanol in a test tube. Mix well.
B. Add 2 drops of Sudan III stain to the stool-ethanol mixture. Mix well and let stand a few minutes.
C. Using a pipette, remove a drop from the test tube and place it on a slide. Cover with a coverslip.
D. Using the microscope, examine the slide for globules of fat stained red (neutral fat) and needle-like crystals (fatty acid).
E. Add several drops of glacial acetic acid to the test tube. Remove a drop from the test tube and place it on a slide. Cover with a coverslip.
F. Gently heat the slide over a flame. (Do not boil.)
G. Observe again on the microscope for globules of fat stained red.

VI. RESULTS
Red-stained fat globules or fatty acid crystals will be seen microscopically. Report specimen as “Fat not increased” or “Fat increased,” depending on the number of globules seen.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS
A. If fewer than 100 globules/high-power field (HPF) are seen before or after heating, report “Fat not increased.”
B. If more than 100 globules/HPF are seen before or after heating, report “Fat increased.”
C. If fatty acid crystals have been seen before heating and globules appear after heating, report “Fatty acids increased.”

VIII. LIMITATIONS OF THE PROCEDURE
A. Just because large needle-like crystals form as the preparation cools after heating, it does not mean that the original globules were fatty acid. Sudan III will form very short needle-like crystals in bunches as it dries.
B. A very few, if any, neutral fat globules are seen in a normal stool specimen. The presence of large amounts of neutral fat should make one suspicious that the patient has ingested mineral oil or castor oil, thus causing a false positive.
C. Do not count the fat that is present in vegetable cells.

SUPPLEMENTAL READING
9.5.8 Reducing Substances (Clinitest)

[Procedure added March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE
Clinitest is a reagent tablet test based on the classic Benedict’s copper sulfate reduction reaction combining ingredients with an integral heat-generating system. Clinitest provides clinically useful information about carbohydrate metabolism by determining the amount of reducing substance in urine or stool. Reducing substances convert the cupric (Cu^{2+}) to cuprous (Cu^{+}) oxide and a change in solution color ranging from green to orange.

II. SPECIMEN
Unpreserved stool.
A. Place in the refrigerator in case there is a delay in testing.
B. Specimens >48 h old or dried out should be discarded and new specimens should be collected.

III. MATERIALS
A. Reagents
1. Clinitest reagent tablets. (Store tablets at room temperature in a plastic bag.)
2. Deionized water
3. Chek-Stix positive control
B. Supplies
1. Disposable plastic test tubes with round bottoms
2. Applicator sticks
3. Disposable plastic transfer pipettes
4. Biohazard discard container
5. Disposable gloves
C. Equipment
Biological safety cabinet

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL
A. Run controls with each batch of patient tests as in the procedure below.
B. Positive control: Chek-Stix. The development of green, yellow, or orange color with yellow or red precipitate is considered a positive result.
C. Negative control: 0.5 ml of deionized water. A blue color development is considered a negative result.
D. If QC is unacceptable, the test must be repeated and documented on the corrective action sheet.
V. PROCEDURE
   A. All testing on clinical specimens should be performed in a biological safety
      cabinet, wearing gloves and a lab coat.
   B. Add 1 volume of stool to 2 volumes of deionized water. Mix thoroughly.
   C. Using a disposable transfer pipette, transfer 15 drops of this suspension into a
      clean test tube.
   D. Drop one Clinitest tablet reagent into the test tube.
   E. Observe the reaction. Do not shake the tube while the chemical reaction is
      occurring.
   F. Wait 15 s after the reaction stops, and gently shake contents to mix.
   G. Compare the color of the liquid to the color chart in the package insert of the
      Clinitest tablet reagent.
   H. Discard supplies in appropriate biohazard containers.

VI. RESULTS
   A. Negative: clear to cloudy blue color
   B. Positive: Compare the liquid color to the color chart. Grade the degree of color
      development to the color chart (trace, 1+, 2+, 3+, or 4+). These results
      equate to the grams per deciliter of the reducing substance present in the sample.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS
   A. Positive: report as trace (0.25 g/dl), 1+ (0.5 g/dl), 2+ (0.75 g/dl), 3+ (1.0 g/
      dl), or 4+ (greater than or equal to 2 g/dl).
   B. Negative
      Report as negative.

VIII. PROCEDURE NOTES
   A. Clinitest is not specific for glucose and will react with any reducing substance
      in stool, including lactose, fructose, galactose, and pentoses.
   B. Interfering substances may affect results. Some interfering substances include
      salycilates, penicillin, large quantities of ascorbic acid, nalidixic acid, and cephe-
      alosporins.
   C. Failure to observe the reaction at all times may lead to erroneously low results
      if reducing substances are present in large amounts. If more than 2% sugar is
      present, a rapid color change may occur during boiling, causing the color to
      pass rapidly through bright orange to a dark brown or greenish brown.

SUPPLEMENTAL READING

Bayer Corporation Diagnostic Division. September 1995. Clinitest package insert. Bayer Cor-
poration Diagnostic Division, Elkhart, Ind.
Davidson, G., and M. Mullinger. 1970. Reduc-
ing substances in neonatal stool detected by Clini-
Kerry, K. R., and C. M. Anderson. 1964. A
ward test for sugar in feces. Lancet I:981.
### Examination for Pinworm: Cellulose Tape Preparation

#### Preanalytical Considerations

##### I. Principle
The clear-cellulose-tape preparation is the most widely used procedure for the detection of human pinworm infections (2, 4). Adult *Enterobius vermicularis* worms inhabit the large intestine and rectum; however, the eggs are not normally found in fecal material. The adult female migrates out the anal opening and deposits the eggs on the perianal skin, usually during the night. The eggs, and occasionally the adult female worms, stick to the sticky surface of the cellulose tape. These cellulose tape preparations are submitted to the laboratory, where they are examined under the microscope. Commercial collection systems are also available (pinworm collection kit; Evergreen Scientific, Los Angeles, Calif.) (1).

##### II. Specimen
The specimen is collected from the skin of the perianal area first thing in the morning, before the patient has bathed or used the toilet. Preparations should be taken for at least 4 to 6 consecutive days with negative results before a patient is considered free of the infection.

##### III. Materials

<table>
<thead>
<tr>
<th>A. Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indicate the expiration date on the label and in the work record or on the manufacturer’s label.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. Supplies</th>
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<tbody>
<tr>
<td>1. Clear cellulose tape (not Magic mend tape)</td>
</tr>
<tr>
<td>2. Tongue depressors</td>
</tr>
<tr>
<td>3. Glass slides (1 by 3 in., or larger if you prefer)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>C. Equipment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)</td>
</tr>
<tr>
<td>2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.</td>
</tr>
</tbody>
</table>

#### Analytical Considerations

##### IV. Quality Control

A. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (see procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.
IV. QUALITY CONTROL (continued)

B. Pictures of Enterobius eggs (with measurements) should be available for comparison with the clinical specimen.

C. Record all QC results.

V. PROCEDURE

A. Place a strip of clear cellulose tape (adhesive side down) on a microscope slide as follows: starting ca. 1.5 cm from one end, run the tape toward the same end, and wrap the tape around the slide to the opposite end. Tear the tape even with the end of the slide. Attach a label to the tape at the end torn flush with the slide.

B. To obtain a sample from the perianal area, peel back the tape by gripping the labeled end, and, with the tape looped (adhesive side outward) over a wooden tongue depressor that is held firmly against the slide and extended about 2.5 cm beyond it, press the tape firmly several times against the right and left perianal folds (see Fig. 9.6.1–1).

C. Smooth the tape back on the slide, adhesive side down.

D. Label with patient name and date.

E. Submit the tapes and slides to the laboratory in a plastic bag.

F. Examine the slide directly under the low-power objective (10×) of the microscope. To make the eggs more visible, lift the tape from the slide and add a drop of xylene or toluene to the slide. Press the tape back down on the slide. Examine with low light intensity on low power.

VI. RESULTS

A. Typical pinworm eggs are thick shelled and football shaped, with one flattened side, and may contain a partially or fully developed larva.

B. Adult female worms are occasionally seen on the tape preparation. The worms are approximately 1 cm in length, are white or cream colored, and have a pointed tail.

POSTANALYTICAL CONSIDERATIONS

A. Report the organism and stage. Do not use abbreviations.

Example: Enterobius vermicularis eggs present.

B. Report adult worms.

Example: Enterobius vermicularis adult worm present.

VII. REPORTING RESULTS

Figure 9.6.1–1 Collection of E. vermicularis eggs by the cellulose tape method (from reference 1).
VIII. PROCEDURE NOTES

A. Pinworm eggs are usually infectious. The use of glass slides and tapes may expose laboratory personnel to these eggs.

B. Some recommend the use of the Swube (paddle with sticky adhesive coat; Becton Dickinson) as a safer alternative. The petrolatum swab is another alternative (3). Another option is the collection system available from Evergreen Scientific (Fig. 9.6.1–2).

C. If opaque tape is submitted by mistake, a drop of immersion oil on the top of the tape will clear it enough to proceed with the microscopic examination.

IX. LIMITATIONS OF THE PROCEDURE

A. The female pinworm deposits eggs on the perianal skin only sporadically.

B. Without multiple tapes (taken consecutively, one each morning), it is not possible to determine if the patient is positive or negative for the infection.

C. Occasionally, a parent will bring in an adult worm collected from the perianal skin or from the surface of the stool. The identification of the adult worm (almost always the female) confirms the infection.

REFERENCES


9.6.2 Sigmoidoscopy Specimen: Direct Wet Smear

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The direct smear (1, 2) is primarily used to detect motile parasites that are found in the colon (the organism in question is usually Entamoeba histolytica or Entamoeba histolytica/E. dispar). Specific ulcerated areas should always be sampled; in the absence of specific lesions, the mucosa is randomly sampled. On low-power (×100) examination of the smear, motility of trophozoites and/or human cells might be detected. At high dry power (×430), organisms might be tentatively identified on the basis of size, nucleus/cytoplasm ratio, appearance of the cytoplasm, and motility (saline only). The direct smear can be prepared either with 0.85% NaCl or with iodine (Lugol’s or D’Antoni’s). Presumptive findings using this procedure must be confirmed by some type of permanent stained smear.

II. SPECIMEN

A. The specimen may consist of mucosal lining, mucus, stool, and/or a combination of the three. The specimen will be taken by the physician and either prepared at bedside for immediate review or submitted to the laboratory for subsequent examination.

B. Prepare direct wet mounts on clean, new glass slides (1 by 3 in.). Depending on the specimen type, the following amounts should be used.

1. For mounts of mucus or similar material, place approximately 1 or 2 drops on the slide.
2. For mounts of stool, place approximately 1 or 2 drops on the slide.
3. If the material is very wet (watery), place 1 or 2 drops on the slide.

C. If the specimen must be transported to the laboratory, the material can be placed in a small amount of 0.85% NaCl (0.5 to 1.0 ml) to keep the specimen from drying out. These specimens should be transported to the laboratory within no more than 30 min from collection time and should be examined immediately. If this is not possible, then the material can be preserved and processed as a permanent stained smear (see procedure 9.6.3).

III. MATERIALS

A. Reagents (see Appendix 9.6.2–1)

B. Supplies

1. Disposable glass or plastic pipettes
2. Glass slides (1 by 3 in., or larger if you prefer)
3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)
4. Small tubes containing 0.5 to 1.0 ml of 0.85% NaCl

C. Equipment

1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
3. Tabletop centrifuge (for tubes containing specimen and 0.85% NaCl if the specimen is submitted in saline)
IV. QUALITY CONTROL

A. Check the direct-mount reagents each time they are used.
   1. The saline should be clear, with no visible contamination.
   2. The iodine should be a strong-tea color, and there should be crystals in the
      bottom of the bottle. Small aliquots of the stock solution should always be
      strong-tea color. If not, then discard and aliquot some stock solution into
      your dropper bottle.

B. The microscope should be calibrated, and the objectives and oculars used for
   the calibration procedure should be used for all measurements on the micro-
   scope. Post the calibration factors for all objectives on the microscope for easy
   access (multiplication factors can be pasted right on the body of the microscope)
   (see procedures 9.1 and 9.3.2). Although there is not universal agreement, the
   microscope should probably be recalibrated once each year. This recommen-
   dation should be considered with heavy use or if the microscope has been
   bumped or moved multiple times. If the microscope does not receive heavy use,
   then recalibration is not required on a yearly basis.

C. Record all QC results.

V. PROCEDURE

A. Wear gloves when performing this procedure.

B. To the drop or two of patient material on the slide, add 1 or 2 drops of 0.85%
   NaCl, mix with the corner of the coverslip or an applicator stick, and mount
   with a no. 1 coverslip (22 by 22 mm). The amount of saline will be determined
   by the specimen (less saline if the material is very liquid).

C. Examine the smear with the low-power (10×) objective with low light. View
   each field for a few seconds, looking for any organism motility. Any suspicious
   objects can be examined with the high dry (40×) power objective with low
   light.

D. Prepare a wet mount with Lugol’s or D’Antoni’s iodine (working solution)
   rather than saline. Another option would be to add a small drop of iodine at the
   side of the coverslip on the saline wet preparation. The iodine will diffuse into
   the saline suspension under the coverslip. However, if the specimen is thick or
   contains mucus, then capillary action pulling the iodine under the coverslip and
   into the saline may not occur, and a separate iodine mount may be required.
   The addition of iodine will give the material some color (organisms may be
   easier to see), but motility will be lost.

VI. RESULTS

A. Protozoan trophozoites may be confused with human cells (macrophages), so
   report any identification as presumptive until the permanent stained smears have
   been examined.

B. Also, the presumptive identification and quantitation of the human cells (mac-
   rophages, PMNs, eosinophils, RBCs) could be obtained from the wet prepara-
   tions. Consider this information presumptive also until the permanent stained
   smears have been examined.
POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the organism and stage (trophozoite, cyst, oocyst, etc.; do not use abbreviations); however, confirmation of species may require some type of permanent stained smear.

Example: Entamoeba histolytica trophozoites

B. Note and quantitate the presence of human cells.

Examples: Moderate WBCs, many RBCs, few macrophages

C. Call the physician if pathogenic organisms are identified.

D. Report negative results as a presumptive report (based on wet examination only) prior to the examination of the permanent stained smear.

E. See Table 9.6.2–1 for correct method of quantitation.

VIII. PROCEDURE NOTES

A. Remember that the iodine working solution should be a strong-tea color; if it is not, discard and prepare new working solution.

B. Final identification of some of the intestinal protozoa may be difficult (small size, confusion between organisms and human cells), and a permanent stained smear must be used as a confirmatory method and examined at ×1,000 to see morphological details.

C. In saline, human cells and/or protozoan trophozoites may exhibit some motility.

D. In iodine, human cells and/or protozoan trophozoites may be seen (no motility).

E. Confirm presumptive findings (either positive or negative) with a permanent stained smear.

IX. LIMITATIONS OF THE PROCEDURE

A. Multiple areas of the mucosa should be examined (six smears are often recommended), and this technique should not take the place of the routine ova and parasite examination.

B. Wet preparations are normally not examined with oil immersion power (100×). Consequently, use permanent stained smears to confirm morphology and organism (or human cell) identification.

C. If the specimen amount is limited, then do not do the wet preparation, but process the specimen that is available by using the permanent-stained-smear protocol (see procedure 9.6.3) to maximize the amount and clinical relevance of the information obtained.

D. Morphologic differentiation between the Entamoeba histolytica/E. dispar group and Entamoeba coli can be difficult, in addition to the problem of differentiating human cells from protozoa. Also, unless RBCs are seen, it will be impossible

<table>
<thead>
<tr>
<th>Quantity</th>
<th>No. of protozoa, human cells, yeast cells, artifacts Per 10 oil immersion fields (×1,000)</th>
<th>Per 10 40× fields (×400)</th>
<th>No. of helminths/cover slip</th>
</tr>
</thead>
<tbody>
<tr>
<td>Few</td>
<td>≤2</td>
<td>≤2</td>
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</tr>
<tr>
<td>Moderate</td>
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</tr>
<tr>
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<td>≥10</td>
<td>≥10</td>
<td>≥10</td>
</tr>
</tbody>
</table>

* In general, protozoa are not quantitated on the laboratory slip (exception: Blastocystis hominis); but human cells, yeast cells, and artifacts like Charcot-Leyden crystals are normally reported and quantitated.

* Wet preparation. Coverslips were 22 by 22 mm.

* Wet preparation.
IX. LIMITATIONS OF THE PROCEDURE (continued)

to differentiate organisms in the *E. histolytica/E. dispar* group from the actual pathogen, *E. histolytica*.

REFERENCES


APPENDIX 9.6.2–1

Reagents

Include QC information on reagent container and in QC records.

A. 0.85% NaCl

1. Dissolve in distilled water in an appropriate glass flask by using a magnetic stirrer.

   - sodium chloride (NaCl) ....................... 850 mg
   - distilled water ................................ 100 ml

2. Store in a glass bottle.

3. Label as 0.85% NaCl with the preparation date and an expiration date of 6 months.

   Store at room temperature.

B. Modified D’Antoni’s stock iodine

1. Dissolve in distilled water in an appropriate glass flask, using a magnetic stirrer.

   - potassium iodide (KI) ...................... 1.0 g
   - powdered iodine crystals .................. 1.5 g
   - distilled water ............................ 100 ml

2. The D’Antoni’s solution should be saturated with iodine, with some excess crystals left in the bottle. Store in a brown bottle at room temperature. The stock solution remains good as long as an excess of iodine crystals remains on the bottom of the bottle.

3. Label as D’Antoni’s stock iodine with the preparation date and an expiration date of 1 year.

4. Small amounts of stock iodine solution can be aliquoted into brown dropper bottles for routine daily use. The expiration date will be from 30 to 60 days, depending on the amount of fading of the solution from the normal strong-tea color (smaller dropper bottles and the use of clear glass result in a shorter expiration time). The use of a brown bottle will lengthen the expiration time.

C. Lugol’s iodine solution

1. Dissolve in distilled water in an appropriate glass flask by using a magnetic stirrer.

   - potassium iodide (KI) .................... 10.0 g
   - iodine crystals ........................... 5.0 g
   - distilled water .......................... 100 ml

2. The Lugol’s solution should be saturated with iodine, with some excess crystals left in the bottle. Store in a brown bottle at room temperature. The stock solution remains good as long as an excess of iodine crystals remains on the bottom of the bottle.

3. Label as Lugol’s stock iodine with the preparation date and an expiration date of 1 year.

4. Small amounts of stock iodine solution can be aliquoted into brown dropper bottles for routine daily use. The expiration date will be from 30 to 60 days, depending on the amount of fading of the solution from the normal strong-tea color (smaller dropper bottles and the use of clear glass result in a shorter expiration time). The use of a brown bottle will lengthen the expiration time.
9.6.3 Sigmoidoscopy Specimen: Permanent Stained Smear

**PREANALYTICAL CONSIDERATIONS**

I. **PRINCIPLE**
The permanent stained smear is used primarily to detect parasites in the colon (the primary organism in question is usually *Entamoeba histolytica* or *Entamoeba histolytica*/E. dispar). Specific ulcerated areas are always sampled; in the absence of specific lesions, the mucosa would be randomly sampled. On oil immersion (magnification of \( \times 1,000 \)) examination of the smear, protozoan trophozoites and/or cysts might be detected. Coccidian oocysts, helminth eggs or larvae, and/or human cells are also detected with this procedure. The permanent smear can be stained with trichrome or iron hematoxylin stains. Permanent stained smears usually confirm structures seen on wet specimen examinations (1).

II. **SPECIMEN**
The specimen may consist of mucosal lining, mucus, stool, and/or a combination of the three.

- A. Prepare smears on clean, new glass slides (1 by 3 in.).
- B. For mounts of mucus or similar material, place approximately 1 or 2 drops of specimen on the slide.
- C. For mounts of stool, place approximately 1 drop on the slide.
- D. If the material is very wet (watery), place 1 or 2 drops on the slide.

III. **MATERIALS**

- A. **Reagents**
  - Indicate the expiration date on the label and in the work record or on the manufacturer’s label.
  - 1. Schaudinn’s fixative
     - See Appendix 9.2.2–1, item A, for preparation instructions.
  - 2. Polyvinyl alcohol (PVA) fixative
     - See Appendix 9.2.2–1, item B, for preparation instructions.

- B. **Supplies**
  - 1. Disposable glass or plastic pipettes
  - 2. Glass slides (1 by 3 in., or larger if you prefer)
  - 3. Coverslips (22 by 22 mm or larger; no. 1 thickness)

- C. **Equipment**
  - 1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
  - 2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
  - 3. Tabletop centrifuge

*Observe standard precautions. Include QC information on reagent container and in QC records.*
**ANALYTICAL CONSIDERATIONS**

IV. QUALITY CONTROL

A. Check the fixatives weekly or when a new lot number is used (visual inspection). Use either fresh stool containing protozoa or negative stool seeded with human buffy coat cells to evaluate the efficacy of the fixatives after staining (see procedures 9.3.6 and 9.3.7). Cultured protozoa can also be used.

B. The Schaudinn’s fixative should appear clear, without floating debris or crystals. Some crystal sediment on the bottom of the Coplin jar or dish is acceptable.

C. The PVA should be clear (slight milky or smoky color is acceptable). A slight precipitate on the bottom of the container is acceptable. The fluid should easily move in the bottle when it is inverted, and the viscosity of many of the available formulations will actually approach that of water.

D. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (see procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

E. Record all QC results.

V. PROCEDURE

A. Wear gloves when performing this procedure.

B. If the specimen does not contain blood and/or mucus or is not “wet,” gently smear a drop or two of patient material onto the slide, and immediately immerse the slide into the Schaudinn’s fixative. There should be no problem with adherence to the slide. The fixation and staining times are identical to those for routine fecal smears (see procedures 9.2.2. V, 9.3.6, and 9.3.7 for specific directions).

C. If the material is bloody, contains a lot of mucus, or is a wet specimen, gently mix 1 or 2 drops of patient material with 3 or 4 drops of PVA fixative directly on the slide. Allow the smear to air dry for at least 2 h prior to staining. The fixation and staining times are identical to those for routine fecal smears (see procedures 9.2.2.V, 9.3.6, and 9.3.7 for specific directions).

D. Examine the stained smear with the oil immersion lens (100×) with maximum light. Examine at least 300 oil immersion fields of the smear.

VI. RESULTS

A. With either the trichrome or iron hematoxylin stain, the protozoan trophozoites and cysts will be easily seen. Oocysts will not be clearly delineated; if you see suspect organisms, then use additional procedures for confirmation (see procedures 9.4.1 and 9.4.2).

B. Helminth eggs or larvae may not be easily identified on the permanent stained smear, and you may need to do wet-mount examinations.

C. Human cells are readily identified (macrophages, PMNs, RBCs, etc.). Yeast cells (single cells, budding, presence of pseudohyphae) can also be easily seen.
POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the organism and stage. Do not use abbreviations.
   Example: *Entamoeba histolytica* trophozoites (containing ingested RBCs)

B. Note and quantitate the presence of human cells.
   Examples: Moderate WBCs, many RBCs, few macrophages, etc.

C. Report and quantitate yeast cells.
   Example: Moderate budding yeast cells and few pseudohyphae

D. Call the physician if pathogenic organisms are identified.

E. See Table 9.6.3–1 for correct method of quantitation.

VIII. PROCEDURE NOTES

A. Sigmoidoscopy specimens are submitted in order to help differentiate between inflammatory bowel disease and amebiasis. It is critical that the specimens be preserved immediately after being taken. Any delay could result in the disintegration of amebic trophozoites or distortion of human cells.

B. It is critical that permanent stained smears of this material be carefully examined with the oil immersion lens (100×).

IX. LIMITATIONS OF THE PROCEDURE

A. The more areas of the mucosa sampled, the more likely the organisms will be found. If only one or two smears are submitted for examination, the physician must be informed (recommendation is six smears from representative areas of the mucosa).

B. The examination of smears prepared at sigmoidoscopy does not take the place of routine ova and parasite examinations but serves as a supplemental procedure. Stools for routine examinations should also be submitted (a minimum of three specimens collected every other day or within no more than 10 days).

C. Morphologic differentiation between the *Entamoeba histolytica/E. dispar* group and *Entamoeba coli* can be difficult, in addition to the problem of differentiating human cells from protozoa. Also, unless RBCs are seen, it will be impossible to differentiate organisms in the *E. histolytica*/*E. dispar* group from the actual pathogen, *E. histolytica*.

REFERENCE


Table 9.6.3–1 Quantitation of parasites, human cells, yeast cells, and artifacts in specimens from the intestinal tract

<table>
<thead>
<tr>
<th>Quantity</th>
<th>No. of protozoa, human cells, yeast cells, artifacts</th>
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<tr>
<td></td>
<td>No. of oil immersion fields (×1,000)</td>
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a In general, protozoa are not quantitated on the laboratory slip (exception: *Blastocystis hominis*); but human cells, yeast cells, and artifacts like Charcot-Leyden crystals are normally reported and quantitated.

b Wet preparations. Coverslips were 22 by 22 mm.

c PVA smear.

d Wet preparation.
Duodenal Contents: String Test
(Entero-Test Capsule)

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The Entero-Test capsule is usually administered and the string is retrieved by a physician. This test is used to procure specimens from the duodenum that are then examined for the presence of parasites. The Entero-Test is a gelatin capsule lined with silicone rubber that contains a spool of nylon string and a weight (Fig. 9.6.4–1). The end of the string is taped to the back of the patient’s neck or the patient’s cheek just before the capsule is swallowed with water. After swallowing the capsule, the patient is allowed to relax for 4 h. The patient is not allowed to eat during this time but is allowed to drink a small amount of water. As the capsule dissolves, the string unwinds and is carried by peristalsis to the duodenum, and the duodenal mucus adheres to the string. Any Strongyloides larvae (2), Giardia trophozoites (1–6), or Cryptosporidium (1, 5, 6) or Isospora (1, 5, 6) oocysts that are present will also adhere to the string and will be pulled up with the string when it is removed. The specimen can be examined as a wet preparation or as a permanent stained smear. In rare instances, Clonorchis sinensis eggs may be recovered (1–3). This test is a less invasive substitute for duodenal aspiration (1–6).

Figure 9.6.4–1 Diagram of Entero-Test capsule (from reference 2).
II. SPECIMEN

A. The physician must notify the laboratory when a capsule is swallowed so that a parasitologist is available to read the test 4 h later, when the string is removed.

B. The string is placed in a small, securely covered container that is transported in a plastic bag. Unless the specimen container is hand delivered to the microbiology unit, a petri dish should not be used because the top cannot be securely attached. A urine container with a screw-cap top is practical for this purpose.

C. The specimen must be transported immediately and read within 1 h (2).

D. If there is any delay anticipated in transport to the laboratory, a small amount of saline (~1 ml) can be added to keep the string moist.

III. MATERIALS

A. Reagents

1. Schaudinn’s fixative.
   See Appendix 9.2.2–1, item A, for preparation instructions.

2. Polyvinyl alcohol (PVA) fixative
   See Appendix 9.2.2–1, item B, for preparation instructions.

3. 10% Formalin
   See Appendix 9.2.2–1, item G, for preparation instructions.

B. Supplies

1. Disposable glass or plastic pipettes
2. Glass slides (1 by 3 in., or larger if you prefer)
3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)

C. Equipment

1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)

2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.

IV. QUALITY CONTROL

A. Check the fixatives weekly or when a new lot number is used (visual inspection). Use either fresh stool containing protozoa or negative stool seeded with human buffy coat cells to evaluate the efficacy of the fixatives after staining (see procedures 9.3.6 and 9.3.7). Cultured protozoa can also be used.

1. The Schaudinn’s fixative should be clear, without floating debris or crystals. Some crystal sediment on the bottom of the Coplin jar or dish is acceptable.

2. The PVA should be clear (slightly milky or smoky color is acceptable). A slight precipitate on the bottom of the container is acceptable. The fluid should easily move in the bottle when it is inverted. The viscosity of many of the available formulations will actually approach that of water.

3. The 10% formalin should be clear, with no visible contamination.

B. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (see procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

C. Record all QC results.
V. PROCEDURE

A. *Gloves must be worn when handling this specimen.* Infectious *Strongyloides* larvae can penetrate the intact skin.

B. Record the color of the string. Yellow bile stain indicates that the string did reach the duodenum.

C. Place the specimen under the biosafety cabinet, hold the dry white end in one hand, and strip all the mucus off the string by gripping it between the thumb and index finger of the other hand and squeezing it all the way down to the end, so that the mucus goes into the screw-cap container.

D. Place 1 drop of mucus on a clean slide, and cover with a coverslip (22 by 22 mm). If the mucus is very viscous, add a drop of saline before adding the coverslip.

E. Store the remaining mucus in a transfer pipette placed in a labeled test tube (16 by 125 mm) so that it will not dehydrate.

F. Examine the entire coverslip under low power (100×) for larvae or motile trophozoites, looking especially carefully at the mucus, where *Giardia lamblia* may be entangled.

G. Examine the mucus under high dry power (400×), since *G. lamblia* may be detectable only by the flutter of the flagella rather than by motility.

H. If there is enough specimen, gently smear a drop or two of patient material on two slides, and immediately immerse the slides in Schaudinn’s fixative so that permanent stained slides may be made. If the specimen is not adequate for this, place the wet mount slide in a Coplin jar containing Schaudinn’s solution after it has been read. The coverslip will float off and sink to the bottom, allowing the remaining material to be stained. The fixation and staining times are identical with those for routine fecal smears (see procedure 9.2.2 for specific directions).

I. If the material contains a lot of mucus or is a watery specimen, gently mix 1 or 2 drops of patient material with 3 or 4 drops of PVA fixative directly on the slide. Let the smear air dry for at least 2 h prior to staining. The fixation and staining times are identical to those for routine fecal smears (see procedure 9.2.2 for specific directions).

J. Place a drop of the mucus on one or more slides to be stained for *Cryptosporidium* and *Isospora* species, and then repeat the wet-mount procedure (steps, V.D through G above) until all the remaining mucus is used.

K. Stain the *Cryptosporidium* and *Isospora* slide(s) with modified acid-fast stain, and examine as usual (see procedures 9.4.1 and 9.4.2 for specific directions).

L. Examine the permanent stained smear with the oil immersion lens (100×) with maximum light. Examine at least 300 oil immersion fields on each smear.

M. If *Strongyloides* larvae are found, preserve the rest of the specimen in 10% formalin for teaching purposes.

VI. RESULTS

A. With either the trichrome or iron hematoxylin stain, the protozoan trophozoites and cysts will easily be seen. Oocysts will not be clearly delineated; if suspect organisms are seen, use additional procedures for confirmation (see procedures 9.4.1 and 9.4.2).

B. *Cryptosporidium* and *Isospora* oocysts will be visible on permanent stained smears (modified acid-fast procedures) (see procedures 9.4.1 and 9.4.2 for specific directions).

C. Helminth eggs or larvae may not be easily identified on the permanent stained smear but will be visible in the wet preparations.
Postanalytical Considerations

VII. REPORTING RESULTS

A. Report the organism and stage (trophozoite, cyst, oocyst, etc.). Do not use abbreviations. Confirmation of species may require some type of permanent stained smear.

Examples: *Giardia lamblia* trophozoites, *Strongyloides stercoralis* larvae

B. Call the physician if pathogenic organisms are identified.

C. Quantitate *C. sinensis* eggs if they are recovered.

D. If the results are negative on the wet smear examination, a preliminary report can be sent (based on wet examination only) prior to the examination of the permanent stained smear.

E. See Table 9.6.4–1 for correct method of quantitation.

**Table 9.6.4–1** Quantitation of parasites, human cells, yeast cells, and artifacts in specimens from the intestinal tract

<table>
<thead>
<tr>
<th>Quantity</th>
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<th>No. of helminths/coverslip</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Per 10 oil immersion fields (× 1,000)¹</td>
<td>Per 10 40× fields (× 400)²</td>
</tr>
<tr>
<td>Few</td>
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¹ In general, protozoa are not quantitated on the laboratory slip (exception: *Blastocystis hominis*); however, human cells, yeast cells, and artifacts like Charcot-Leyden crystals are normally reported and quantitated.

² Wet preparation. Coverslip was 22 by 22 mm.

³ PVA smear.

⁴ Wet preparation.

VIII. PROCEDURE NOTES

Modified acid-fast methods (or immunoassay detection methods) will have to be used for the identification of *Cryptosporidium parvum*. *Isospora belli* can be identified on the wet examination or from smears stained by modified acid-fast methods.

IX. LIMITATIONS OF THE PROCEDURE

A. Many of the parasites will be caught up in the mucus; therefore, it is very important to examine the specimen carefully under high dry power (400×) with low light in order to see the flutter of the *Giardia* flagella.

B. If the duodenal specimens from the Entero-Test capsule are normally examined as wet preparations, remember that some of the organisms (*Cryptosporidium* and *Isospora* species) may be missed without additional permanent stains.

REFERENCES


9.6.5 Duodenal Contents: Duodenal Aspirate

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE
Some organisms may be difficult to recover in the stool, particularly those normally found in the duodenum. An alternative approach to routine stool examinations is to sample the duodenal contents. Samples are obtained through the use of nasogastric intubation or the Entero-Test capsule (string test). Fluid from the duodenum is examined for the presence of *Strongyloides stercoralis* larvae (1, 2), *Giardia lamblia* trophozoites (1, 2), and *Cryptosporidium parvum* and *Isospora belli* oocysts. The specimen can be examined as a wet preparation or as a permanent stained smear. In rare instances, *Clonorchis sinensis* eggs may be recovered (1, 2). Material can also be examined as permanent stained smears (e.g., modified trichrome stain) for the presence of microsporidial spores.

II. SPECIMEN
Observe standard precautions.

Duodenal fluid must be transported immediately in a securely covered container placed in a plastic bag. A screw-cap urine container or plastic centrifuge tube with no preservatives is practical for this purpose.

III. MATERIALS
Include QC information on reagent container and in QC records.

A. Reagents
   1. Schaudinn’s fixative
      See Appendix 9.2.2–1, item A, for preparation instructions.
   2. Polyvinyl alcohol (PVA) fixative
      See Appendix 9.2.2–1, item B, for preparation instructions.
   3. 10% Formalin
      See Appendix 9.2.2–1, item G, for preparation instructions.

B. Supplies
   1. Disposable glass or plastic pipettes
   2. Glass slides (1 by 3 in., or larger if you prefer)
   3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)
   4. Gloves
   5. Urine container
   6. Centrifuge tubes (15 or 50 ml)

C. Equipment
   1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
   2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
   3. Tabletop centrifuge

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL
A. Check the fixatives weekly or when a new lot number is used (visual inspection). Use either fresh stool containing protozoa or negative stool seeded with human buffy coat cells to evaluate the efficacy of the fixatives after staining (see procedures 9.3.6 and 9.3.7). Cultured protozoa can also be used.
   1. The Schaudinn’s fixative should be clear, without floating debris or crystals. Some crystal sediment on the bottom of the Coplin jar or dish is acceptable.
IV. QUALITY CONTROL
(continued)

2. The PVA should be clear (slightly milky or smoky color is acceptable). A slight precipitate on the bottom of the container is acceptable. The fluid should easily move in the bottle when it is inverted. The viscosity of many of the available formulations will actually approach that of water.

3. The 10% formalin should be clear, with no visible contamination.

B. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (see procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

C. Record all QC results.

V. PROCEDURE

A. Gloves must be worn when handling this specimen. Infectious Strongyloides larvae can penetrate the intact skin.

B. Examine the specimen within 1 h after it is taken. Note the amount of yellow color, which indicates bile staining and confirms that the specimen is actually from the duodenum.

C. Centrifugation may be necessary to concentrate the mucus and any organisms present (500 × g for 10 min). Centrifugation should be routinely performed if the volume of fluid is ≥2 ml.

D. Place 1 drop of fluid on a clean slide, and cover with a coverslip (22 by 22 mm). If the specimen is very viscous, add a drop of saline before adding the coverslip.

E. Examine the entire coverslip under low power (100×) for larvae or motile trophozoites, looking especially carefully around the mucus, where G. lamblia may be entangled.

F. Examine the mucus present under high dry power (400×), since G. lamblia may be detectable only by the flutter of the flagella rather than by motility.

G. If there is enough specimen, gently smear a drop or two of patient material on two slides, and immediately immerse the slides in Schaudinn’s fixative so that permanent stained slides may be made. If the specimen is not adequate for this, place the wet-mount slide in a Coplin jar containing Schaudinn’s solution, after it has been read. The coverslip will float off and sink to the bottom, allowing the remaining material to be stained. Fixation and staining times are identical to those for routine fecal smears (see procedure 9.2.2 for specific directions).

H. If the material contains a lot of mucus or is a watery specimen, gently mix 1 or 2 drops of patient material with 3 or 4 drops of PVA fixative directly on the slide. Let the smear air dry for at least 2 h prior to staining. The fixation and staining times are identical to those for routine fecal smears (see procedure 9.2.2 for specific directions).

I. Place a drop of the duodenal fluid on one or more slides to be stained for Cryptosporidium and Isospora species, and then repeat the wet mount procedure (steps V.D through G above) until all the remaining mucus (after centrifugation) or sediment is gone.

J. Stain the Cryptosporidium and Isospora slide(s) with modified acid-fast stain, and examine as usual (see procedures 9.4.1 and 9.4.2 for specific directions).

K. Examine the permanent stained smear with the oil immersion lens (100×) with maximum light. Examine at least 300 oil immersion fields on each smear.
V. PROCEDURE (continued)

L. If Strongyloides larvae are found, preserve the rest of the specimen in 10% formalin for teaching purposes.

VI. RESULTS

A. With either the trichrome or iron hematoxylin stain, the protozoan trophozoites and cysts will easily be seen. Oocysts will not be clearly delineated; if suspect organisms are seen, use additional procedures for confirmation (see procedures 9.4.1 and 9.4.2).

B. Cryptosporidium and Isospora oocysts will be visible on permanent stained smears (modified acid-fast procedures) (see procedures 9.4.1 and 9.4.2 for specific directions).

C. Helminth eggs or larvae may not be easily identified on the permanent stained smear but will be visible in the wet preparations.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the organism and stage (trophozoite, cyst, oocyst, etc.). Do not use abbreviations. Confirmation of species may require some type of permanent stained smear.

Examples: Giardia lamblia trophozoites, Strongyloides stercoralis larvae

B. Call the physician if pathogenic organisms are identified.

C. Quantitate C. sinensis eggs if they are recovered.

D. If the results are negative on the wet smear examination, a preliminary report can be sent (based on wet examination only) prior to the examination of the permanent stained smear.

E. See Table 9.6.5–1 for the correct method of quantitation.

VIII. PROCEDURE NOTES

A. If you receive more than 2 ml of specimen, you must centrifuge the specimen (500 × g for 10 min) and examine the mucus or material in the bottom of the tube.

B. Modified acid-fast methods (or immunoassay detection methods) will have to be used for the identification of C. parvum. I. belli can be identified by wet examination or from smears stained with the modified acid-fast methods.

Table 9.6.5–1 Quantitation of parasites, human cells, yeast cells, and artifacts in specimens from the intestinal tract

| Quantity | No. of protozoa, human cells, yeast cells, artifacts | No. of helminths/coverslip
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per 10 oil immersion fields (×1,000)</td>
<td>Per 10 40× fields (×400)</td>
</tr>
<tr>
<td>Few</td>
<td>≤2</td>
<td>≤2</td>
</tr>
<tr>
<td>Moderate</td>
<td>3–9</td>
<td>3–9</td>
</tr>
<tr>
<td>Many</td>
<td>≥10</td>
<td>≥10</td>
</tr>
</tbody>
</table>

* In general, protozoa are not quantitated on the laboratory slip (exception: Blastocystis hominis), but human cells, yeast cells, and artifacts like Charcot-Leyden crystals are normally reported and quantitated.

† Wet preparation. Coverslips are 22 by 22 mm.

‡ PVA smear.

§ Wet preparation.
IX. LIMITATIONS OF THE PROCEDURE

A. Many of the parasites will be caught up in the mucus; therefore, it is very important to centrifuge the specimen and concentrate this mucous material for examination. Centrifugation is mandatory if the specimen is more than 2 ml in volume.

B. Although duodenal aspirate specimens are normally examined as wet preparations, remember that some of the organisms may be missed without additional permanent stains (Cryptosporidium and Isospora species).

REFERENCES


9.6.6 Urogenital Specimens: Direct Saline Mount

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Trichomonas vaginalis infections are primarily diagnosed by detecting live motile flagellates from direct saline (wet) mounts. Microscope slides made from patient specimens can be examined under low and high power for the presence of actively moving organisms.

II. SPECIMENS

A. Vaginal discharge
B. Urethral discharge
C. Penile discharge
D. Urethral-mucosa scrapings
E. First-voided urine with or without prostatic massage

Collect specimens with a platinum loop, cotton or Dacron swab, or speculum. Place these specimens in a small amount (<1.0 ml) of 0.85% NaCl in a test tube or on a microscope slide, and dilute them with a drop of 0.85% NaCl. If the specimen cannot be examined immediately, place the swab in Amies transport medium, which will keep the organisms viable for approximately 24 h. Collect urine specimens in a clean-catch urine collection container. Centrifuge the urine at 500 × g, and examine the sediment for T. vaginalis. Hold all specimens at room temperature, because refrigerator temperatures inhibit motility and have a deleterious effect on the organisms. Returning the specimen to room temperature will not reverse these deleterious morphological changes. Reject any specimens more than 24 h old.

III. MATERIALS

A. Reagents (see Appendix 9.6.6–1)
B. Supplies
1. Disposable glass or plastic pipettes
2. Glass slides (1 by 3 in., or larger if you prefer)
3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)
4. Small tubes containing 0.5 to 1.0 ml of 0.85% NaCl
C. Equipment
1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
3. Tabletop centrifuge
4. Staining rack

Observe standard precautions.
ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Check the direct-mount reagents each time they are used.
   1. The saline should be clear, with no visible contamination.
   2. The iodine should be a strong-tea color, and there should be crystals in the bottom of the bottle. Small aliquots of the stock solution should always be strong-tea color. If not, then discard, and aliquot some stock solution into your dropper bottle.

B. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (see procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

C. Record all QC results.

V. PROCEDURE (1)

A. Apply the patient’s specimen to a small area on a clean microscope slide.
B. Immediately before the specimen dries, add 1 or 2 drops of saline with a pipette.
   If urine sediment is used, the addition of saline may not be necessary.
C. Mix the saline and specimen together with the pipette tip or the corner of the coverslip.
D. Cover the specimen with the no. 1 coverslip.
E. Examine the wet mount with the low-power ($10\times$) objective and low light.
F. Examine the entire coverslip for motile flagellates. Suspicious objects can be examined with the high-power ($40\times$) objective.
G. The organism is usually slightly larger than a PMN, and you should see flagellar movement.

VI. RESULTS

A. If motile flagellates (axostyle and undulating membrane) are seen, then the trophozoites of *T. vaginalis* are present.
B. If nonmotile organisms (axostyle) are visible after staining with D’Antoni’s iodine, then the trophozoites of *T. vaginalis* are present.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the organism. Do not report the organism stage, since there is no known cyst stage for the trichomonads. The organisms do not need to be quantitated.
   Example: *Trichomonas vaginalis* present.
B. If no flagellated organisms are seen, report the specimen as negative for *T. vaginalis*.
   Example: No *Trichomonas vaginalis* seen.

VIII. PROCEDURE NOTES

A. It is very important that specimens to be examined for *T. vaginalis* be delivered to the laboratory within 1 h after collection.
B. After 1 h, organisms will lose their motility, particularly when they begin to dry out.
C. If a dry smear is delivered to the laboratory, salvage it by fixing the dry smear as you would a thin blood film (absolute methanol) and stain it with Giemsa at a 1:20 dilution for at least 20 min (see procedure 9.8.5). The stained organisms may be difficult to see, but if you can actually see and identify the organisms as *T. vaginalis*, that information may be clinically relevant.

D. Calgiswabs are not recommended (tight adherence of specimen to swab). Reject the specimen if submitted on this type of swab.

E. Swabs received in charcoal transport media are unacceptable because the charcoal particles make it difficult to visualize organisms.

F. When the specimen is examined microscopically, always confirm that no fecal contamination (artifacts, vegetable debris, etc.) is present. This type of contamination is rare and would probably be limited to a urine specimen. However, if a urine or other urogenital specimen was contaminated with fecal material, it is possible that *Pentatrichomonas hominis* (nonpathogen found in the intestinal tract) could be misidentified as *T. vaginalis*, an identification that implies sexual transmission.

IX. LIMITATIONS OF THE PROCEDURE

A. If the specimen is left at room temperature or held at refrigerator temperature for a prolonged period (usually >1 h), the organisms will round up, lose their motility, and eventually die. Motility may occasionally be enhanced by warming the specimen to 37°C, but this will not revive dying organisms.

B. Wet mounts have been reported to detect *T. vaginalis* in 75 to 85% of infected patients. **Alternative diagnostic methods may include culture, use of monoclonal antigen detection kits, use of permanent stained slides, and collection of a second sample for examination.**

C. If the patient has a *P. hominis* intestinal infection and the urogenital specimen becomes contaminated with fecal material, a false-positive *T. vaginalis* result may be reported, because *P. hominis* and *T. vaginalis* are similar in shape.

REFERENCE


SUPPLEMENTAL READING


APPENDIX 9.6.6–1

Reagents

Provide QC information on reagent container and in QC records.

A. 0.85% NaCl

1. Dissolve in distilled water in an appropriate glass flask by using a magnetic stirrer.

   
   
   sodium chloride (NaCl) ..................... 850 mg
   
   distilled water ........................... 100 ml

2. Store in a glass bottle.

3. Label as 0.85% NaCl with a preparation date and an expiration date of 6 months. Store at room temperature.
B. Modified D’Antoni’s stock iodine

1. Dissolve in distilled water with an appropriate glass flask by using a magnetic stirrer.

   potassium iodide (KI) .................. 1.0 g
   powdered iodine crystals ............. 1.5 g
   distilled water ........................ 100 ml

2. The D’Antoni’s solution should be saturated with iodine, with some excess crystals left in the bottle. Store in a brown bottle at room temperature. The stock solution remains good as long as an excess of iodine crystals remains on the bottom of the bottle.

3. Label as D’Antoni’s stock iodine with the preparation date and an expiration date of 1 year.

4. Small amounts of stock iodine solution can be aliquoted into brown dropper bottles for routine daily use. The expiration date will be from 30 to 60 days, depending on the amount of fading of the solution from the normal strong-tea color (small dropper bottles and the use of clear glass will result in a shorter expiration time). The use of a brown bottle will lengthen the expiration time.
9.6.7 Urogenital Specimens: Permanent Stained Smear (Giemsa)

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

_Trichomonas vaginalis_ infections are primarily diagnosed from direct saline (wet) mounts by detecting live motile flagellates. Permanently stained smears can be made from patient specimens for specific identification of the organism. Although a number of stains can be used, Giemsa and Papanicolaou stains are the ones most frequently used to diagnose _T. vaginalis_ infections.

II. SPECIMEN

A. Vaginal discharge
B. Urethral discharge
C. Penile discharge
D. Urethral-mucosa scrapings
E. First-voided urine with or without prostatic massage

Collect specimens with a platinum loop, cotton-Dacron swab, or speculum. Place these specimens in a small amount (<1.0 ml) of 0.85% NaCl in a test tube, or smear them directly onto a microscope slide. Use a drop of 0.85% NaCl to dilute the direct smear when it is placed on the slide. Air dry slides prepared in this manner before transporting them to the laboratory. Place specimens collected with a cotton-Dacron swab in Amies transport medium if the specimen cannot be processed immediately. Organisms will remain viable for approximately 24 h in Amies transport medium. Collect urine specimens in a clean-catch urine collection container. Centrifuge the urine at 500 × g for 5 min, and examine the sediment for _T. vaginalis_. Hold all specimens at room temperature, because refrigerator temperatures have a deleterious effect on the organisms. Returning the specimen to room temperature will not reverse these deleterious morphological changes. Reject any specimens more than 24 h old.

III. MATERIALS

A. Reagents (see Appendix 9.6.7–1)
B. Supplies
   1. Disposable glass or plastic pipettes
   2. Glass slides (1 by 3 in., or larger if you prefer)
   3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)
   4. Small tubes containing 0.5 to 1.0 ml of 0.85% NaCl
   5. 2 Coplin jars
C. Equipment
   1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
   2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
   3. Tabletop centrifuge (for tubes containing specimen and 0.85% NaCl if the specimen is submitted in saline)
**ANALYTICAL CONSIDERATIONS**

### IV. QUALITY CONTROL

A. Check the direct-mount reagents each time they are used.
   1. The saline should be clear, with no visible contamination.
   2. Giemsa stain
      A peripheral blood film may be used to quality control the Giemsa stain. For staining characteristics, see procedure 9.8.5.
   3. Phosphate buffer
      Check the buffer each time you use it. The buffer should be clear, with no signs of visible contamination or precipitates. The pH should be between 6.8 and 7.2.
   4. Review the Giemsa-stained control slide before searching the patient’s specimen for the organism. If there was potential fecal contamination of the specimen, you may have to differentiate *T. vaginalis* from *Pentatrichomonas hominis*.

B. The microscope should have been calibrated within the last 12 months, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (see procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

C. Record all QC results.

### V. PROCEDURE (1)

A. Apply patient’s specimen to a small area of a clean microscope slide.
B. Immediately before the specimen dries, add 1 or 2 drops of saline with a pipette.
   If urine sediment is used, the addition of saline may not be necessary.
C. Mix the saline and specimen together with the pipette tip.
D. Air dry the slide.
E. Fix in absolute methanol for 1 min (Coplin jar or slide staining rack).
F. Place the slide in the Giemsa solution, and stain for the desired time, depending on the stain dilution used (20 min at 1:20 dilution).
G. Rinse the slide with tap water (gently running or in a Coplin jar) to remove excess stain solution.
H. Air dry the slide. Do not apply a coverslip.
I. Examine the slide with the oil (100×) objective.
J. Examine the entire smear for flagellates.
K. The organism is usually slightly larger than a PMN. The organism is 7 to 23 μm long and 5 to 15 μm wide. Differential characteristics to be observed include anterior flagella, undulating membrane, axostyle, and nucleus.

### VI. RESULTS

If organisms (axostyle) are visible after staining with Giemsa stain, then the trophozoites of *T. vaginalis* are present.
POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the organism. Do not report the organism stage, since there is no known cyst stage for the trichomonads. The organisms do not need to be quantitated.
   
   Example: *Trichomonas vaginalis* present.

B. If no flagellated organisms (axostyle and undulating membrane) are seen, report the specimen as negative for *T. vaginalis*.
   
   Example: No *Trichomonas vaginalis* seen.

VIII. PROCEDURE NOTES

A. It is very important that specimens to be examined for *T. vaginalis* be delivered to the laboratory within 1 h after collection.

B. After 1 h, organisms will lose their motility, particularly when they begin to dry out, and the morphology on the permanent stained smear will be difficult to see.

C. If a dry smear is delivered to the laboratory, salvage it by fixing the dry smear as you would a thin blood film (absolute methanol) and staining it with Giemsa at a 1:20 dilution for at least 20 min (see procedure 9.8.5). The stained organisms may be difficult to see, but if you can actually see and identify the organisms as *T. vaginalis*, that information may be clinically relevant.

D. Calgiswabs are not recommended (tight adherence of specimen to swab). Reject the specimen if submitted on this type of swab.

E. Smears made from swabs received in charcoal transport media are unacceptable for staining. The charcoal particles make it difficult to visualize the organisms.

F. When the specimen is examined microscopically, always confirm that no fecal contamination (artifacts, vegetable debris, etc.) is present. This type of contamination is rare and would probably be limited to a urine specimen. However, if a urine or other urogenital specimen was contaminated with fecal material, it is possible that *P. hominis* (nonpathogen found in the intestinal tract) could be misidentified as *T. vaginalis*, an identification that implies sexual transmission.

IX. LIMITATIONS OF THE PROCEDURE

A. If the specimen is left at room temperature or held at a refrigerator temperature for a prolonged period (usually >1 h), the organisms will round up and eventually die.

B. If the patient has a *P. hominis* intestinal infection and the urogenital specimen becomes contaminated with fecal material, a false-positive *T. vaginalis* result may be reported, because *P. hominis* and *T. vaginalis* are similar in shape. The position of the undulating membrane will allow differentiation between *T. vaginalis* and *P. hominis*.

REFERENCE


SUPPLEMENTAL READING


APPENDIX 9.6.7–1

Include QC information on reagent container and in QC records.

APPENDIX 9.6.7–2

Reagents

- Indicate the expiration date on the label and in the work record or on the manufacturer’s label.

A. 0.85% NaCl

1. Dissolve in distilled water in an appropriate glass flask by using a magnetic stirrer.
   
   sodium chloride (NaCl) ..................... 850 mg
   distilled water ................................. 100 ml

2. Store in a glass bottle.
3. Label as 0.85% NaCl with a preparation date and an expiration date of 6 months.
   Store at room temperature.

B. Giemsa stain
   For preparation of Giemsa stain and phosphate buffer solutions, see procedure 9.8.5.

C. Absolute methanol

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>T. vaginalis</th>
<th>P. hominis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>7–23 μm long</td>
<td>5–15 μm long</td>
</tr>
<tr>
<td></td>
<td>5–15 μm wide</td>
<td>7–10 μm wide</td>
</tr>
<tr>
<td>Shape</td>
<td>Pear</td>
<td>Pear</td>
</tr>
<tr>
<td>Flagella</td>
<td>4 anterior</td>
<td>3–5 anterior</td>
</tr>
<tr>
<td>Undulating membrane</td>
<td>Extends half of organism length</td>
<td>Extends entire length with free trailing flagella</td>
</tr>
<tr>
<td>Axostyle</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Nucleus</td>
<td>Anterior end, oval</td>
<td>Anterior end, oval</td>
</tr>
</tbody>
</table>
# Urine Concentration: Centrifugation

## PREANALYTICAL CONSIDERATIONS

### I. PRINCIPLE

Helminthic larval stages and eggs and some protozoa infecting humans may be found in the urine whether or not they cause pathologic sequelae in the urinary tract. Filariasis can be diagnosed from urine samples, and *Trichomonas vaginalis* and *Schistosoma haematobium* eggs can be detected in the urine. Microfilariae may be detected in the urine of heavily infected patients or of patients recently treated with diethylcarbamazine. Some of the microsporidia, such as *Encephalitozoon* (*Septata*) *intestinalis*, can be found in the urine, particularly that of immunosuppressed patients, including those with AIDS.

### II. SPECIMENS

*Urine*

Observe standard precautions.

**A. T. vaginalis**

Collection of first-voided urine, particularly after prostatic massage in male patients, is useful for the diagnosis of this infection.

**B. S. haematobium**

Collection of a midday urine specimen or a 24-h collection in a container without preservatives is recommended. Peak egg excretion occurs between noon and 3 p.m. In patients with hematuria, eggs may be found trapped in the blood and mucus in the terminal portion (last-voided portion) of the urine specimen.

**C. Filariasis**

Microfilariae may be detected in urine of patients with chyluria, of patients with very heavy filarial infections, and of patients treated with diethylcarbamazine.

**D. Microsporidia**

Microsporidial spores may be detected in concentrated urine of patients who are immunosuppressed, including those with AIDS. A number of different stains can be used, such as modified trichrome and optical brightening agents.

### III. MATERIALS

**A. Reagent** *(see Appendix 9.6.8–1)*

**B. Supplies**

1. Disposable glass or plastic pipettes
2. Glass slides (1 by 3 in., or larger if you prefer)
3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)
4. Small tubes containing 0.5 to 1.0 ml of 0.85% NaCl
5. Conical centrifuge tubes (15 ml)
6. Erlenmeyer flask (1,000 ml)

**C. Equipment**

1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
3. Tabletop centrifuge (for tubes containing specimen and 0.85% NaCl if the specimen is submitted in saline)
IV. QUALITY CONTROL

A. Check the direct-mount reagent each time it is used. The saline should be clear, with no visible contamination.
B. The microscope should have been calibrated within the last 12 months, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (see procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.
C. Record all QC results.

V. PROCEDURE

A. If a 24-h urine sample was collected, allow the specimen to sediment for 2 h, and decant the major portion of the supernatant. There may be 100 to 200 ml of sediment left. If a first-voided urine specimen is received, use the entire specimen.
B. Place the remaining urine specimen (sediment) in centrifuge tubes.
C. Centrifuge the specimen at 500 × g for 5 min.
D. Decant the supernatant fluid.
E. With a pipette, mix and aspirate the sediment.
F. Place 1 drop of the sediment on a microscope slide.
G. Place a coverslip on top of the sediment.
H. Observe the specimen under the coverslip at magnifications of ×100 and ×400. Examine the entire coverslip at ×100 and at least half the coverslip at ×400.

VI. RESULTS

A. If motile flagellates are seen (axostyle and undulating membrane), then the trophozoites of *T. vaginalis* are present.
B. If live microfilariae are seen, then confirm or accomplish species identification by using permanent stains (1) (see procedures 9.8.5, 9.8.6, and 9.8.8).
C. If eggs of *S. haematobium* are seen, observe the eggs for live miracidia. If flame cell activity (motile cilia) is detected inside the miracidium larva, the miracidium is viable. A hatching test may also be used to determine if the eggs are viable (1) (see procedure 9.5.5).

VII. REPORTING RESULTS

A. *T. vaginalis*
   Report the organism. Do not report the stage, since there is no known cyst stage for the trichomonads. The organisms do not need to be quantitated.
   Example: *Trichomonas vaginalis* present.

B. Filariae
   Report the presence of microfilariae. Genus and species should be reported if possible. The organisms do not need to be quantitated.
   Example: *Wuchereria bancrofti* microfilariae present.
C. *S. haematobium*

If eggs are present, report the genus and species and whether the eggs are viable or nonviable.

**Examples:** *Schistosoma haematobium* eggs present (viable eggs seen). *Schistosoma haematobium* eggs present (nonviable, eggshells only).

**VIII. PROCEDURE NOTES**

A. Specimens to be examined for *T. vaginalis* should be delivered to the laboratory as soon as possible after collection. Hold all specimens at room temperature because refrigerator temperatures will have a deleterious effect on *T. vaginalis*.

B. Species identification of the microfilariae from unstained preparations (urine sediment) may not be possible, and permanent stains may be necessary for further identification (1).

C. It is very important that all urine specimens (24-h and single-voided specimens) be collected with no preservatives. It is clinically important to determine whether the eggs are viable. This can be accomplished by examining eggs in the wet preparations at ×400.

D. It is possible, although much less likely, that *Schistosoma mansoni* or *Schistosoma japonicum* eggs could also be recovered in urine. Therefore, the egg morphology must be carefully examined for accurate identification to species.

E. For the detection of *T. vaginalis*, filariae, and schistosomes, reject specimens, including midday urine samples, that are more than 24 h old.

F. Reject all 24-h urine specimens that are more than 48 h old.

G. When the specimen is examined microscopically, always confirm that no fecal contamination (artifacts, vegetable debris, etc.) is present. This type of contamination is rare and would probably be limited to a urine specimen. However, if a urine or other urogenital specimen was contaminated with fecal material, it is possible that *Pentatrichomonas hominis* (nonpathogen found in the intestinal tract) could be misidentified as *T. vaginalis*, an identification that implies sexual transmission.

**IX. LIMITATIONS OF THE PROCEDURE**

A. If the urine specimen is left at room temperature or held at a low temperature for a prolonged period, *T. vaginalis* may round up, become nonmotile, and eventually die.

B. If the patient has a *P. hominis* intestinal infection and the urogenital specimen becomes contaminated with fecal material, a false-positive *T. vaginalis* result may be reported because *P. hominis* and *T. vaginalis* are similar in shape.

C. Microfilariae can be identified to the species level only by making a permanent stained slide from the specimen.

**REFERENCE**


**SUPPLEMENTAL READING**


Reagent

- Indicate the expiration date on the label and in the work record or on the manufacturer's label.

0.85% NaCl

A. Dissolve in distilled water in an appropriate glass flask by using a magnetic stirrer.

- sodium chloride (NaCl) ................ 850 mg
- distilled water .......................... 100 ml

B. Store in a glass bottle.
C. Label as 0.85% NaCl with a preparation date and an expiration date of 6 months. Store at room temperature.

Include QC information on reagent container and in QC records.
**9.6.9**

**Urine Concentration: Membrane Filter (Nuclepore)**

**PREANALYTICAL CONSIDERATIONS**

**I. PRINCIPLE**

Microfilariae may be detected in the urine of heavily infected patients or of patients recently treated with diethylcarbamazine. Eggs of *Schistosoma haematobium* can also be recovered in urine specimens (2). Microfilariae and *S. haematobium* eggs can be easily concentrated by passing the specimen through a membrane filter. The filter can then be observed through a microscope.

**II. SPECIMENS**

Urine

**A. Filariasis**

Microfilariae may be detected in urine of patients with chyluria, of patients with very heavy filarial infections, and of patients treated with diethylcarbamazine. Collect specimens as first-voided specimens or as a 24-h collection in a container without preservatives.

**B. S. haematobium**

Collection of a midday urine specimen or a 24-h collection in a container without preservatives is recommended. Peak egg excretion occurs between noon and 3 p.m. In patients with hematuria, eggs may be found trapped in the blood and mucus in the terminal portion (last-voided portion) of the urine specimen.

**III. MATERIALS**

**A. Reagent** (see Appendix 9.6.9–1)

**B. Supplies**

1. Disposable glass or plastic pipettes
2. Glass slides (1 by 3 in., or larger if you prefer)
3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)
4. Small tubes containing 0.5 to 1.0 ml of 0.85% NaCl
5. Nuclepore membrane filters (25 mm) of 3-, 5-, and 8-μm pore sizes
6. Filter holder (25 mm) that attaches to syringe
7. Syringe (10 ml)
8. Forceps

**C. Equipment**

1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.

**Observe standard precautions.**
ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Check the direct-mount reagent each time it is used. The saline should be clear, with no visible contamination.

B. The microscope should have been calibrated within the last 12 months, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (see procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

C. Record all QC results.

V. PROCEDURE

A. If a midday urine sample is used, proceed to step V.B; if a 24-h urine sample was collected for S. haematobium diagnosis, allow the specimen to sediment for 2 h, and decant the major portion of the supernatant. There may be 100 to 200 ml of sediment left.

B. Thoroughly mix the urine specimen.

C. Draw 10 ml of urine into the syringe. If the urine is excessively cloudy or turbid, 10 ml may have to be used.

D. Attach the filter holder containing the filter to the syringe. For S. haematobium, use 8-μm-pore-size filters; for Wuchereria bancrofti, Brugia malayi, and Loa loa, use 5-μm-pore-size filters; and for Mansonella species, use 3-μm-pore-size filters.

E. Express the urine through the filter.

F. Wash the membrane with physiological saline by removing the filter holder, drawing 10 ml of saline into the syringe, reattaching the filter holder, and expressing the saline through the filter.

G. Repeat step V.F, but fill the syringe with air instead of saline, and express the air through the filter.

H. Remove the filter holder from the syringe.

I. Disassemble the filter holder to expose the filter.

J. Remove the filter from the holder with forceps.

K. Place the filter upside down on a microscope slide.

L. With a Pasteur pipette, add 1 drop of saline to moisten the filter.

M. Examine the filter for microfilariae and eggs at a magnification of ×100.

VI. RESULTS

A. If live microfilariae are seen, then confirm or accomplish species identification by using permanent stains (1) (see procedures 9.8.5, 9.8.6, and 9.8.8).

B. If eggs of S. haematobium are seen, observe the eggs for live miracidia. If flame cell activity (motile cilia) is detected inside the miracidium larva, the miracidium is viable. A hatching test may also be used to determine if the eggs are viable (1).
POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Filariae
   Report the presence of microfilariae. Genus and species should be reported if possible. The organisms do not need to be quantitated.
   Example: *Wuchereria bancrofti* microfilariae present.

B. *S. haematobium*
   If eggs are present, report the genus and species and whether the eggs are viable or nonviable.
   Examples: *Schistosoma haematobium* eggs present (viable eggs seen).
               *Schistosoma haematobium* eggs present (nonviable, egg shells only).

VIII. PROCEDURE NOTES

A. Species identification of the microfilariae may not be possible from unstained preparations (urine sediment), and permanent stains may be necessary for further identification (1). Microfilariae will measure 3 to 10 μm in width by 160 to 330 μm in length. Depending on the species, a sheath may or may not be present.

B. It is very important that all urine specimens (24-h and single-voided specimens) be collected with no preservatives. It is clinically important to determine whether the eggs are viable. This can be accomplished by examining eggs in the wet preparations at ×400.

C. For the detection of filariae and schistosomes, reject specimens, including midday urine samples, that are more than 24 h old.

D. Reject all 24-h urine specimens that are more than 48 h old.

E. If you accidentally put the filter right side up, don’t add more than 1 drop of saline (the organisms may accidentally float off the filter and onto the glass slide).

IX. LIMITATIONS OF THE PROCEDURE

A. Microfilariae can be identified to the species level only by making a permanent stained slide from the specimen. For a method that uses the membrane filter, refer to page 807 in Garcia (1).

B. A hatching test may also be used to determine if the eggs are viable. For a hatching method, refer to procedure 9.5.5.

C. Infrequently, eggs of other *Schistosoma* species may be recovered in the urine.

REFERENCES


SUPPLEMENTAL READING

APPENDIX 9.6.9–1

Include QC information on reagent container and in QC records.

Reagent

- Indicate the expiration date on the label and in the work record or on the manufacturer’s label.

0.85% NaCl

A. Dissolve in distilled water in an appropriate glass flask by using a magnetic stirrer.

sodium chloride (NaCl) ................. 850 mg
distilled water ................................ 100 ml

B. Store in a glass bottle.

C. Label as 0.85% NaCl with a preparation date and an expiration date of 6 months. Store at room temperature.

APPENDIX 9.6.9–2

Diagram of Nuclepore filtration system for the recovery of microfilariae. From top to bottom: syringe in sterile container (sterile syringe is not necessary) (top left) and needle (top right), slide (center left), package of filtration membranes (center middle), filter holder (center right, two pieces), and complete apparatus (bottom). (Illustration by Sharon Belkin.)

APPENDIX 9.6.9–3

Characterization of schistosome eggs

<table>
<thead>
<tr>
<th>Species</th>
<th>Size</th>
<th>Shape</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schistosoma haematobium</td>
<td>112–170 by 40–70 μm</td>
<td>Elongate, terminal spine</td>
</tr>
<tr>
<td>Schistosoma japonicum</td>
<td>55–85 by 40–60 μm</td>
<td>Oval, minute lateral spine</td>
</tr>
<tr>
<td>Schistosoma mansoni</td>
<td>114–180 by 45–73 μm</td>
<td>Elongate, prominent lateral spine</td>
</tr>
</tbody>
</table>
9.7.1 Expectorated Sputum: Direct-Mount and Stained Preparations

**PREANALYTICAL CONSIDERATIONS**

I. **PRINCIPLE**

A. A direct smear can be used to detect large or motile organisms from the lung. Parasites which can be detected and may cause pneumonia, pneumonitis, or Loeffler’s syndrome include *Entamoeba histolytica*, *Paragonimus* spp., *Strongyloides stercoralis*, *Ascaris lumbricoides*, and hookworm. The smears can be examined with and without the addition of D’Antoni’s or Lugol’s iodine (see procedure 9.3.3).

B. Trichrome stains of material may aid in differentiating *E. histolytica* from *Entamoeba gingivalis*, and Giemsa stain may better define larvae and juvenile worms. Prepare a stain of material if organisms are found in examination of direct mounts which require additional differentiation.

C. Although *Cryptosporidium parvum* will be difficult to see in a direct mount, examination of smears stained with modified acid-fast stains (hot or cold method) may provide confirmation of pulmonary cryptosporidiosis.

D. In order to see microsporidial spores, centrifuged specimens stained with modified trichrome stains or optical brightening agents will be required (500 \( \times \) for 10 min).

II. **SPECIMEN**

**Observe standard precautions.**

A. Expectorated sputum specimens are collected after patient instruction in the appropriate measures to take to ensure quality specimens, including mouth wash before expectorating and exclusion of saliva from specimens.

B. Transport specimens to the laboratory in clean, closed containers. Select any blood-tinged, viscous areas for sampling.

C. If the specimen is uniformly mucoid, do the following.

1. Remove a 1.0-ml portion to a 15-ml conical tube.
2. Add 1.0 ml of mucolytic agent such as Sputolysin that has been prepared according to the manufacturer’s instructions.
3. Incubate at room temperature for 15 min.
4. Add 2.0 ml of phosphate buffer (pH 6.8, 0.067 M).
5. Centrifuge the material at 1,000 \( \times \) for 5 min.
6. Decant supernatant, and use pelleted material to prepare wet mounts and smears.
III. MATERIALS

A. **Reagents** *(see Appendix 9.7.1–1)*

B. **Supplies**
   1. Pasteur pipettes
   2. 1- and 10-ml pipettes
   3. Glass slides (2 by 3 in.)
   4. Glass slides (1 by 3 in.)
   5. 22- by 22-mm coverslips; no. 1 thickness
   6. 15-ml conical centrifuge tubes
   7. Coplin jars with lids
   8. Protective gloves

C. **Equipment**
   1. Centrifuge with carriers for 15-ml tubes with safety caps
   2. Binocular microscope with 10×, 40×, and 100× (oil immersion) objectives; 50× oil objective optional but very helpful
   3. Oculars should be 10×. Some may prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Saline (0.85% NaCl), clear and free of particulate material. If cloudy, discard.
B. Mucolytic agent free of contamination as determined by clear appearance. If cloudy, discard, and make new working solution.
C. Do a control trichrome stain for each new set of reagents with a specimen containing blood to see that WBCs stain with purple nuclei and blue-green cytoplasm *(see procedure 9.3.6)*. If cells do not stain appropriately, change reagents.
D. With each new lot of Giemsa stain or new buffer, check stain with a specimen containing blood to see that RBCs stain grayish, WBC nuclei stain red-purple, and WBC cytoplasm stains bluish. If cells do not stain appropriately, check stock stain and buffer to find cause.
E. The microscope(s) should be calibrated, and the original optics used for the calibration should be in place on the microscope(s). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.
F. Clean and check centrifuge for speed, and clean brushes once a year.
G. Maintain temperature of refrigerator where mucolytic-agent working solution is stored at 4°C (range, 2 to 8°C).
   Make fresh working solution each day. Use a clean, dry pipette to remove stain from stock stain container.
I. Record all QC results.

V. PROCEDURE

A. **Wear gloves when performing this procedure.**
B. Expectorated sputum (untreated with mucolytic agent)
   1. With a Pasteur pipette, place 1 or 2 drops (50 μl) on one side of a glass slide (2 by 3 in.), and cover with a no. 1 coverslip (22 by 22 mm).
   2. Place a second drop on the slide, add 1 drop of saline, and cover with a coverslip.
C. Material that has been treated with a mucolytic agent can be suspended in 100 μl of saline. Place 1 drop of the mixture on a slide (2 by 3 in.), and cover with a coverslip.
D. Reserve the specimen and remaining treated specimen for preparation of smears for staining should stains be required.
V. PROCEDURE (continued)

E. Examine the wet preparations field by field with low light and the 10× objective to detect eggs, larvae, oocysts, or amebic trophozoites.

F. If inconclusive, prepare smears of material for staining.
   1. Place 1 drop of sediment in the center of each of three glass slides (1 by 3 in.), and spread the material with the tip of the pipette.
   2. Place one slide in Schaudinn’s fixative while wet, and dry the other two thoroughly.
   3. Trichrome stain the slide fixed in Schaudinn’s fixative (see procedure 9.3.6).
   4. Fix the air-dried smears in methanol. Stain one with Giemsa (see procedure 9.8.5) and the other with a modified acid-fast stain (see procedures 9.4.1 and 9.4.2).
   5. Put immersion oil on stained smears, and examine Giemsa-stained smear with the 10× objective and trichrome-stained smear with the 50× oil objective if available. Otherwise, use the 100× oil objective.

VI. RESULTS

A. Helminth larvae (rare) are more likely to be seen in the wet preparation with and/or without iodine (see procedures 9.3.3 and 9.3.4).

B. Protozoan trophozoites will generally be visible in the trichrome-stained smears.

C. Oocysts will be seen on the modified acid-fast preparation.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

Record any amebae, oocysts, larvae, or eggs detected by wet mounts.

A. Give genus and species of amebae after confirmation with permanent stain.

B. “No parasites found” in expectorated sputum are considered “normal flora”; therefore, call the physician if any organisms are detected.

VIII. PROCEDURE NOTES

In the United States, detection of parasites in expectorated sputum is rare. If characteristics of suspected parasites are not “classic,” request another specimen for confirmation.

IX. LIMITATIONS OF THE PROCEDURE

A. Although there are rare reports of detection of Pneumocystis carinii in expectorated sputum, this method cannot be recommended for detection of this organism (1).

B. If C. parvum is suspected, remember to centrifuge the sputum specimen for 10 min at 500 × g. Otherwise, the oocysts may not be found in the sediment used to prepare smears for modified acid-fast stains.

REFERENCES


SUPPLEMENTAL READING

Reagents

Indicate the expiration date on the label and in the work record or on the manufacturer’s label.

A. Mucolytic agent such as Sputolysin Stat-Pack dithiothreitol solution (Behring Diagnostics, Inc.)
   1. Store unopened at room temperature until expiration date published on package.
   2. Store working solution at 4°C. Include the date made and the expiration date. Discard working solution after 48 h. Prepare working solution by removing 1.0 ml from the 10-ml bottle and diluting with 9.0 ml of sterile water.

B. Iodine reagents
   1. D’Antoni’s iodine (see procedure 9.3.3)
   2. Lugol’s iodine (see procedure 9.3.3)

C. Trichrome stain reagents
   1. Schaudinn’s fixative (see procedure 9.2.2)
   2. Trichrome stain (see procedure 9.3.6)

D. Giemsa stain reagents
   1. Giemsa stain (see procedure 9.8.5)
   2. Phosphate buffer, pH 7.0 to 7.2 (see procedure 9.8.5)
   3. Phosphate buffer plus Triton X-100 at 0.01% (see procedure 9.8.5)

Phosphate buffer solutions

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<tr>
<th>pH</th>
<th>Na₂HPO₄ (9.5 g/liter) (anhydrous) (ml)</th>
<th>NaH₂PO₄·H₂O (9.2 g/liter) (ml)</th>
<th>Distilled water (ml)</th>
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<tr>
<td>6.6</td>
<td>37.5</td>
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<td>900</td>
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<tr>
<td>6.8</td>
<td>49.6</td>
<td>50.4</td>
<td>900</td>
</tr>
<tr>
<td>7.0</td>
<td>61.1</td>
<td>38.9</td>
<td>900</td>
</tr>
<tr>
<td>7.2</td>
<td>72.0</td>
<td>28.0</td>
<td>900</td>
</tr>
</tbody>
</table>

Include QC information on reagent container and in QC records.
9.7.2 Aspirates and Bronchoscopy Specimens

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The examination of aspirated material for the diagnosis of parasitic infections is useful when routine specimens and methods have failed to demonstrate the organisms. Aspirates include liquid specimens collected from a variety of anatomic sites that delineate the types of organisms to be expected. Aspirates most commonly processed in the parasitology laboratory include fine-needle aspirates and duodenal aspirates. Fluid specimens collected by bronchoscopy include bronchoalveolar lavage (BAL) fluids and bronchial washings.

II. SPECIMENS

A. Fine-needle aspirates

When specimens are collected and sent to the laboratory for processing, slides must be stained appropriately for suspected organisms and examined microscopically. Suggested stains are Giemsa and methenamine-silver nitrate for Pneumocystis carinii, Giemsa for Toxoplasma gondii, trichrome for amebae, and modified acid fast for Cryptosporidium parvum.

B. Aspirates of cysts and abscesses

Aspirates to be evaluated for amebae may require concentration by centrifugation, digestion (streptokinase; see Appendix 9.7.2–1), microscopic examination for motile organisms in direct preparations, and cultures and microscopic evaluation of stained preparations (12).

C. Duodenal aspirates

Aspirates to be evaluated for Strongyloides stercoralis, Giardia lamblia, or Cryptosporidium may require concentration by centrifugation prior to microscopic examination for motile organisms and permanent stains. In order to see microsporidial spores, centrifuged sediment stained with modified trichrome stains or optical brightening agents will be required.

D. Bone marrow aspirates

Aspirates to be evaluated for Leishmania amastigotes, Trypanosoma cruzi amastigotes, or Plasmodium spp. require Giemsa staining.

E. Fluid specimens collected by bronchoscopy

Specimens may be lavage fluids or washings, with BAL fluids preferred (11). Specimens are usually concentrated by centrifugation prior to microscopic examination of stained preparations (Sputolysin; see Appendix 9.7.2–1). Organisms discussed here which may be detected are P. carinii, T. gondii, C. parvum, and the microsporidia (4, 9).

Observe standard precautions.
III. MATERIALS

A. Reagents (see Appendix 9.7.2–1)

B. Supplies
1. Pasteur pipettes
2. 1- and 10-ml pipettes
3. Glass slides (2 by 3 in.)
4. Glass slides (1 by 3 in.)
5. Coverslips (22 by 22 mm; no. 1 thickness)
6. Centrifuge tubes, 15 and 50 ml, conical
7. Glass Coplin jars
8. Plastic Coplin jar with lid
9. Stain rack
10. Graduated cylinder
11. Bleach
12. Protective gloves
13. Nocardia asteroides ATCC 19247

C. Equipment
1. Centrifuge with carriers for 15- and 50-ml tubes with safety caps
2. Binocular microscope with 10×, 40× or 50× oil, and 100× oil immersion objectives
3. Fluorescence microscope with filters to obtain appropriate wavelength of light for fluorescent label used if immunostaining
4. Microwave oven or water bath and thermometer

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Incorporate control slides in all stain procedures.
1. Yeast-containing material may be used as a positive control for silver staining.
2. Material containing RBCs and WBCs should be used as a control for Giemsa staining.
3. Both P. carinii-positive and P. carinii-negative but yeast-positive material should be used for immunospecific staining.
4. Stains cannot be evaluated if controls do not stain appropriately.

B. Material containing RBCs and WBCs should be used as control for Giemsa (see procedure 9.8.5) and trichrome (see procedure 9.3.6) stains.

C. Alcohol-fixed slides of N. asteroides ATCC 19247 culture serve as a control for modified acid-fast stain.

D. Use both P. carinii-positive and -negative controls for immunospecific staining.

E. Both Sputolysin and streptokinase working reagents should be stored at 4°C and made fresh after 48 h.

F. Incubate new lots of media to check for sterility, and inoculate samples with stock amebae to see that medium supports growth (see procedures 9.9.1 to 9.9.3 and 9.9.5).

G. Clean and check centrifuge for speed, and clean brushes once a year.

H. Maintain temperature of refrigerator where mucolytic agent working solution is stored at 4°C (range, 2 to 8°C).

I. With each new lot of Giemsa stain or new buffer, check stain with a specimen containing blood to see that RBCs stain grayish, WBC nuclei stain red-purple, and WBC cytoplasm stains bluish. If cells do not stain appropriately, check stock stain and buffer to find cause (see procedure 9.8.5).

J. Fluorescence microscope can be evaluated for correct light wavelength for label with commercially available QC slides.

K. Check incubator for temperature maintenance at 35 ± 2°C.

L. Record all QC results.

V. PROCEDURE

A. Wear gloves when performing this procedure.

B. Specimens that contain mucus may be treated with a mucolytic agent by adding a volume of agent equal to or one-half to two-thirds of the volume of specimen and incubating at room temperature for 15 min. Centrifuge at 1,000 × g for 5 min, and use sediment to prepare wet mounts and smears for staining.
V. PROCEDURE (continued)

C. Specimens that contain cell debris and proteinaceous material and that require digestion should be treated with streptokinase (1 part enzyme solution to 5 parts specimen) for 1 h at 35°C. Shake at intervals (every 15 min). Centrifuge at 1,000 × g for 5 min, and use sediment to prepare wet mounts and smears for staining.

D. Specimens that include significant amounts of blood require treatment with an agent to lyse RBCs. Add 1 volume of lysing agent per volume of specimen, and incubate at room temperature for 5 min.

E. Place representative samples of untreated and Lyse-treated specimens in 15-ml conical centrifuge tubes, and centrifuge at 1,000 × g for 5 min. For BAL or bronchial-washing specimens, which usually are 50 ml or more, place 20 to 24 ml of each specimen in a 50-ml conical centrifuge tube, and centrifuge as described above.

F. Decant supernatants from centrifuged samples into a disposal container containing disinfectant.

G. With a Pasteur pipette, remove drops of sediment for wet mounts, stain preparations, and culture.

H. For duodenal aspirates and aspirates from cysts or abscesses, place 1 drop of sediment on a glass slide (2 by 3 in.), add a drop of 0.85% NaCl, and cover with a no. 1 coverslip (22 by 22 mm).

I. Examine preparation field by field with low light until the entire mount has been examined.

J. If the wet mount is equivocal for a protozoan, place a drop of sediment on a glass slide (1 by 3 in.), and add a drop of polyvinyl alcohol (PVA) fixative. Mix the drops with a pipette, and spread the mixture into an even film (about 22 by 22 mm). Dry the preparation thoroughly, and stain with trichrome stain.

K. Trichrome stain (see procedure 9.3.6)

L. For material from cysts or abscesses, prepare cultures by adding 0.5 ml of material to a tube of culture medium for the recovery of amebae in accordance with procedure 9.9.1.

M. Aspirates of bone marrow may be submitted for diagnosis of leishmaniasis, trypanosomiasis, and occasionally for malaria. Material should be stained with Giemsa stain and examined with the 100× oil immersion objective.

N. Sediments from BAL or bronchial washings are examined in stained preparations. Three slides should be stained.

1. Use a Pasteur pipette to place drops of sediment from each specimen on at least four slides. With the pipette, spread the sediment into a thin, even film. For specimens treated with an agent to lyse RBCs, use sediment from both treated and untreated samples for smears.

2. Air dry slides, and fix in methanol. If slides are to be stained with immuno-specific stains, fix according to package instructions.

3. Stain one slide with rapid Giemsa stain (Diff-Quik or Giemsa Plus). Rapid Giemsa stain procedure
   a. Stain solutions should be kept in dropper bottles to avoid bacterial contamination. Place 1 or 2 drops of red stain solution 1 on specimen smear and control slide, hold for 10 s, and drain.
   b. Add 1 or 2 drops of blue solution 2, hold for 10 s, drain, and rinse very briefly with deionized water.
   c. Stand slides on end to drain and air dry.
   d. Slides must be examined with oil or mounted with mounting medium.

4. Stain one slide with modified acid-fast stain (see procedure 9.4.1).

5. Stain one slide with methenamine-silver nitrate (4) or other cyst wall stain (3, 8, 14) (see procedure 8.3.1).
V. PROCEDURE (continued)

6. Staining with immunospecific stain may be desirable (see Appendix 9.10.2–1 at the end of this section).
   a. Follow package directions exactly.
   b. If negative control slide containing yeast cells exhibits fluorescence or if specimen slide is at all equivocal, do not report specimen as positive for *P. carinii* on the basis of this stain.
   c. Perform cyst wall and organism stains.

O. Put oil on stained slides or mount. Examine stained slides with 50× oil immersion objective, if available; otherwise, use 100× oil objective. If any slides are equivocal, stain additional slides.

P. Slides of fine-needle aspirates from lung are stained as described above (step V.N) for BAL and bronchial washings. Fine-needle aspirates of lesions likely to contain amebae are stained as described above (steps V.H through L) for cysts and abscesses.

Q. Generally portions of aspirate specimens are shared with bacteriology, and it is always important to consider the importance of a Gram stain in ruling out bacterial etiology.

VI. RESULTS

A. Helminth larvae (rare) are more likely to be seen in the wet preparation (specimens from bronchoscopy) with and/or without iodine.

B. Protozoa will be visible in the trichrome- or Giemsa-stained smears; trophozoites and cysts of *P. carinii* can be detected in Giemsa-stained smear.

C. Cysts of *P. carinii* will be seen on the methenamine-silver-stained smears.

D. Oocysts will be seen on the modified acid-fast preparation.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Organisms detected should be reported as follows.

1. From fine-needle aspirates and aspirates of cysts and abscesses, report *P. carinii*, *T. gondii*, and *Entamoeba histolytica* or *Acanthamoeba* spp. if morphology in stained preparations is classic. If ameba cultures are positive, species can be determined. Since the only *Entamoeba* sp. causing disseminated disease is *E. histolytica*, identification to the species level does not require positive culture.

2. From bone marrow aspirates, report *Leishmania donovani*, *T. cruzi*, or *Plasmodium* spp. if forms demonstrated in stains are classic.

3. From BAL specimens and bronchial washings, report *P. carinii* if characteristic trophozoite and cysts are detected (cysts alone are sufficient); report *T. gondii* if characteristic tachyzoites are detected in Giemsa stain and *C. parvum* if characteristic oocysts are detected in modified acid-fast or immunospecific stains. Note the quality of the specimen. If only a few alveolar cells are present, failure to find organisms may not rule out infection.

B. Notify clinician if any organisms described are detected.

VIII. PROCEDURE NOTES

A. Fine-needle aspirates often vary from stick to stick. Examine Giemsa-stained slides with a low-power (10×) objective to find cellular areas of slides to screen carefully. If one stick has better material than others, use slides of this specimen for other staining.

B. With aspirates of cysts and abscesses, material may vary from thin, with few cells, to very thick. In thick material, select several samples, including bloody
VIII. PROCEDURE NOTES (continued)

areas, for digestion. Organisms will often be found in the bloody material aspirated last.

C. With duodenal aspirates, if strongyloidiasis is expected, examine all of the specimen sediment in wet mounts with the 10× objective.

D. Aspirates of bone marrow are rarely submitted for examination for parasites. In the United States, splenic aspirations are very rarely performed.

E. With BAL specimens and bronchial washings, specimens are usually submitted for detection of *P. carinii*, although other organisms may be found. If *C. parvum* oocysts are found, report their detection, although their presence may not be clinically significant. *T. gondii* is being detected more frequently in BAL specimens, particularly those from human immunodeficiency virus-positive individuals (6).

IX. LIMITATIONS OF THE PROCEDURE

Lack of detection of *P. carinii* in BAL samples from patients given prophylaxis with or treated with aerosolized pentamidine has been reported (10). As more individuals are placed on prophylaxis, fluid specimens from bronchoscopy may be less useful.

REFERENCES


SUPPLEMENTAL READING


SUPPLEMENTAL READING
(continued)


APPENDIX 9.7.2–1

Reagents

- Include QC information on reagent container and in QC records.

<table>
<thead>
<tr>
<th>A. Staining reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Giemsa stain reagents (see procedure 9.8.5)</td>
</tr>
<tr>
<td>a. Giemsa stain (see procedure 9.8.5)</td>
</tr>
<tr>
<td>b. Phosphate buffer, pH 7.0 to 7.2 (see procedure 8.3.1)</td>
</tr>
<tr>
<td>c. Phosphate buffer plus Triton X-100 at 0.01% (see procedure 9.8.5)</td>
</tr>
<tr>
<td>d. Rapid Giemsa</td>
</tr>
<tr>
<td>1) Diff-Quik (Baxter Scientific)</td>
</tr>
<tr>
<td>2) Giemsa Plus (Trend Scientific)</td>
</tr>
<tr>
<td>2. Trichrome stain reagents (see procedure 9.3.6)</td>
</tr>
<tr>
<td>a. Schaudinn’s fixative (see procedure 9.2.2)</td>
</tr>
<tr>
<td>b. PVA fixative (see procedure 9.2.2)</td>
</tr>
<tr>
<td>3. Acid-fast stain reagents</td>
</tr>
<tr>
<td>a. Kinyoun’s acid-fast stain (cold) (see procedure 9.4.1)</td>
</tr>
<tr>
<td>b. Modified acid-fast stain (hot) (see procedure 9.4.2)</td>
</tr>
<tr>
<td>4. Methenamine-silver nitrate stain (see procedure 8.3.1)</td>
</tr>
</tbody>
</table>

| B. Culture media for *E. histolytica* (see procedure 9.9.1) |

<table>
<thead>
<tr>
<th>C. Materials for digestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sputolysin Stat-Pack dithiothreitol (Behring Diagnostics)</td>
</tr>
<tr>
<td>Prepare (according to package directions) a 1:10 dilution with distilled water (include expiration date). Store no longer than 48 h in refrigeration.</td>
</tr>
<tr>
<td>2. Lysing agent (available from most major laboratory suppliers)</td>
</tr>
<tr>
<td>3. Streptokinase</td>
</tr>
<tr>
<td>Reconstitute streptokinase and streptodornase (Veridase; Lederle Laboratory) as directed by manufacturer.</td>
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<table>
<thead>
<tr>
<th>D. Diluent</th>
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<td>0.85% NaCl</td>
</tr>
</tbody>
</table>
Biopsy Specimens

[Updated March 2007]

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Biopsy specimens are recommended for the diagnosis of tissue parasites. The procedures that may be used for this purpose in addition to standard histological preparations are impression smears and teased and squash preparations of biopsy tissue from skin, muscle, cornea, intestine, liver, lung, and brain. Tissue to be examined by permanent sections or electron microscopy should be fixed as specified by the laboratories which will process the tissue.

II. SPECIMENS

Tissue submitted in a sterile container on a sterile sponge dampened with saline may be used for protozoan cultures after mounts for direct examination or impression smears for staining have been prepared. If cultures for parasites will be made, use sterile slides for smear and mount preparation.

A. To prepare sterile slides for impression smears, lay six to eight glass slides (1 by 3 in.) on a paper towel, fold the towel (containing the slides) accordion fashion, tape, and autoclave using routine temperature and time frames (see procedure 9.8.4). An alternative method would be to soak the slides in 95% ethyl alcohol and flame them prior to use.

B. Use sterile (autoclaved or flamed) forceps for handling tissue.

C. Place tissue in a sterile petri dish to examine macroscopically and to select sample for microscopic evaluation. Minced tissue can be used if it is kept sterile.

1. If biopsy tissue is several millimeters to a centimeter in size, select from area differing from normal. For example, select gray consolidated or granulomatous portion of lung or ulcerated area of intestinal tissue.

2. If tissue is one or several small fragments a few millimeters in size that look alike, use one fragment. If tissue fragments look different, use one of each type for microscopic examination.

D. Prepare impression smears.

1. Blot tissue sample on sterile toweling. If the sample is large enough, cut the tissue, and use the cut surface to touch the slide.

2. Press tissue against slide, lift, and press again. Turn sample over and press against slide to make two more impressions. Keep impressions close together to speed screening. If several tissue samples were selected, make impressions in a row with each sample. For example, place sample 1 on top, sample 2 in the middle, and sample 3 at bottom, making impressions from left to right with each sample rather than from top to bottom. This scheme avoids differences in sample staining related to location.

Observe standard precautions.
II. SPECIMENS (continued)

3. Air dry smears, and fix in methanol for 1 min for subsequent Giemsa, methenamine-silver nitrate, and modified acid-fast staining. If the amount of tissue is sufficient, prepare multiple smears for each stain selected.

4. Place wet slide in Schaudinn’s fixative for subsequent trichrome staining.

5. Fix slide according to manufacturer’s directions for immunospecific staining.

E. Teased preparations

1. Place sample in the bottom of a plastic petri dish. Cover with 2 to 4 drops of saline (100 to 200 μl).

2. Gently tease tissue with needles, or hold tissue with forceps while pulling apart with a scalpel.

3. Put cover on dish, and leave at room temperature for 30 min.

F. Squash preparation

Cut selected tissue portions into very fine fragments with a scalpel. Place a fragment on a slide (1 by 3 in.), add 1 drop of saline, cover with a second slide (1 by 3 in.), and hold together with membrane clips (surgical supply company). If these are not available, use paper clips, but they are not as efficient.

G. Skin scrapings

Request that scrapings be sent between two glass slides or in a small vial.

H. Culture

Prepare cultures to demonstrate the following organisms.

1. Entamoeba histolytica (see procedure 9.9.1) (18)

2. Acanthamoeba spp. and Naegleria spp. (see procedure 9.9.2)

3. Leishmania spp. (see procedure 9.9.5)

I. Mouse passage for Toxoplasma gondii (15)

1. Grind tissue in 0.85% NaCl until a fine suspension results.

2. Intraperitoneally inject three to five mice of any laboratory strain that weigh ~20 g each with 0.2 to 0.4 ml of suspension.

3. House mice in isolation.

4. Check daily for signs of central nervous system (CNS) dysfunction. If CNS symptoms are detected, proceed to animal autopsy (see item V.F.4 below).

III. MATERIALS

A. Reagents (see Appendix 9.7.3–1)

B. Supplies

1. Pasteur pipette

2. Sterile glass slides (1 by 3 in.)

3. Petri dishes

4. Glass Coplin jars with lids

5. Plastic Coplin jars with lids

6. Pipettes, 1 and 10 ml

7. Scalpels

8. Forceps

9. Graduated cylinders

10. Stain rack

11. Protective gloves

12. Tissue grinder

C. Equipment

1. Microwave oven or water bath (80°C) and thermometer

2. Binocular microscope with 10×, 40×, or 50× oil, and 100× oil immersion objectives

3. Fluorescence microscope with filters to obtain appropriate wavelength of light for fluorescent label used if immunostaining

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Incorporate control slides in all stain procedures.

1. Yeast-containing material may be used as a positive control for silver staining (see procedure 8.3.1).

2. Material containing RBCs and WBCs may be used as a control for Giemsa staining (see procedure 9.8.5).

3. Both Pneumocystis carinii-positive and P. carinii-negative but yeast-positive material should be used for immunospecific staining for P. carinii.

4. Stains cannot be evaluated if controls do not stain appropriately.
IV. QUALITY CONTROL (continued)

B. To check for sterility, incubate new lots of media and inoculate stock ameba cultures of *E. histolytica* to see that medium supports growth (see procedure 9.9.1).

C. In refrigerator where culture medium is stored, maintain temperature at 4°C. Remember, daily temperature recording is required by Clinical Laboratory Improvement Amendments of 1988 regulations.

D. With each new lot of Giemsa stain or new buffer, check stain with specimen containing blood to see that RBCs stain grayish, WBC nuclei stain red-purple, and WBC cytoplasm stains bluish. If cells do not stain appropriately, check stock stain and buffer to find cause.

E. Fluorescence microscope can be evaluated for correct light wavelength for label with commercially available QC slides (see Appendix 9.10.6–1).

F. Check incubator for temperature maintenance at 35°C.

G. Record all QC results.

V. PROCEDURE

A. Impression smears

Stain and examine to detect possible organisms according to specimen and clinical history (Table 9.7.3–1).

1. Giemsa stain (see procedure 9.8.5)
2. Methenamine-silver nitrate procedure (see procedure 8.3.1) (16)
3. Modified acid-fast stain (see procedures 9.4.1 and 9.4.2)
4. Trichrome stain (see procedure 9.3.6)
5. Staining with immunospecific stain for *P. carinii* may be desirable (see Appendix 9.10.6–1 at the end of this section).
6. Modified trichrome stain (see procedures 9.4.3, 9.4.4, and 9.4.5). However, many biopsy specimens for the microsporidia are sent for routine histology processing and staining with hematoxylin and eosin (H&E), tissue Gram stains (Brown and Brenn, Brown and Hopps), silver stains (Warthin-Starry), or periodic acid-schiff (PAS).
   a. Follow package directions exactly.
   b. If negative control slide containing yeast cells exhibits fluorescence or if specimen slide is at all equivocal, do not report specimen as positive for *P. carinii* on basis of this stain.
   c. Examine stained slides microscopically for suspected organisms. Send slides to referral laboratory if necessary.

B. Teased preparation

Skin snips for detection of microfilariae of *Onchocerca volvulus* and *Mansonella streptocerca*

1. Tease the small bit of tissue apart in a few drops of saline to release the microfilariae.
2. Remove drops of saline to a glass slide (1 by 3 in.), cover with no. 1 coverslip, and examine with low light for microfilariae.
3. For a permanent record, run alcohol under coverslip to fix filariae, partially dry, remove coverslip, and stain with Giemsa.

C. Squash preparation for muscle detection of *Trichinella spiralis*

Examine microscopically with low power (100×) and low light.

D. Scrapings of skin for scabies

Examine scrapings microscopically at low power (100×) and low light.

E. Inoculate cultures with ground tissue suspensions (to release organisms from the cells).

   1. Place small tissue sample in sterile tissue grinder (Ten Broeck or Dounce) in 0.5 ml of sterile saline, and grind until tissue is dispersed.
Table 9.7.3–1 Stains for identifying parasites in various tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Possible parasite(s)</th>
<th>Stain(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td><em>P. carinii</em></td>
<td>Giemsa (27), methenamine-silver (4) or other cyst wall (3, 8, 12, 21, 23), immunospecific (2, 11, 17, 19, 26)</td>
</tr>
<tr>
<td>Lung</td>
<td><em>T. gondii</em></td>
<td>Giemsa, immunospecific (9)</td>
</tr>
<tr>
<td>Lung</td>
<td><em>E. histolytica</em></td>
<td>Trichrome (28), Giemsa</td>
</tr>
<tr>
<td>Liver</td>
<td><em>T. gondii, Leishmania donovani</em></td>
<td>Giemsa</td>
</tr>
<tr>
<td>Liver</td>
<td><em>C. parvum</em></td>
<td>Modified acid fast (14), immunospecific (1, 25)</td>
</tr>
<tr>
<td>Liver</td>
<td><em>P. carinii</em></td>
<td>Giemsa, silver or other cyst wall, immunospecific</td>
</tr>
<tr>
<td>Liver</td>
<td><em>E. histolytica</em></td>
<td>Giemsa, trichrome</td>
</tr>
<tr>
<td>Brain</td>
<td><em>Naegleria</em> sp.</td>
<td>Giemsa, trichrome</td>
</tr>
<tr>
<td>Brain</td>
<td><em>E. histolytica</em></td>
<td>Giemsa, trichrome</td>
</tr>
<tr>
<td>Brain</td>
<td><em>T. gondii</em></td>
<td>Giemsa, immunospecific</td>
</tr>
<tr>
<td>Brain</td>
<td>Microsporidia</td>
<td>Acid fast, Giemsa, Gram (confirm with EM) (24)</td>
</tr>
<tr>
<td>Brain</td>
<td><em>Encephalitozoon cuniculi</em></td>
<td>Giemsa (confirm with EM)</td>
</tr>
<tr>
<td>Skin</td>
<td><em>Leishmania</em> spp.</td>
<td>Giemsa</td>
</tr>
<tr>
<td>Skin</td>
<td><em>O. volvulus</em></td>
<td>Giemsa</td>
</tr>
<tr>
<td>Skin</td>
<td><em>M. streptocerca</em></td>
<td>Giemsa</td>
</tr>
<tr>
<td>Intestine</td>
<td><em>C. parvum</em></td>
<td>Modified acid fast (10), immunospecific (1, 25)</td>
</tr>
<tr>
<td></td>
<td><em>Enterocytozoon bieneusi</em></td>
<td>Giemsa (confirm with EM) (5, 6, 22)</td>
</tr>
<tr>
<td>Duodenum</td>
<td><em>Giardia lamblia</em></td>
<td>Trichrome</td>
</tr>
<tr>
<td>Colon</td>
<td><em>E. histolytica</em></td>
<td>Giemsa, trichrome</td>
</tr>
<tr>
<td></td>
<td><em>C. parvum</em></td>
<td>Modified acid fast (10), immunospecific (1, 25)</td>
</tr>
<tr>
<td>Cornea, conjunctiva</td>
<td><em>Microsporidium</em> spp., <em>Nosema</em> sp.</td>
<td>Acid fast, Giemsa (confirm with EM) (7)</td>
</tr>
<tr>
<td>Cornea, conjunctiva</td>
<td><em>Acanthamoeba</em> sp.</td>
<td>Giemsa, trichrome, calcofluor for cysts (13)</td>
</tr>
<tr>
<td>Muscle</td>
<td><em>T. spiralis</em></td>
<td></td>
</tr>
</tbody>
</table>

*Note: In disseminated infections with microsporidia, spores and developing stages from a number of different species could be found throughout the body. Routine histology processing and staining (H&E, silver, Gram, PAS) are used, as are Giemsa, modified trichrome stains, and optical brightening agents. However, the modified trichrome stains (chromotrope) are more commonly used and recommended for clinical specimens other than tissues, such as stool, urine, eye specimens, etc.*

*EM, electron microscopy.

V. PROCEDURE (continued)

2. Add several drops of ground tissue to culture medium as follows.
   a. To NNN medium (see procedure 9.9.5), add drops at bottom of slant, where they will “pool” with condensed moisture on slant. Incubate at room temperature (isolation of *Leishmania* spp.).
   b. To TYSGM-9 medium (see procedure 9.9.1), add several drops to the liquid medium, add 3 drops of the starch suspension, and incubate at a 45° to 50° angle at 35°C for 48 h (isolation of *E. histolytica*).
   c. Add drops to center of seeded agar plate, and incubate at 35 to 37°C (isolation of *Acanthamoeba* spp. or *Naegleria* spp.).
V. Procedure (continued)

F. Examine cultures.

1. TYSGM-9 medium for amebae (see procedure 9.9.1)
   With a Pasteur pipette, remove 1 or 2 drops of material adhering to sides or
   bottom of tube, place on glass slide, cover with coverslip, and examine micro-
   scopically (100×) with low light.

2. Agar plate with lawn of Escherichia coli or Enterobacter for detection of
   free-living amebae (see procedure 9.9.2)
   Examine microscopically at low power (100×) for changes in bacterial
   lawn, particularly patches and “tracks.”

3. NNN agar for promastigotes of Leishmania spp. (20) (see procedure 9.9.5)
   a. With a Pasteur pipette, remove a drop of fluid from interface of agar slant
      and condensed moisture.
   b. Place on glass slide, and cover with coverslip.
   c. Examine microscopically (400×) with low light for motile promastigotes.

4. Mouse passage for detection of T. gondii (15)
   a. Wear canvas gloves to handle mice. Gardening gloves work well.
   b. Sacrifice mice.
   c. Pin mouse to board, spray with 70% ethyl alcohol, and open peritoneal
      cavity with sterile scissors.
   d. Remove fluid from the peritoneal cavity with sterile Pasteur pipette, and
      place in small tube or make slide directly.
   e. Make Giemsa-stained smears of peritoneal exudate, and examine micro-
      scopically at 1,000× for T. gondii tachyzoites.
   f. Place mice and all contaminated disposable materials in bag to be auto-
      claved and destroyed. Place nondisposable materials (scissors) in bag to
      be autoclaved prior to washing.

G. Correlate all examination results (wet mount, stains, and cultures) to determine
   presence of organisms.

VI. Results

A. The majority of the protozoa will be found on the specimen permanent stained
   smears (impression smears, touch or squash preparations, teased preparations).

B. When culture is used, permanent stained smears of cultural material may also
   reveal some protozoa (18).

C. Although infrequently used, material from animals (at autopsy) can be examined
   as both wet and permanent stained preparations for confirmation of protozoa.

D. Filarial infections may be confirmed by the recovery and identification of mi-
   crofilariae in skin scrapings and/or biopsies.

Postanalytical Considerations

VII. Reporting Results

Report organisms detected if identifications are certain. If presumed parasites for
which the laboratory has no corresponding reference material are found, confirm-
ation by another laboratory is suggested.

VIII. Procedure Notes

A. Immunospecific staining reagents are rapidly becoming more available. Eval-
   uations of monoclonal antibodies and staining techniques such as indirect fluo-
   rescence, direct fluorescence, and enzyme tagging have been variable. In using
   newly developed tests, closely follow directions, and include all possible control
   measures. In addition to new tests, there are “new” organisms.

B. Daily, more is being learned about the microsporidia. Most individuals working
   with new pathogens or developing new tests will gladly accept referral material.
   Check references for workers to whom material might be sent for confirmatory
   identification.
IX. LIMITATIONS OF THE PROCEDURE

Success in detection of parasites in tissue depends in part on the adequacy of the specimen. It has been reported that patients being treated with aerosolized pentamidine may have localized (peripheral) P. carinii (16). Biopsy specimens are often very tiny and may not be representative of the whole infectious process. The availability of more than one tissue sample taken by transbronchial biopsy enhances diagnosis. To optimize the yield from any tissue specimen, examine all areas, and use as many means of organism detection as possible. The tissue sample results from an invasive costly procedure and deserves the most exhaustive examination possible.

REFERENCES

REFERENCES (continued)

9.7.3.7


SUPPLEMENTAL READING


APPENDIX 9.7.3–1

Reagents

Indicate the expiration date on the label and in the work record or on the manufacturer’s label.

A. NaCl, 0.85%

B. Giemsa stain (see procedure 9.8.5)

C. Rapid Giemsa

1. Diff-Quick (Baxter Scientific)
2. Giemsa Plus (Trend Scientific)

D. Giemsa stain buffer with 0.01% Triton X-100 (see procedure 9.8.5)

E. Giemsa buffer (see procedure 9.8.4)

F. Methenamine-silver nitrate stain reagents (see procedure 8.3.1)

G. Trichrome stain reagents (see procedure 9.3.6)

H. Schaudinn’s fixative (see procedure 9.2.2)

I. Kinyoun’s modified acid-fast stain (see procedure 9.4.1)

J. Bleach

K. Culture media

1. E. histolytica (see procedure 9.9.1)
2. Acanthamoeba spp. and Naegleria spp. (see procedure 9.9.2)
3. Leishmania spp. (see procedure 9.9.5)

L. Modified trichrome stain (see procedures 9.4.3, 9.4.4, and 9.4.5)
I.  PRINCIPLE

During some stages in their life cycle, *Plasmodium* spp. (malaria), *Babesia* spp., *Trypanosoma* spp., *Leishmania donovani,* and the filaria are detectable in human blood. *Plasmodium* and *Babesia* species are found within the RBCs; trypanosomes and microfilariae, the larval stage of filariae, are found outside the RBCs; and *Leishmania* amastigotes are occasionally found within monocytes. Trypanosomes and microfilariae, which frequently are present in low numbers, exhibit motility in freshly collected blood films, and this can aid in their detection. However, species identifications of all blood parasites are usually made from either or both of two types of stained blood films: a thin film and a thick film. These films can be made from whole or anticoagulated blood or from the sediment of a variety of procedures designed to concentrate trypanosomes and microfilariae in the blood. Although the films are clearest when stained with Giemsa stain, many infections are detected and diagnosed by using Wright’s stain. Delafield’s hematoxylin is used to enhance the morphological features of microfilariae.

Microscopic examination of stained blood films is best accomplished by beginning with a thorough search of both the thin and thick films with low-power magnification for microfilariae. If larvae are found, magnification at a higher power will reveal the finer morphological details necessary to make a definitive identification. Other blood parasites require examination with oil immersion magnification of both the thin and thick films. Trypanosomes, even those detected in thick films, are more frequently identified in the thicker portion of the thin film. *Plasmodium* and *Babesia* spp., being intracellular parasites, are detected in the thick film but are more readily identified in the thinner portion of the thin film. Depending on the experience of the microscopist, satisfactory examination usually requires 5 to 10 min for the thick film (about 100 fields) and 15 to 20 min for the thin film (about 200 to 300 fields) at 1,000 (oil immersion) magnification. All species of parasites found in a blood specimen should be reported to the attending physician as soon as possible. Notification of appropriate governmental authorities should be made expeditiously where required by law.

II.  THIN AND THICK BLOOD FILMS

A.  Purpose

To date, stained blood films are the most reliable and efficient means for definitive diagnosis of nearly all blood parasites. They provide a permanent record and can be sent to a reference laboratory for consultation or verification of diagnosis. Ordinarily, when a laboratorian tests for blood parasites, two types of blood films are prepared: a thin film and a thick film. These can be made on the same microscope slide, that is, with the thin film on one end of the slide and the thick film on the other, or they can be made on separate slides. When malaria is suspected, the recommended procedure is to prepare a thin film on one slide, a thick film on another, and a combination of thin and thick films on a third slide. The thin film can be stained within a few minutes and will afford a quick diagnosis of malaria if the patient has a high degree of parasitemia; the thick film can be stained in a few hours and will afford a diagnosis of lighter infection; and the combination film is stained several hours later when the blood has dried longer, thus resulting in a better differential stain. The combination film is then used to verify the quick diagnosis and is kept as the permanent record. This three-slide procedure can be used for detecting all blood parasites.
II. THIN AND THICK BLOOD FILMS (continued)

B. Thin blood film (see procedure 9.8.2)
The thin film is identical to a differential WBC count film. It provides a good area for examining the morphology of parasites and RBCs and is used to confirm the identity of parasites that cannot be identified in thick films. Most parasitologists concur that a thin film must be used to differentiate *Plasmodium ovale* from *Plasmodium vivax* and *Babesia* species from the ring forms of *Plasmodium falciparum*. The thin film is also better for identifying *Trypanosoma cruzi*, because these organisms become distorted in thick films. The thin film, however, is less sensitive than the thick film in light parasitemias.

C. Thick blood film (see procedure 9.8.3)
The thick film essentially condenses into an area suitable for examination about 20 times more blood than the thin film. In this respect, the thick film is a concentration procedure. Here, the RBCs are lysed during the staining process so that only parasites, platelets, and WBCs remain visible. The thick film, then, has two advantages over the thin film: it saves time in examining the blood and increases the chance of detecting light infections. Therefore, the thick film is recommended for the routine detection of all parasites when the diagnostic stages occur in blood, and it can be used, in most instances, for identifying to the species level all microfilariae, the African trypanosomes, and all *Plasmodium* spp. except *P. ovale*.

D. Blood specimen
Blood for detecting parasites is obtained by either finger puncture or venipuncture. These procedures are described in detail in NCCLS publications (1, 2) and procedures 9.8.2 and 9.8.3. Standard precautions must be used in the collecting, handling, and disposing of these blood samples. If blood is obtained by finger puncture, care must be taken not to squeeze tissue juice into the sample and risk diluting a possible light parasitemia to below the level of detection.

E. Use of anticoagulants
Blood samples for malaria are preferably collected without anticoagulants, but if anticoagulants must be used for other testing, films for reliable staining of malaria parasites should be made immediately or within 1 h of collection. Trypanosomes and microfilariae are usually not affected by the use of anticoagulants. Although several anticoagulants have been used, EDTA (0.020 g/10 ml of blood) is recommended by most parasitologists for use with malaria parasites. Heparin (0.002 g/10 ml of blood) and sodium citrate (0.050 g/10 ml of blood) are often used for concentration procedures for trypanosomes and microfilariae.

F. Collection guidelines
1. The blood sample must be labeled with the date and time of collection so that findings can be correlated with symptoms and with all other pertinent information. Some parasites appear more frequently in the blood during certain periods than during others. To accommodate this, blood for detection of parasites is usually collected as follows.
   a. On admission or when first suspected of parasite infection
   b. If no parasites are found in the first sample, blood is collected every 6 to 12 h until a diagnosis is made or infection is no longer suspected (usually 3 to 5 days). *Infection with a blood parasite cannot be ruled out by a single blood sample.*
   c. If either trypanosomes or microfilariae are suspected, each sample should be concentrated by an appropriate technique in an effort to detect a low parasitemia.
   d. The examination of blood for malaria should be considered a stat request. Preparation and examination of smears and reporting of results should be performed on a stat basis.
II. THIN AND THICK BLOOD FILMS (continued)

2. Blood films for parasites should be made on clean, standard, glass microscope slides (1 by 3 in.). All slides, even new “precleaned” ones, should be dipped in alcohol and polished with a lint-free towel to remove any grease or dirt before the preparations are made.

3. Instrument methods, for either preparing thin films or staining films, have not been reliable for blood parasites. At this time, manual techniques yield better results.

G. Staining of blood films

1. All blood films, regardless of the stain which will be used, should be stained as soon as possible after they are thoroughly dry. Prolonged storage may cause erratic staining.

2. After staining and thorough drying, films may be examined by using oil directly on the film, or a no. 1-thickness coverslip may be applied to the film by using a mounting medium (pH 7.0).

REFERENCES


9.8.2 Preparation of Thin Blood Films

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The thin film is prepared like that for a differential WBC count and provides an area for examination where the RBCs are neither overlapping nor distorted. Here the morphologies of parasites and infected RBCs are most typical.

II. SPECIMEN

The specimen usually consists of fresh whole blood collected by finger puncture or of whole blood containing EDTA (0.020 g/10 ml of blood) that was collected by venipuncture and is less than 1 h old (1). Occasionally a buffy coat (for leishmaniasis) or the sediment from a special concentration procedure (triple centrifugation for trypanosomes) is spread into a thin film.

III. MATERIALS

A. Reagent

- Indicate the expiration date on the label and in the work record or on the manufacturer’s label.
- Absolute methanol (for Giemsa stain)

B. Supplies

1. Glass microscope slides (1 by 3 in.), alcohol washed
2. Glass marker
3. Blood collection supplies (if applicable)

C. Equipment

None

Include QC information on reagent container and in QC records.

Observe standard precautions.

IV. QUALITY CONTROL

A. Visually, the thin film should be rounded, feathered, and progressively thinner toward the middle of the slide.

B. There should not be any clear areas or smudges in the film itself (indicating that grease or fingerprints were on the glass).
V. PROCEDURE

A. Wear gloves when performing this procedure.

B. Blood from finger puncture

   After wiping off the first drop of blood, touch a clean glass microscope slide (1 by 3 in.) about ½ in. from the end to a small drop of blood (10 to 15 μl) standing on the finger, remove the slide from the finger, turn it blood side up, and place it on a horizontal surface.

   Blood from venipuncture

   Place a clean glass microscope slide (1 by 3 in.) on a horizontal surface. Place a small drop (10 to 15 μl) of specimen onto the center of the slide about ½ in. from the end.

C. Holding a second clean glass slide at a 40° angle, touch the angled end to the midlength area of the specimen slide.

D. Pull the angled slide back into the blood, and allow the blood to almost fill the end area of the angled slide.

E. Continuing contact with the blood under the lower edge, quickly and steadily move the angled slide toward the opposite end of the specimen slide until the blood is used up.

F. The result will be a thin film that is rounded, feathered, and progressively thinner toward the center of the slide.

G. Label the slide appropriately, and allow it to air dry while protected from dust for at least 10 min.

H. If the film will be stained with Giemsa, then after the film is completely dry, fix it by dipping the slide into absolute methanol, and allow the film to air dry in a vertical position. If the film will be stained with Wright’s stain, it does not need to be fixed. Wright’s stain contains the fixative and stain in one solution.

POSTANALYTICAL CONSIDERATIONS

VI. PROCEDURE NOTES

A. A diamond marking pen is recommended.

B. An indelible ink pen can be used.

C. Pencil can be used if the information is actually written in the thick part of the smear (where the original drop of blood was placed).

D. Do not use wax pencils; the material may fall off during the staining procedure.

E. Make sure the films are protected from dust (while drying).

VII. LIMITATIONS OF THE PROCEDURE

A. A light infection may be missed in a thin film, whereas the increased volume of blood present on a thick film may allow the detection of the infection, even with a low parasitemia.

B. If the smears are prepared from anticoagulated blood which is more than 1 h old, the morphology of both parasites and infected RBCs may not be typical.

C. You should be able to identify Plasmodium vivax and Plasmodium ovale, even in the absence of Schüffner’s dots (stippling).

REFERENCE

9.8.3 Preparation of Thick Blood Films

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE
The thick film samples more blood than the thin film and therefore is more likely to demonstrate a low parasitemia. RBCs are lysed during staining, making the preparation more or less transparent and leaving only parasites, platelets, and WBCs for examination (1, 2).

II. SPECIMEN
The specimen usually consists of fresh whole blood collected by finger puncture or of whole blood containing EDTA (0.020 g/10 ml of blood) that was collected by venipuncture and is less than 1 h old. Heparin (2 mg/10 ml of blood) or sodium citrate (0.050 g/10 ml of blood) may be used as an anticoagulant if trypanosomes or microfilariae are suspected. The sediment from a concentration procedure for trypanosomes or microfilariae is frequently spread into a thick film that is stained, examined, and kept as a permanent record (see procedures 9.8.11 and 9.8.12).

Although both EDTA and heparin have been mentioned as anticoagulants, it is very important to request EDTA, rather than heparin, for the preparation and staining of blood films for malaria. However, heparin can be used for the concentration methods related to the recovery of trypanosomes and microfilariae. As a general rule, EDTA is the recommended anticoagulant for all parasitology blood work.

III. MATERIALS

A. Reagents
None

B. Supplies
1. Glass microscope slides (1 by 3 in.), alcohol washed
2. Glass marker

C. Equipment
None

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL
A. Visually, the thick smear should be round to oval and approximately 2.0 cm across.
B. You should barely be able to read newsprint through the wet or dry film.
C. The film itself should not have any clear areas or smudges (indicating that grease or fingerprints were on the glass).
V. PROCEDURE

A. Wear gloves when performing this procedure.

B. Blood from finger puncture

After wiping off the first drop of blood, touch a clean glass microscope slide (1 by 3 in.) to a large drop of blood standing on the finger, and rotate the slide on the finger until the circle of blood is nearly the size of a dime or a nickel (1.8 to 2.0 cm). Without breaking contact with the blood, rotate the slide back to the center of the circle. Remove the slide from the finger, quickly turn it blood side up, and place it on a horizontal surface. The blood should spread out evenly over the surface of the circle and be of a thickness that fine print (newsprint size) can just barely be read through it. If it is thicker than this, take the corner of a second clean slide or an applicator stick, and expand the size of the circle until the print is just readable. The final thickness of the film is important. If too thick, it might flake off while drying or wash off while staining. If too thin, the amount of blood available for examination is insufficient to detect a low parasitemia.

Blood from venipuncture

Place a clean glass microscope slide (1 by 3 in.) on a horizontal surface. Place a drop (30 to 40 µl) of blood onto the center of the side about ½ in. from the end. Using either the corner of another clean glass slide or an applicator stick, spread the blood into a circle about the size of a dime or a nickel (1.8 to 2.0 cm). Immediately place the thick film over some small print, and be sure that the print can just barely be read through it. If not, expand the size of the film until the print can be read. Three or four small drops of blood may be used in place of the large drop, and the small ones can be pooled into a thick film by using the corner of a clean slide or an applicator stick. Be sure that small print can be read through the film.

C. Allow the film to air dry in a horizontal position protected from dust for several hours (6 to 8 h) or overnight. Do not attempt to speed the drying process by applying any type of heat, because heat will fix the RBCs and they will not lyse in the staining process.

D. Do not fix the thick film. If thin and thick films are made on the same slide, do not allow the methanol or its vapors to contact the thick film by slightly tilting the slide when fixing the thin film.

E. Label the slide appropriately.

F. If staining with Giemsa will be delayed for more than 3 days or if the film will be stained with Wright’s stain, lyse the RBCs in the thick film by placing the film in buffered water (pH 7.0 to 7.2) (see procedure 9.7.1) for 10 min. Then remove it from the water, and place it in a vertical position (thick film down) to air dry.

VI. PROCEDURE NOTES

A. A diamond marking pen is recommended.

B. An indelible ink pen can be used.

C. Do not use wax pencils; the material may fall off during the staining procedure.

D. Make sure the films are protected from dust (while drying).

VII. LIMITATIONS OF THE PROCEDURE

A. If the smears are prepared from anticoagulated blood that is more than 1 h old, morphology of the parasites may not be typical, and the film may wash off the slide during the staining procedure.
VI. LIMITATIONS OF THE PROCEDURE (continued)

B. Identification to the species level, particularly between *Plasmodium ovale* and *Plasmodium vivax* and between the ring forms of *Plasmodium falciparum* and *Babesia* spp., may be impossible without examining the stained thin blood film. Also, *Trypanosoma cruzi* trypomastigotes are frequently distorted in thick films.

C. Excess stain on the film may be confusing and make the detection of organisms difficult.

REFERENCES


SUPPLEMENTAL READING


9.8.4 Combination Thick and Thin Blood Films (Can Be Stained as Either)

PREANALYTICAL CONSIDERATIONS

I. **PRINCIPLE**

The combination thick-thin blood film provides both options on one glass slide, and the slide can be stained as either a thick or thin blood film. If fixed prior to staining, then the smear will be read as a thin blood film; if RBCs are lysed during staining, the preparation will be read as a thick blood film (parasites, platelets, WBCs). This combination blood film dries more rapidly than the traditional thick blood film, thus allowing staining and examination to proceed with very little waiting time for the slide(s) to dry (1, 2).

II. **SPECIMEN**

![Observe standard precautions.]

The specimen usually consists of fresh whole blood collected by finger puncture or of whole blood containing EDTA (0.020 g/10 ml of blood) that was collected by venipuncture and is less than 1 h old. Heparin (2 mg/10 ml of blood) or sodium citrate (0.050 g/10 ml of blood) may be used as an anticoagulant if trypanosomes or microfilariae are suspected.

III. **MATERIALS**

| A. Reagents  | None |
| B. Supplies  | 1. Glass slides (1 by 3 in., or larger if you prefer), alcohol washed |
|             | 2. Glass marker |
| C. Equipment | None |

| 3. Blood collection supplies (if applicable) |
| 4. Paper with newsprint-size print |
| 5. Applicator sticks |

IV. **QUALITY CONTROL**

A. Visually, the smear should consist of alternating thick and thin portions throughout the length of the glass slide.

B. One should be able to barely read newsprint through the wet or dry film.

C. The film itself should not have any clear areas or smudges, indicating that grease or fingerprints were on the glass.

D. Blood from a finger puncture is not recommended, since the procedure does not lend itself to “stirring” to prevent fibrin strands.

V. **PROCEDURE** (Fig. 9.8.4–1)

A. Wear gloves when performing this procedure.

B. Place a clean 1- by 3-in. glass microscope slide on a horizontal surface.

C. Place a drop (30 to 40 μl) of blood onto one end of the slide about 0.5 in. from the end.
Combination Thick and Thin Blood Films

9.8.4.2

V. PROCEDURE (continued)

D. Using an applicator stick lying across the glass slide and keeping the applicator in contact with the blood and glass, rotate (do not “roll”) the stick in a circular motion while moving the stick down the glass slide to the opposite end.

E. The appearance of the blood smear should be alternate thick and thin areas of blood that cover the entire slide.

F. Immediately place the film over some small print and be sure that the print is just barely readable.

G. Allow the film to air dry horizontally and protected from dust for at least 30 min to 1 h. Do not attempt to speed the drying process by applying any type of heat, because the heat will fix the RBCs and they subsequently will not lyse in the staining process.

H. This slide can be stained as either a thick or thin blood film.

I. Label the slide appropriately.

J. If staining with Giemsa (as a thick film) will be delayed for more than 3 days or if the film will be stained with Wright’s stain, lyse the RBCs on the thick film by placing the slide in buffered water (pH 7.0 to 7.2) for 10 min, remove it from the water, and place it in a vertical position to air dry.

K. If staining with Giemsa (as a thin film), after the film is completely dry, fix it by dipping the slide into absolute methanol, and allow the film to air dry in a vertical position. If the film will be stained with Wright’s stain, it does not need to be fixed. Wright’s stain contains the fixative and stain in one solution.

POSTANALYTICAL CONSIDERATIONS

VI. PROCEDURE NOTES

A. A diamond marking pen is recommended.

B. An indelible ink pen can be used.

C. Pencil can be used if the information is actually written in the thickest part of the smear (where the original drop of blood was placed).

D. Do not use wax pencils; the material may fall off during the staining procedure.

E. Make sure the films are protected from dust (while drying).
VII. LIMITATIONS OF THE PROCEDURE

A. A light infection may be missed in a thin film, whereas the increased volume of blood present on a thick film may allow the detection of the infection, even with a low parasitemia.

B. If the smears are prepared from anticoagulated blood which is more than 1 h old, the morphology of both parasites and infected RBCs may not be typical.

C. If a tube of blood containing EDTA cools to room temperature and the cap has been removed, several parasite changes can occur. The parasites within the RBCs will respond as if they were now in the mosquito after being taken in with a blood meal. The morphology of these changes in the life cycle and within the RBCs can cause confusion when examining blood films prepared from this blood.
   1. Stippling (Schüffner’s dots) may not be visible.
   2. The male gametocyte (if present) may exflagellate.
   3. The ookinetes of *Plasmodium* species other than *Plasmodium falciparum* may develop as if they were in the mosquito and may mimic the crescent-shaped gametocytes of *P. falciparum*.

D. Identification to the species level, particularly between *Plasmodium ovale* and *Plasmodium vivax* and between the ring forms of *P. falciparum* and *Babesia* spp., may be impossible without examining one of the slides stained as a thin blood film. Also, *Trypanosoma cruzi* trypomastigotes are frequently distorted in thick films.

E. Excess stain deposition on the film may be confusing and make the detection of organisms difficult.

REFERENCES


9.8.5 Giemsa Stain

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE
Giemsa stain is used to differentiate nuclear and/or cytoplasmic morphology of platelets, RBCs, WBCs, and parasites (1–4). The most dependable stain for blood parasites, particularly in thick films, is Giemsa stain containing azure B. Liquid stock is available commercially, or the stock can be made from dry stain powder. Either must be diluted for use with water buffered to pH 6.8 or 7.0 to 7.2, depending on the specific technique used. Either should be tested for proper staining reaction before use. The stock is stable for years, but it must be protected from moisture because the staining reaction is oxidative. Therefore, the oxygen in water will initiate the reaction and ruin the stock stain. The aqueous working dilution of stain is good only for 1 day.

Although not essential, the addition of Triton X-100, a nonionic surface-active agent, to the buffered water used to dilute the stain enhances the staining properties of Giemsa (3) and helps to eliminate possible transfer of parasites from one slide to another (1). For routine staining of thin films and combination thin and thick films, a 0.01% (vol/vol) final concentration of Triton X-100 is best. For staining thick films for microfilariae, use a 0.1% (vol/vol) concentration.

II. SPECIMEN

Observe standard precautions.

The specimen may consist of a thin blood film that has been fixed in absolute methanol and allowed to dry, a thick blood film that has been allowed to dry thoroughly and is not fixed, or a combination of a fixed thin film and an adequately dried thick film (not fixed) on the same slide.

III. MATERIALS

A. Reagents (see Appendix 9.8.5–1)

B. Supplies (including those for preparing stock stain)

1. 3 or 4 Coplin jars, 50 ml
2. Pipette, 2 ml
3. 3 graduated cylinders, 50 ml
4. Mortar and pestle
5. Flask, Erlenmeyer, 500 ml with cotton plug
6. 2 flasks, volumetric, 1 liter
7. Bottle, brown, 150 to 200 ml
8. Bottles, clear, 100 ml and 3 1-liter
9. Bottle, airtight, 50 ml
10. Filter paper, Whatman no. 1
11. Funnel (glass) to hold filter paper

C. Equipment

1. Microscope, binocular with mechanical stage; low-power (10×), high dry power (40×), and oil immersion (100×) objectives; 10× oculars; calibrated ocular micrometer; light source equivalent to 20-W halogen or 100-W tungsten bulb; blue and white ground-glass diffuser filters
2. Timer, 1 h or more in 1-min increments
3. Water bath, 55 to 60°C
4. pH meter
**ANALYTICAL CONSIDERATIONS**

### IV. QUALITY CONTROL

A. The stock buffer solutions and buffered water should be clear, with no visible contamination.

B. Check the Giemsa stain reagents, including the pH of the buffered water, before each use. If Triton X-100 has been added to the buffered water, do not use a colorimetric method to determine the pH, because Triton X-100 interferes with the color indicators. Use a pH meter to test buffered water that contains Triton X-100. The buffered water is usable as long as the pH is within the limits listed for the procedure.

C. Prepare and stain films from “normal” blood, and microscopically evaluate the staining reactions of the RBCs, platelets, and WBCs.

1. Macroscopically, blood films appear purplish. If blue, the buffered water was too alkaline; if pink to red, the buffered water was too acid.

2. Microscopically, RBCs appear pinkish gray, platelets appear deep pink, and WBCs have purple-blue nuclei and lighter cytoplasm. Eosinophilic granules are bright purple-red, and neutrophilic granules are purple. Basophilic stippling within uninfected RBCs is blue.

3. Slight variations may appear in the colors described above depending on the batch of stain used and the character of the blood itself, but if the various morphological structures are distinct, the stain is satisfactory.

D. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (see procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

E. Record all QC results.

### V. PROCEDURE

A. *Wear gloves when performing any of these procedures.*

B. Thin blood films (only)

1. Fix air-dried film in absolute methyl alcohol by dipping the film briefly (two dips) in a Coplin jar containing methyl alcohol.

2. Remove, and let air dry.

3. Stain with diluted Giemsa stain (1:20, vol/vol) for 20 min. For a 1:20 dilution, add 2 ml of stock Giemsa to 40 ml of buffered water containing 0.01% Triton X-100 in a Coplin jar (Triton X-100 optional).

4. Wash by briefly dipping the slide in and out of a Coplin jar of buffered water (one or two dips).

   ![NOTE: Excessive washing will decolorize the film.]

5. Let air dry in a vertical position.

C. Thick blood films (only)

1. Allow film to air dry thoroughly for several hours or overnight. Do not dry films in an incubator or by heat, because this will fix the blood and interfere with the lysing of the RBCs.

   ![NOTE: If a rapid diagnosis of malaria is needed, thick films can be made slightly thinner than usual, allowed to dry for 1 h, and then stained.]

2. *Do not fix.*

3. Stain with diluted Giemsa stain (1:50, vol/vol) for 50 min. For a 1:50 dilution, add 1 ml of stock Giemsa to 49 ml of buffered water containing 0.01% Triton X-100 in a Coplin jar (Triton X-100 optional).
V. PROCEDURE (continued)

Triton X-100 (if staining microfilariae, use 0.1% Triton X-100) in a Coplin jar.

4. Wash by placing film in buffered water for 3 to 5 min.

5. Let air dry in a vertical position.

D. Combination thin and thick blood films

1. Allow the thick film to air dry thoroughly.

2. Fix the thin film by placing only the thin film in methyl alcohol (two dips).
   Be sure not to get the alcohol or its fumes on the thick film by slightly tilting the slide.

3. Let air dry in a vertical position with the thick film up. Be sure slide is thoroughly dry before staining. Introducing even a minute amount of methyl alcohol into the stain dilution will interfere with the lysing of the RBCs in the thick films.

4. Stain the entire slide with diluted Giemsa stain (1:50, vol/vol) for 50 min. Place the slide in the stain, thick film down to prevent the debris caused by dehemoglobinization from falling onto the thin film. For a 1:50 dilution, add 1 ml of stock Giemsa to 50 ml of buffered water containing 0.01% Triton X-100 in a Coplin jar.

5. Rinse the thin film by briefly dipping the film in and out of a Coplin jar of buffered water (one or two dips). Wash the thick film for 3 to 5 min. Be sure that the thick film is immersed but do not allow the water to cover any part of the thin film.

6. Let dry in a vertical position with the thick film down.

VI. RESULTS

A. If *Plasmodium* organisms are present, the cytoplasm stains blue and the nuclear material stains red to purple-red.

B. Schüffner’s stippling and other inclusions in the RBCs infected by *Plasmodium* spp. stain red.

C. Nuclear and cytoplasmic colors that are seen in the malarial parasites will also be seen in the trypanosomes and any intracellular leishmaniae that are present.

D. The sheath of microfilariae may or may not stain with Giemsa, while the body will usually appear blue to purple.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report any parasite, including the stage(s) seen (do not use abbreviations).

   **Examples:** *Plasmodium falciparum* rings and gametocytes, rings only
   *Plasmodium vivax* rings, trophozoites, schizonts, and gametocytes
   *Wuchereria bancrofti* microfilariae
   *Trypanosoma brucei gambiense/rhodesiense* trypomastigotes
   *Trypanosoma cruzi* trypomastigotes
   *Leishmania donovani* amastigotes

B. Any laboratory providing malaria diagnoses should be able to identify *Plasmodium vivax* and *Plasmodium ovale*, even in the absence of Schüffner’s stippling.

VIII. PROCEDURE NOTES

A. Blood films prepared from venipuncture blood when an anticoagulant is used must be prepared within 1 h of collection. Otherwise, certain morphological characteristics of both parasites and infected RBCs may be atypical. Also, thick blood films may wash off the slide during the staining procedure.
VIII. PROCEDURE NOTES

(continued)

B. The correct pH for all buffered-water and staining solutions is also important. Solutions with the incorrect pH will prevent certain morphological characteristics (stippling) from being visible and will not give typical nuclear and cytoplasmic colors on the stained film.

C. Stain a QC slide each time patient blood films are stained. If several patient specimens are stained on the same day (using the same reagents), only one control slide need be stained and examined. The patient slide can serve as the QC slide; if the WBCs and RBCs exhibit typical colors, any parasites present would also stain correctly.

IX. LIMITATIONS OF THE PROCEDURE

A. Finding no parasites in one set of blood films does not rule out a parasitic infection.

B. Examine a minimum of 300 oil immersion (×1,000) fields before reporting no parasites found.

C. Examine the entire smear under low power (100×) for the presence of microfilariae. Remember that the sheath may not be visible (W. bancrofti).

REFERENCES


SUPPLEMENTAL READING


APPENDIX 9.8.5–1

Reagents

A. Stock Giemsa stain

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Giemsa stain (azure B type)</td>
<td>0.6 g</td>
</tr>
<tr>
<td>glycerin</td>
<td>50.0 ml</td>
</tr>
<tr>
<td>methyl alcohol, absolute (acetone free)</td>
<td>50.0 ml</td>
</tr>
</tbody>
</table>

1. Grind 0.6 g of stain powder with 10 to 15 ml of glycerin (freshly opened bottle, neutral) in a clean mortar. Pour the top third into a clean, dry 500-ml flask. Add more glycerin, and grind again. The grinding process should be thorough, to ensure mixing of stain powder and glycerin. Repeat until most of the powder has been mixed with the 50 ml of glycerin and the mixture has been poured into the flask.

2. Stopper or plug the flask, and place and secure it upright in a 55 to 60°C water bath for 2 h. The water in the bath should be above the level of the stain mixture. Shake gently at half-hour intervals.

3. After grinding powder and glycerin together in mortar, measure 50 ml of methyl alcohol (acetone free, neutral), and use it to wash the last bit of stain from the mortar. Pour the washing into a small, airtight bottle.

4. After 2 h, remove glycerin and stain powder mixture from water bath. Allow to come to room temperature, add alcohol washing from the mortar, and shake well.
5. Filter before use through Whatman no. 1 paper into a brown bottle. The stain can be used immediately, but it is preferable to let it stand about 2 weeks with intermittent shakings before the initial filtering.

6. Label appropriately, and store protected from light at room temperature. The shelf life is 36 months, providing QC criteria are met (stock solution).

B. Stock solution of Triton X-100 (10%)

<table>
<thead>
<tr>
<th>Triton X-100</th>
<th>10 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>distilled water</td>
<td>90 ml</td>
</tr>
</tbody>
</table>

1. Combine above liquids in a bottle.
2. Mix thoroughly, label appropriately, and store at room temperature. This solution will keep indefinitely if tightly stoppered.

C. Stock buffers (for preparing buffered water)

1. Alkaline buffer, 0.067 M solution

<table>
<thead>
<tr>
<th>Na$_2$HPO$_4$ (anhydrous)</th>
<th>9.5 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>distilled water, to make</td>
<td>1.0 liter</td>
</tr>
</tbody>
</table>

   In a 1-liter volumetric flask, dissolve Na$_2$HPO$_4$ in about three-fourths of the water. Add water to make 1 liter of solution. Store in tightly stoppered bottle, and label appropriately. The shelf life is 24 months.

2. Acid buffer, 0.067 M solution

<table>
<thead>
<tr>
<th>NaH$_2$PO$_4$·H$_2$O</th>
<th>9.2 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>distilled water, to make</td>
<td>1.0 liter</td>
</tr>
</tbody>
</table>

   In a 1-liter volumetric flask, dissolve NaH$_2$PO$_4$·H$_2$O in about three-fourths of the water. Add water to make 1 liter of solution. Store in tightly stoppered bottle, and label appropriately. The shelf life is 24 months.

D. Buffered water (for diluting stain and washing films), pH 7.0 to 7.2

| alkaline buffer (Na$_2$HPO$_4$) | 61 ml |
| acid buffer (NaH$_2$PO$_4$·H$_2$O) | 39 ml |
| distilled water | 900 ml |

1. Combine above liquids in a 1-liter bottle.
2. Mix thoroughly, and test pH of solution. If pH is not 7.0 to 7.2, discard and remake.

The solution can be used as long as the pH remains between 7.0 and 7.2.

E. Buffered water, pH 6.8 (called for by some commercial stains for diluting stain and washing films)

| alkaline buffer (Na$_2$HPO$_4$) | 50 ml |
| acid buffer (NaH$_2$PO$_4$·H$_2$O) | 50 ml |
| distilled water | 900 ml |

1. Combine above liquids in a 1-liter bottle.
2. Mix thoroughly, and test pH of solution. If pH is not 6.8 ± 0.1, discard and remake.

The solution can be used as long as the pH is within the limits listed for the procedure.

F. Triton-buffered water solutions (optional)

1. For thin blood films or combination thin and thick blood films

   After determining the pH of the buffered water, add 1 ml of the stock 10% aqueous dilution of Triton X-100 to 1 liter of buffered water (pH 7.0 to 7.2, 0.01% final concentration). Label appropriately, and store in tightly stoppered bottle. The solution can be used as long as the pH is within limits listed for the procedure.

2. For thick blood films

   After determining the pH of the buffered water, add 10 ml of the stock 10% aqueous dilution of Triton X-100 to 1 liter of buffered water (pH 7.0 to 7.2, 0.1% final concentration). Label appropriately, and store in tightly stoppered bottle. The solution can be used as long as the pH is within limits listed for the procedure.

G. Methyl alcohol, absolute
Wright’s Stain

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE
Wright’s stain can be used to stain thin blood films for detecting blood parasites, but it is inferior to Giemsa for staining thick films. The liquid, ready-to-use stain is available commercially, or the stain can be made from dry stain powder and is ready for use in about a week. The staining reaction is somewhat similar to that of Giemsa and is achieved by using buffered water with a pH of 6.8. The stain contains the fixative and stain in one solution (1, 2).

II. SPECIMEN
A. The specimen usually consists of a dry, unfixed thin blood film.
B. Thick blood films may be stained with Wright’s stain if the RBCs are lysed before staining.

III. MATERIALS
A. Reagents (see Appendix 9.8.6–1)
B. Supplies
   1. 2 Coplin jars, 50 ml, for dehemoglobinizing thick films
   2. Mortar and pestle
   3. Flask, volumetric, 1 liter
   4. Bottle, brown, 500 ml
   5. Bottle, tight stopper, 1 liter
   6. Staining rack
   7. 3 or 4 pipettes, disposable
   8. 3 or 4 pipette bulbs
   9. Gauze for wiping slides
   10. Filter paper, Whatman no. 1
   11. Funnel (glass) to hold filter paper
C. Equipment
   1. Microscope, binocular with mechanical stage; low-power (10×), high dry power (40×), and oil immersion (100×) objectives; 10× oculars; calibrated ocular micrometer; light source equivalent to 20-W halogen or 100-W tungsten bulb; blue and white ground-glass diffuser filters
   2. Timer, 1 h or more in 1-min increments
   3. pH meter

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL
A. The stock buffer solutions and buffered water should be clear, with no visible contamination.
B. Check the Wright’s stain reagents, including the pH of the buffered water, before each use for diagnosis of blood parasites. The buffered water is usable as long as the pH is within the limits listed for the procedure.
C. Prepare and stain films from “normal” blood, and microscopically evaluate the staining reactions of RBCs, platelets, and WBCs.
   1. Macroscopically, blood films appear pinkish purple. If blue, the buffered water was too alkaline; if pink to red, the buffered water was too acid.
IV. QUALITY CONTROL
(continued)

2. Microscopically, RBCs appear tan to pinkish red, platelets appear deep pinkish red, and WBCs have bright blue nuclei and lighter cytoplasm.

3. Slight variations may appear in the colors described above depending on the batch of stain used and the character of the blood itself, but if the various morphological structures are distinct, the stain is satisfactory.

D. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (see procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

E. Record all QC results.

V. PROCEDURE

A. Wear gloves when performing this procedure.

B. Thin blood films (only) (1)
   1. Place air-dried films on a level staining rack.
   2. Use a pipette to cover the surface of the slide with stain, adding stain drop by drop. Count the number of drops needed to cover the surface. Let stand 1 to 3 min (optimal staining time [to obtain correct color range and intensity] will vary with each batch of stain).
   3. Add to the slide the same number of drops of buffered water as were used of stain for step V.B.2 and mix the stain and water by blowing on the surface of the fluid.
   4. After 4 to 8 min, flood the stain from the slide with buffered water. Do not pour the stain off before flooding, or a precipitate will be deposited on the slide.
   5. Wipe the underside of the slide to remove excess stain.

C. Combination thin and thick blood films
   1. Lyse the RBCs in the thick film by immersing it for 10 min in buffered water. Be sure that the water does not touch the unfixed thin film.
   2. Remove the slide, and rinse the thick film by dipping in additional buffered water (two or three dips).
   3. Let film air dry thoroughly.
   4. Stain both thin and thick films with Wright’s stain as directed for thin films.

VI. RESULTS

A. If Plasmodium organisms are present, the cytoplasm stains pale blue and the nuclear material stains red.

B. Schüffner’s stippling in RBCs infected by malaria species usually does not stain or stains very pale red with Wright’s stain.

C. Nuclear and cytoplasmic colors that are seen in the malarial parasites will also be seen in the trypanosomes and any intracellular leishmaniae that are present.

D. The sheath of microfilariae may or may not stain with Wright’s stain, while the body will usually appear pale to dark blue.
POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report any parasite, including the stages found (do not use abbreviations).
   Examples: *Plasmodium falciparum* rings and gametocytes, rings only
   *Plasmodium vivax* rings, trophozoites, schizonts, and gametocytes
   *Plasmodium ovale* rings and schizonts
   *Plasmodium malariae* rings and developing trophozoites
   *Babesia* sp. rings
   *Wuchereria bancrofti* microfilariae
   *Trypanosoma brucei gambiense/rhodesiense* trypanostigotes
   *Trypanosoma cruzi* trypanostigotes
   *Leishmania donovani* amastigotes

B. Any laboratory providing malaria diagnoses should be able to identify *Plasmodium vivax* and *Plasmodium ovale*, even in the absence of Schüffner’s stippling.

VIII. PROCEDURE NOTES

A. Blood films prepared from venipuncture blood when an anticoagulant is used must be prepared within 1 h of collection. Otherwise, certain morphological characteristics of both parasites and infected RBCs may be atypical.

B. The correct pH for all buffered-water and staining solutions is also important. Solutions with the incorrect pH will prevent certain morphological characteristics (stippling) from being visible and will not give typical nuclear and cytoplasmic colors on the stained film.

C. Stain QC slide each time patient blood films are stained. If several patient specimens are stained on the same day (using the same reagents), only one control slide need be stained and examined.

D. If at any time the stain appears to contain particulate matter or stain, precipitate, filter prior to use.

IX. LIMITATIONS OF THE PROCEDURE

A. *Finding no parasites in one set of blood films does not rule out a parasitic infection.*

B. Examine a minimum of 300 oil immersion (×1,000) fields before reporting no parasites found.

C. Examine the entire smear under low power (100×) for the presence of microfilariae. Remember that the sheath may not be visible (*W. bancrofti*).

REFERENCES


SUPPLEMENTAL READING


APPENDIX 9.8.6–1

Include QC information on reagent container and in QC records.

Reagents

- Indicate the expiration date on the label and in the work record or on the manufacturer’s label.

A. Wright’s stain

Wright’s stain .................................. 0.9 g
methyl alcohol, absolute
(acetone free) ............................. 500.0 ml

1. Grind 0.9 g of stain powder with 10 to 15 ml of methyl alcohol (anhydrous, acetone free) in a clean mortar. Gradually add methyl alcohol while grinding. As the dye is dissolved in the alcohol, pour that solution off and add more alcohol to the mortar. Repeat the process until the 500 ml of alcohol is used up.
2. Store the stain solution in a tightly stoppered glass bottle (1 liter) at room temperature.
3. Shake the bottle several times daily for at least 5 days (aging process).
4. Before use, filter through Whatman no. 1 paper into a brown bottle (stock stain).
5. Label appropriately, and store protected from light at room temperature. The shelf life is 36 months, providing QC criteria are met.

B. Stock buffers (for preparing buffered water)

1. Alkaline buffer, 0.067 M solution

\[
\text{Na}_2\text{HPO}_4 \text{ (anhydrous)} \quad 9.5 \, \text{g}
\]

\[
\text{distilled water, to make} \quad 1.0 \, \text{liter}
\]

In a 1-liter volumetric flask, dissolve Na$_2$HPO$_4$ in about three-fourths of the water. Add water to make 1 liter of solution. Store in tightly stoppered bottle, and label appropriately. The shelf life is 24 months. Note the date on label and in work record.

2. Acid buffer, sodium dihydrogen phosphate (monobasic), 0.067 M solution

\[
\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O} \quad 9.2 \, \text{g}
\]

\[
\text{distilled water, to make} \quad 1.0 \, \text{liter}
\]

In a 1-liter volumetric flask, dissolve NaH$_2$PO$_4$•H$_2$O in about three-fourths of the water. Add water to make 1 liter of solution. Store in tightly stoppered bottle, and label appropriately. The shelf life is 24 months. Note date on label and in work record.

C. Buffered water, pH 6.8 (called for by some commercial stains for diluting stain and washing films)

\[
\text{alkaline buffer (Na}_2\text{HPO}_4) \quad 50 \, \text{ml}
\]

\[
\text{acid buffer (NaH}_2\text{PO}_4\cdot\text{H}_2\text{O)} \quad 50 \, \text{ml}
\]

\[
\text{distilled water} \quad 900 \, \text{ml}
\]

1. Combine above liquids in a 1-liter bottle.
2. Mix thoroughly, and test pH of solution. If pH is not 6.8 ± 0.1, discard and remake. The solution can be used as long as the pH is within limits listed for the procedure.
9.8.7 Determination of Parasitemia

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE
It is important to report the level of parasitemia when blood films are examined and found to be positive for malaria parasites. Because of the potential for drug resistance in some of the Plasmodium species, particularly Plasmodium falciparum, it is important that every positive smear be assessed and the parasitemia reported exactly the same way for follow-up specimens as for the initial specimen (1–6). This allows the parasitemia to be monitored after therapy has been initiated. In cases where the patient is hospitalized, monitoring should be performed at 24, 48, and 72 h after initiating therapy. Generally the parasitemia will drop very quickly within the first 2 h; however, in cases of drug resistance, the level may not decrease but actually increase over time.

II. SPECIMEN
The specimen consists of stained thick or thin blood films that have been examined a minimum of 300 oil immersion fields per blood film to determine that the film is positive for malaria parasites.

Observe standard precautions.

III. MATERIALS
A. Reagents
   None
B. Supplies
   None
C. Equipment
   Microscope, binocular with mechanical stage; low-power (10×), high dry power (40×), and oil immersion (100×) objectives; 10× oculars; calibrated ocular micrometer; light source equivalent to a 20-W halogen or 100-W tungsten bulb; blue and white ground-glass diffuser filters

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL
A. Prepare and stain films from “normal” blood, and microscopically evaluate the staining reactions of RBCs, platelets, and WBCs (normally done during staining procedure using Giemsa or Wright’s stain).
B. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (see procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.
V. PROCEDURE

A. Thin blood film: counting several hundred to 1,000 RBCs, report the percentage of infected RBCs per 100 RBCs counted (0.5%, 1.0%, etc.).

B. Thick or thin blood film: counting 100 WBCs (or more), report the number of parasites per 100 WBCs on the smear.
   1. This figure can be converted to the number of parasites per microliter of blood; divide the number of parasites per 100 WBCs by 100, and multiply that figure by the number of WBCs per microliter of blood.
   2. Depending on the parasitemia, 200 or more WBCs may have to be counted, so the denominator may vary (it may be 200 or even more).
   3. Using this method, blood for both the peripheral smears and cell counts must be collected at the same time.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS (Table 9.8.7–1)

A. Using the thin blood film method, report the percentage of parasite-infected RBCs per 100 RBCs counted.
   
   Example: *Plasmodium falciparum*, parasitemia = 0.5%

B. Using the thick-thin blood film method, report the number of parasites per microliter of blood.
   
   Example: *Plasmodium falciparum*, parasitemia = 10,000 per µl of blood

| % Parasitemia | No. of parasites/µl | Clinical correlation
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0001–0.0004</td>
<td>5–20</td>
<td>Number of organisms that are required for a positive thick film (sensitivity)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Note: Examination of 100 TBF fields (0.25 µl) may miss infections up to 20% (sensitivity of 80–90%); at least 300 TBF fields should be examined before reporting a negative result.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Note: Examination of 100 THBF fields (0.005 µl); at least 300 THBF should be examined before reporting a negative result. Both TBF and THBF should be examined for every specimen submitted for a suspected malaria case. One set (TBF + THBF) of negative blood films does not rule out a malaria infection.</td>
</tr>
<tr>
<td>0.002</td>
<td>100</td>
<td>Patients may be symptomatic below this level.</td>
</tr>
<tr>
<td>0.2</td>
<td>10,000</td>
<td>Level above which immune patients will exhibit symptoms</td>
</tr>
<tr>
<td>2</td>
<td>100,000</td>
<td>Maximum parasitemia of <em>P. vivax</em> and <em>Plasmodium ovale</em> (infect young RBCs only)</td>
</tr>
<tr>
<td>2–5</td>
<td>100,000–250,000</td>
<td>Hyperparasitemia, severe malaria; increased mortality</td>
</tr>
<tr>
<td>10</td>
<td>500,000</td>
<td>Exchange transfusion may be considered, high mortality</td>
</tr>
</tbody>
</table>

* Adapted from references 2 and 6.

* TBF, thick blood film; THBF, thin blood film.

* World Health Organization criteria for severe malaria are parasitemia of >10,000/µl and severe anemia (hemoglobin <5 g/liter). Prognosis is poor if >20% of parasites are pigment-containing trophozoites and schizonts and/or if >5% of neutrophils contain visible pigment.
VII. PROCEDURE NOTES

A. It is critical that the same reporting method be used consistently for every subsequent set of blood films so that the parasitemia can be tracked for decrease or possible increase, indicating resistance.

B. Remember, drug resistance may not become evident for several days; the parasitemia may even appear to be dropping before it begins to increase again.

C. It is very important that any patient with *P. falciparum* infection be monitored; drug resistance has also been reported for *Plasmodium vivax* infections.

D. It is critical to remember that mixed malarial infections occur, many of which will include *P. falciparum*.

VIII. LIMITATIONS OF THE PROCEDURE

A. A light infection may be missed in a thin film, whereas the increased volume of blood present on a thick film may allow the detection of the infection, even with a low parasitemia.

B. If the smears are prepared from anticoagulated blood that is more than 1 h old, the morphology of both parasites and infected RBCs may not be typical.

C. It is important that good-quality blood films be examined and counted according to directions; poorly prepared and/or stained blood films will lead to incorrect assessments of the parasitemia (both too low and too high).

D. Follow-up counts are critical in monitoring the patient, and it is critical to understand the types of malaria resistance (see Table 9.8.7–2).

<table>
<thead>
<tr>
<th>Table 9.8.7–2 Malaria resistance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistance definition</td>
</tr>
<tr>
<td>Sensitive</td>
</tr>
<tr>
<td>Resistance type I</td>
</tr>
<tr>
<td>Resistance type II</td>
</tr>
<tr>
<td>Resistance type III</td>
</tr>
</tbody>
</table>

* Adapted from references 3 and 4.
REFERENCES


Delafield’s Hematoxylin Stain

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE
Microfilariae in an unstained preparation do not show sufficiently definite morphological characteristics to allow positive identification. Although Giemsa stain, used for the routine staining of blood parasites, can be used for microfilariae, Delafield’s hematoxylin stain is widely used to demonstrate greater structural detail of microfilariae (1, 2). This stain especially enhances the nuclei and the sheath, if present. No one type of stain, however, will reveal all the details of morphology, and in some cases, more than one staining procedure may be necessary for species identification.

II. SPECIMEN
A. Delafield’s hematoxylin stain can be used for thick or thin blood films.
B. It is most frequently used to stain the sediment of a concentration procedure (see procedures 9.8.10 and 9.8.11) designed to recover more microfilariae from the blood.

III. MATERIALS
A. Reagents (see Appendix 9.8.8–1)
B. Supplies
1. 4 or 5 Coplin jars, 50 ml
2. Glass or plastic Pasteur pipettes
3. Pipette, to deliver 0.05 ml
4. Beakers, 250 ml, 25 ml
5. Flask, Erlenmeyer, 500 ml
6. Graduated cylinders, 3 100 ml, 500 ml, 50 ml
7. Bottles, 2 1,000 ml, 100 ml
8. Funnel (glass) to hold filter paper
9. Filter paper, Whatman no. 1
10. Coverslips, 22 by 22 mm or larger, no. 1 thickness
11. Mounting medium, pH 7.0
C. Equipment
1. Microscope, binocular with mechanical stage; low-power (10×), high dry power (40×), and oil immersion (100×) objectives; 10× oculars; calibrated ocular micrometer; light source equivalent to 20-W halogen or 100-W tungsten bulb; blue and white ground-glass diffuser filters
2. Timer, 1 h or more in 1-min increments
3. pH meter (or pH paper) to check ammonia water
4. Hot plate

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL
A. If possible, check the stain procedure before each use for diagnosis of filariasis on blood known to contain microfilariae.
B. If human blood containing microfilariae is not available, try to obtain canine blood containing *Dirofilaria immitis* from a veterinarian. No sheath is present, but nuclei should be distinct, and the colors should be those described below.
C. If positive blood is not available, follow the procedure carefully in testing the specimen submitted for diagnosis. Be sure that the morphological features of the microfilariae found are distinct and that the colors are those described below.

1. Macroscopically, the films appear bluish purple.
2. Microscopically, the nuclei of the microfilariae are blue or purplish, and the sheath, if present, is a light purple. The cytoplasm is reddish. The R, anal, and excretory cells may or may not be visible, but if they are, they do not differ markedly from the nuclei. The inner body may be seen as a whitish structure.

D. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (see procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

E. Record all QC results.

V. PROCEDURE (adapted from reference 3)

A. Wear gloves when performing this procedure.
B. Prepare thick or thin blood films or films from sediments of concentrated blood, and dry thoroughly (see procedures 9.8.10 and 9.8.11).
C. If thick films are to be stained, lyse the RBCs by placing the film in distilled water for 10 min. Allow to air dry. This step is not necessary for thin blood films or films prepared from sediments.
D. Fix all films in absolute methanol for 5 min. Allow to air dry.
E. Stain in undiluted Delafield’s hematoxylin for 10 to 15 min.
F. Destain with acid water (0.05% HCl) for 5 to 10 s.
G. Wash in water containing sufficient ammonia to yield an alkaline pH (~9.0 to 10.0) until a blue color appears in film.
H. Air dry.
I. Mount film with neutral mounting medium and coverslip (no. 1 thickness), or examine unmounted.
J. Examine the entire film with the low-power (10×) objective. Specific morphological details can be observed with the high dry power (40×) and oil immersion (100×) objectives.

VI. RESULTS

A. Microfilariae may be present. Based on the positions of the nuclei and the presence or absence of a sheath, they can be identified to the genus and species levels.
B. If other blood parasites are seen, it is recommended that routine thin and thick films be stained with Giemsa stain.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the genus and species of microfilariae present.
   Example: Wuchereria bancrofti microfilariae present.
B. If sufficient morphological detail is not visible to allow generic and/or specific identification, report as follows.
   Examples: Sheathed microfilariae present.
              Unsheathed microfilariae present.
              Microfilariae present.
VIII. PROCEDURE NOTES

A. It is important that the stock stain age for at least 1 month prior to use. During this time, it should be exposed to sunlight.
B. If the nuclei do not stain blue and the cytoplasm does not stain red, review proper aging of the stain.
C. One of the main benefits of this stain is improved visibility of the sheath.
   1. The sheath of *W. bancrofti* will often not be seen using Giemsa stain.
   2. The sheath of *W. bancrofti* is easily seen when Delafield’s hematoxylin is used.

IX. LIMITATIONS OF THE PROCEDURE

A. If the blood specimen is too old or has not been processed properly, the final stain may not reveal clear nuclear and sheath detail.
B. Regardless of the quality of the stain, a low parasitemia may not be detected.

REFERENCES


SUPPLEMENTAL READING


APPENDIX 9.8.8–1

Reagents

Include QC information on reagent container and in QC records.

A. Delafield’s hematoxylin stain, hematoxylin solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>hematoxylin crystals</td>
<td>4.0 g</td>
</tr>
<tr>
<td>ethyl alcohol, 95%</td>
<td>25.0 ml</td>
</tr>
<tr>
<td>saturated aluminum ammonium sulfate</td>
<td>400.0 ml</td>
</tr>
<tr>
<td>solution</td>
<td></td>
</tr>
<tr>
<td>methyl alcohol (acetone free)</td>
<td>100.0 ml</td>
</tr>
<tr>
<td>glycerin</td>
<td>100.0 ml</td>
</tr>
</tbody>
</table>

1. Dissolve 4.0 g of hematoxylin crystals in 25 ml of 95% ethyl alcohol.
2. Prepare 400 ml of saturated aluminum ammonium sulfate \([\text{AlNH}_2(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}]\) solution by adding the aluminum ammonium sulfate crystals (~90 g) to 400 ml of very hot distilled water, stirring until no more crystals go into solution, cooling to allow excess alum to crystallize, and decanting the solution.
3. Mix the hematoxylin solution with the 400 ml of saturated aluminum ammonium sulfate solution in a loosely capped or cotton-plugged bottle. Keep in a light, airy location for 2 weeks.
4. After 2 weeks, mix 100 ml of methyl alcohol (acetone free) with 100 ml of glycerin, and add this mixture to the mixture from step A.3.
5. Bottle the complete mixture from step A.4., leave cap loose, and expose to direct sunlight for at least 1 month.
6. Filter before use through Whatman no. 1 paper into a clear glass bottle, stopper tightly, and store at room temperature.
7. Label appropriately. The shelf life is 36 months, providing QC criteria are met.
APPENDIX 9.8.8–1 (continued)

B. Acid water destain

\[
\begin{align*}
\text{HCl} & : \quad 0.05 \text{ ml} \\
\text{distilled water} & : \quad 99.95 \text{ ml}
\end{align*}
\]

1. Carefully dispense the concentrated hydrochloric acid into the distilled water.
2. Label and store in a tightly stoppered bottle, and label with an expiration date of 12 months.

C. Ammonia water

\[
\begin{align*}
\text{ammonium hydroxide (NH}_4\text{OH)} & : \quad 3 \text{ to } 5 \text{ drops} \\
\text{tap water} & : \quad 50.0 \text{ ml}
\end{align*}
\]

1. Mix the two components in a Coplin jar. Discard remaining solution after use.
2. Check pH (~9.0 to 10.0).

D. Methyl alcohol (acetone free)

E. Glycerin

F. Distilled water
Concentration Procedures: Buffy Coat Concentration

Concentration procedures increase the number of organisms recovered from blood specimens submitted for diagnosis of trypanosomiasis, filariasis, and leishmaniasis. A concentration procedure should be performed routinely on all blood specimens submitted for examination for trypanosomes or microfilariae when the suspected organisms are not found in thick blood films or when organisms are so few that more are needed to make a positive identification of species (see Appendix 9.8.9–1) (1, 2).

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Leishmania donovani amastigotes are difficult to detect in blood specimens but may occasionally be found within monocytes by fractional centrifugation of comparatively large amounts of blood. The procedure may also be used to recover trypanosomes and microfilariae, both of which are found in the plasma.

II. SPECIMEN

Whole blood collected by using EDTA, heparin, or sodium citrate anticoagulant (regular venipuncture collection tubes are recommended—primarily the lavender/EDTA or green/heparin stoppers)

III. MATERIALS

A. Reagents

1. Methyl alcohol, absolute
2. Giemsa or hematoxylin stain (see procedures 9.8.5 and 9.8.8)

B. Supplies

1. 2 centrifuge tubes, glass, 12 ml (plastic tubes can be used, provided they are clear plastic)
2. Capillary pipette with bulb
3. Glass microscope slides, 1 by 3 in., alcohol washed
4. Coverslips, 22 by 22 mm or larger, no. 1 thickness
5. Microhematocrit tube(s) (for alternate procedure)

6. Blood collection supplies, if applicable

C. Equipment

1. Centrifuge with sealable carrier cups, speed calibrated
2. Microscope, binocular with mechanical stage; low-power (10×), high dry power (40×), and oil immersion (100×) objectives; 10× oculars; calibrated ocular micrometer; light source equivalent to 20-W halogen or 100-W tungsten bulb; blue and white ground-glass diffuser filters
3. Timer, 1 h or more in 1-min increments

Observe standard precautions. Include QC information on reagent container and in QC records.
ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Check calibration of centrifuge.
B. Perform the procedure on “normal” blood. The film should be composed almost exclusively of WBCs, which stain characteristically with Giemsa (see procedure 9.8.5). If parasites are present, they also should stain characteristically (see procedure 9.8.5).
C. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (see procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.
D. Record all QC results.

V. PROCEDURE (adapted from reference 3)

A. Wear gloves when performing this procedure.
B. Centrifuge the anticoagulated blood specimen in a sealed cup at 100 × g for 15 min.
C. Remove the thin creamy layer (buffy coat) between the RBCs and plasma with a capillary pipette, or transfer the creamy layer (buffy coat) and plasma to another tube, and centrifuge in a sealed cup at 300 × g for 15 min.
D. Examine buffy coat directly for motile trypomastigotes and microfilariae.
   1. Place one-half drop of saline on a clean microscope slide.
   2. Remove a drop of sediment, and mix it in the saline.
   3. Add a coverslip, and examine for organism motility with the low-power (10 ×) and high dry power (40 ×) objectives.
E. Prepare thin films, dry, fix, and stain with Giemsa stain (see procedure 9.8.5 or, if for microfilariae, procedure 9.8.8).

VI. RESULTS

A. If present, *L. donovani* amastigotes will be found within the monocytes on a Giemsa-stained film. Nuclear material stains dark purple-red, the cytoplasm is light blue, and the kinetoplast may or may not be visible as a dark bluish purple structure.
B. Trypomastigotes will be found extracellularly (motile in the wet smear). Morphological detail will be seen in the Giemsa-stained film. The stain reaction is like that of *L. donovani*; the kinetoplast will be visible.
C. Microfilariae may be found in the wet smear. Morphological detail will be seen in a Giemsa- or hematoxylin-stained film. The stain reaction is typical for each stain (see procedures 9.8.5 and 9.8.8).

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the presence of organisms from the wet smear.
   Examples: Trypomastigotes present.
   Microfilariae present.
B. Report the genus and species of organisms from the Giemsa-stained film.
   Examples: *Trypanosoma cruzi* trypomastigotes present.
   *Leishmania donovani* amastigotes present.
VIII. PROCEDURE NOTES

A. If you need to add anticoagulant to blood, mix 9 ml of blood and 1 ml of 5% sodium citrate in a glass centrifuge tube. Then proceed with centrifugation.

B. This procedure can be performed in a microhematocrit tube if the tube is carefully scored and broken at the buffy coat interface and if the WBCs are prepared and stained as for a thin blood film.

C. Also, the tube can be examined microscopically (high dry magnification) at the buffy coat layer for motile trypomastigotes and microfilariae before the tube is scored and broken.

IX. LIMITATIONS OF THE PROCEDURE

A. When examined as a wet smear, the intracellular leishmaniae are very difficult to see.

B. Although trypomastigote and microfilarial motility may be visible on the wet smear, specific identification may be difficult.

REFERENCES


APPENDIX 9.8.9–1

QBC Capillary Blood Tube

Recently, a centrifugation procedure which yields a buffy coat has been used to concentrate and detect Plasmodium spp. (1–3). The technique uses a commercially available capillary tube (QBC Capillary Blood Tube; Becton Dickinson, Franklin Lakes, N.J.) which is coated with acridine orange stain and fitted with a buoyant plastic insert. The stain causes the malaria organisms to fluoresce, and the plastic insert forces the RBCs containing stained parasites, because they differ in buoyancy from uninfected RBCs, to be concentrated just under the buffy coat. After centrifugation, the tube is examined under the microscope at the plastic insert level for the presence of malaria parasites. Some laboratorians have been able to accurately identify species by using this method. At this time, however, this technique requires more trials, and appropriate thin and/or thick films are recommended for the positive identification of Plasmodium spp.

References


9.8.10 Concentration Procedures: Membrane Filtration Concentration

**Preanalytical Considerations**

I. **Principle**

Membrane filtration methods for recovering microfilariae from patients with light infections have been developed. These methods have an advantage over simple centrifugation methods in that large samples of blood (20 ml or more) can be used if necessary. The technique described here is one of the most efficient for the clinical laboratory when other procedures used to recover microfilariae are unsatisfactory (1–3).

Membrane filtration recovers most species of microfilariae; however, because of their smallness, *Mansonella perstans* and *Mansonella ozzardi* may not be recovered. Membranes of smaller pore size (3 μm) have been suggested for recovering these two species.

II. **Specimen**

Whole blood collected by using EDTA, heparin, or sodium citrate anticoagulant.

*Observe standard precautions.*

III. **Materials**

Include QC information on reagent container and in QC records.

A. **Reagents**

   - Distilled water
   - Methyl alcohol, absolute
   - Giemsa or hematoxylin stain (see procedures 9.8.5 and 9.8.8)
   - Toluene

B. **Supplies**

   1. Glass syringe, 15 ml (clear plastic is acceptable)
   2. Nuclepore membrane filter, 25 mm, 5-μm porosity
   3. Swinney filter adapter (attaches to syringe, holds filter)
   4. Filter paper pad, 25 mm (used to support the membrane filter)
   5. Glass microscope slides, 1 by 3 in.
   6. Coverslips, 22 by 22 mm or larger, no. 1 thickness
   7. Mounting medium

C. **Equipment**

Microscope, binocular with mechanical stage; low-power (10×), high dry power (40×), and oil immersion (100×) objectives; 10× oculars; calibrated ocular micrometer; light source equivalent to 20-W halogen or 100-W tungsten bulb; blue and white ground-glass diffuser filters.

**Analytical Considerations**

IV. **Quality Control**

A. If possible, check the procedure by using human or canine blood containing microfilariae.

B. If positive blood is not available, follow the procedure carefully in testing the specimen submitted for diagnosis. Examine sediment thoroughly with low- and high-power magnification.
IV. QUALITY CONTROL (continued)

C. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (see procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

D. Record all QC results.

V. PROCEDURE

A. Wear gloves when performing this procedure.

B. Draw 1 ml of fresh whole blood or anticoagulated blood into a 15-ml syringe containing 10 ml of distilled water.

C. Gently shake the mixture for 2 to 3 min to ensure that all blood cells are lysed.

D. Place a 25-mm Nuclepore filter (5-μm porosity) over a moist 25-mm filter paper pad, and place in a Swinney filter adapter (see illustration in Appendix 9.6.9–2 of procedure 9.6.9).

E. Attach the Swinney filter adapter to the syringe containing the lysed blood.

F. With gentle but steady pressure on the piston, push the lysed blood through the filter.

G. Without disturbing the filter, remove the Swinney adapter from the syringe, and draw approximately 10 ml of distilled water into the syringe. Replace the adapter, and gently push the water through the filter to wash the debris from the filter.

H. Remove the adapter again, draw the piston of the syringe to about half the length of the barrel, replace the adapter, and push the air in the barrel through the filter to expel excess water.

I. To prepare the filter for staining, remove the adapter, draw the piston about half the length of the barrel, and then draw 3 ml of absolute methanol into the syringe. Holding the syringe vertically, replace the adapter, and push the methanol followed by the air through the filter to fix the microfilariae and expel the excess methanol, respectively.

J. To stain, remove the filter from the adapter, place it on a slide, and allow it to air dry thoroughly. Stain with Giemsa stain as for a thick film (using 0.1% Triton X-100) (see procedure 9.8.5) or with Delafield’s hematoxylin (see procedure 9.8.8).

K. To cover the stained filter, dip the slide in toluene before mounting the filter with neutral mounting medium and a coverslip. This will lessen the formation of bubbles in or under the filter.

VI. RESULTS

A. If present in the sample, microfilariae are concentrated and will appear on the wet membrane.

B. After being stained with Giemsa or Delafield’s hematoxylin, the microfilariae will stain characteristically. The sheath, if present, may or may not stain with Giemsa.

VII. REPORTING RESULTS

A. Report the presence of organisms from the wet Nuclepore membrane.

  Example: Microfilariae present.

B. Report the genus and species of organisms from the Giemsa- or hematoxylin-stained membrane.

  Example: *Wuchereria bancrofti* microfilariae present.
VIII. PROCEDURE NOTES

A. Gently shake the water-blood mixture to ensure total lysis of blood cells. Some parasitologists prefer to use an aqueous solution of 10% Teepol (Shell Oil Co.) to lyse the blood cells.

B. Motile microfilariae may be seen on the membrane filter after washing with water (step V.G); however, low light will be necessary and the filter must be reassembled before fixing with methanol.

C. The membrane filter must be supported by the moistened filter pad to prevent rupture when the water is expelled through the membrane.

D. If you need to add anticoagulant to blood, mix 9 ml of blood and 1 ml of 5% sodium citrate in a clear plastic centrifuge tube. Then proceed with centrifugation.

IX. LIMITATIONS OF THE PROCEDURE

A. Giemsa or hematoxylin staining may be necessary to identify the organisms to the species level.

B. Identification of microfilariae on filters to the species level may be difficult.

REFERENCES


SUPPLEMENTAL READING


9.8.11 Concentration Procedures: Knott Concentration

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

This technique is used to recover low numbers of microfilariae from blood. A solution is used to lyse the RBCs in a large blood sample, and the organisms are concentrated from the supernatant fluid by centrifugation (1–4). The disadvantage of this technique is that the microfilariae are killed and immobilized and are therefore not readily revealed by any motility.

II. SPECIMEN

Whole blood collected by using EDTA, heparin, or sodium citrate anticoagulant

Observe standard precautions.

III. MATERIALS

A. Reagent

- Indicate the expiration date on the label and in the work record or on the manufacturer’s label.
- Formalin, aqueous, 2% (vol/vol) formaldehyde, liquid ..............2 ml distilled water ..................... 98 ml
- Mix thoroughly. Store in stoppered bottle. Label appropriately. Shelf life is 24 months.

B. Supplies

1. Graduated cylinder, 100 ml
2. Pipette, 5 ml
3. Bottle, 100 ml
4. Capillary pipette with bulb
5. Glass microscope slides, 1 by 3 in.
6. Coverslips, 22 by 22 mm or larger, no. 1 thickness

C. Equipment

1. Centrifuge with sealable carrier cups, speed calibrated
2. Microscope, binocular with mechanical stage; low-power (10×), high dry power (40×), and oil immersion (100×) objectives; 10× oculars; calibrated ocular micrometer; light source equivalent to 20-W halogen or 100-W tungsten bulb; blue and white ground-glass diffuser filters
3. Timer, 1 h or more in 1-min increments

7. Blood collection supplies, if applicable
8. Centrifuge tube, glass, 12 ml
ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Check calibration of centrifuge.
B. If possible, check the procedure by using human or canine blood containing microfilariae with or without a sheath.
C. If positive blood is not available, follow the procedure carefully in testing the specimen submitted for diagnosis. Examine sediment thoroughly with low- and high-power magnification.
D. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (see procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.
E. Record all QC results.

V. PROCEDURE

A. Wear gloves when performing this procedure.
B. Place 1 ml of fresh whole blood or anticoagulated blood in a centrifuge tube containing 10 ml of 2% formalin. Mix thoroughly.
C. Centrifuge for 5 min at 300 × g.
D. Pour off the supernatant fluid without disturbing the sediment.
E. Using a capillary pipette, transfer a portion of the sediment to a slide.
   1. Apply a coverslip, and examine microscopically under low-power (100×) and high-power (400×) magnification.
   2. If microfilariae are present, prepare a thick film from the remainder of the sediment, air dry, fix in absolute methanol for 5 min, air dry again, and stain with Giemsa or Delafield’s hematoxylin (see procedures 9.8.5 and 9.8.8).

VI. RESULTS

A. If present in the sample, microfilariae are concentrated and will appear non-motile in the wet smear.
B. After being stained with Giemsa or Delafield’s hematoxylin, the microfilariae will exhibit diagnostic morphology and typical staining characteristics.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the presence of organisms from the wet smear.
   Example: Microfilariae present.
B. Report the genus and species of organisms from the Giemsa- or hematoxylin-stained film.
   Example: Wuchereria bancrofti microfilariae present.

VIII. PROCEDURE NOTES

A. If you need to add anticoagulant to blood, mix 9 ml of blood and 1 ml of 5% sodium citrate in a glass centrifuge tube. Then proceed with centrifugation.
B. Morphological details may not be visible prior to Giemsa or hematoxylin staining.
IX. LIMITATIONS OF THE PROCEDURE

A. Motility will not be visible after formalin fixation.
B. Identification to the species level may be difficult for most laboratorians without additional staining.
C. The blood-formalin mixture can be sent to a reference laboratory for staining and identification of microfilariae.

REFERENCES

Concentration Procedures: Triple Centrifugation Concentration

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

In many instances, the number of trypomastigotes (trypanosomes) present in peripheral blood is too small to be detected in direct wet mounts or stained films. Fractional centrifugation is an effective method of concentrating the organisms from a relatively large blood sample (1–3).

II. SPECIMEN

Whole blood collected by using EDTA, heparin, or sodium citrate anticoagulant. Make sure to fill the tube (containing the anticoagulant) completely, thus guaranteeing the correct anticoagulant/blood ratio.

Observe standard precautions.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagent
   1. Indicate the expiration date on the label and in the work record or on the manufacturer’s label.
   2. Giemsa or Wright’s stain (see procedures 9.8.5 and 9.8.6)

B. Supplies
   1. 3 centrifuge tubes, 12 or 15 ml
   2. Capillary pipettes with bulb
   3. Glass microscope slides, 1 by 3 in.
   4. Coverslips, 22 by 22 mm or larger, no. 1 thickness
   5. Blood collection supplies, if applicable

C. Equipment
   1. Centrifuge with sealable carrier cups, speed calibrated
   2. Microscope, binocular with mechanical stage; low-power (10×), high dry power (40×), and oil immersion (100×) objectives; 10× oculars; calibrated ocular micrometer; light source equivalent to 20-W halogen or 100-W tungsten bulb; blue and white ground-glass diffuser filters
   3. Timer, 1 h or more in 1-min increments

IV. QUALITY CONTROL

A. Check calibration of centrifuge.
B. If possible, check the procedure by using blood containing trypanosomes. Positive blood may be available at universities and large research laboratories.
C. If positive blood is not available, follow the procedure carefully in testing the specimen submitted for diagnosis.
D. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the micro-
IV. QUALITY CONTROL (continued)

scope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (see procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.
E. Record all QC results.

V. PROCEDURE

A. Wear gloves when performing this procedure.
B. Centrifuge the blood at 300 × g for 10 min.
C. Transfer the supernatant fluid to a second centrifuge tube, and centrifuge at 500 × g for 10 min.
D. Again, transfer the supernatant fluid to a clean tube, and centrifuge at 900 × g for 10 min.
E. Carefully decant the supernatant fluid, and examine about one-half of the sediment directly as a wet preparation for motile trypanosomes. Use the 10× objective for the examination; in order to see greater organism detail, use the 40× objective (do not use the 100× oil immersion objective on wet preparations).
F. From the remaining sediment, prepare thin films, and stain with Giemsa or Wright’s stain.

VI. RESULTS

A. If present in the sample, trypomastigotes (trypanosomes) are concentrated.
B. After being stained with Giemsa or Wright’s stain, the trypomastigotes will exhibit diagnostic morphology and typical staining characteristics.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the presence of organisms from the wet smear.
   Example: Trypomastigotes present.
B. Report the genus and species of organisms from the Giemsa- or Wright-stained films.
   Example: Trypanosoma cruzi or Trypanosoma gambiense/rhodesiense present.

VIII. PROCEDURE NOTES

A. Each time, remove the supernatant fluid carefully (do not remove any sediment).
B. Make sure the centrifuge is calibrated and the timing is begun when the centrifuge reaches the recommended speed.
C. Leave some sediment for the preparation of thin films for staining with Giemsa or Wright’s stain.

IX. LIMITATIONS OF THE PROCEDURE

A. Although organism motility may be visible, morphological detail may not be.
B. Unless dividing trypomastigotes are found in the wet preparation, permanent staining with Giemsa or Wright’s stain will be necessary to differentiate the species.

REFERENCES

Parasite Culture: *Entamoeba histolytica*

**PREANALYTICAL CONSIDERATIONS**

I. **PRINCIPLE**

*Entamoeba histolytica*, the agent of intestinal and hepatic amebiasis, can be cultivated in conjunction with the bacteria voided in feces by the infected patient. Although cultures for *E. histolytica* are not routinely offered by most clinical laboratories, this approach may be helpful when routine procedures have failed to provide a diagnosis. Polyxenic cultured organisms can also be used to produce intestinal and hepatic amebiasis in susceptible experimental hosts such as hamsters, guinea pigs, and rats. Axenic cultivation of organisms is invaluable for the following: (i) to study the biochemistry, physiology, and metabolism of the organisms in order to establish nutritional requirements of the parasites; (ii) to produce antigens of and monoclonal and polyclonal antibodies against *E. histolytica* for serological diagnosis as well as other immunologic studies; (iii) to differentiate pathogenic from nonpathogenic strains by using isoenzyme electrophoresis, monoclonal antibody, and/or DNA probes; (iv) to screen drugs in vitro to identify isolates susceptible and resistant to particular drugs so that advances in chemotherapy can be evaluated; (v) to infect experimental animals to produce the disease so that pathologic processes can be understood; and (vi) to understand the organization of the parasite at the ultrastructural level.

II. **SPECIMEN**

A. The specimen may consist of stool, mucus, or a combination of the two.
B. The specimen should be as fresh as possible but never more than 24 h old.

III. **MATERIALS**

A. **Reagents** *(see Appendix 9.9.1–1)*

B. **Supplies**

1. Graduated Erlenmeyer flasks
2. Graduated cylinders (500 ml)
3. Volumetric flasks (1,000 and 2,000 ml)
4. Microscope slides (1 by 3 in. or larger)
5. Coverslips (no. 1; 22 by 22 mm or larger)
6. Sterile syringes (10 ml)
7. Sterile needles (20 gauge)
8. Sterile screw-cap tubes (16 by 125 mm)
9. Sterile cryovials or screw-cap vials (to hold 1 ml)
10. Box for vial storage in freezer
11. Disposable sterile Pasteur and serological pipettes
12. Filter paper, Whatman no. 1
13. Sterile filtration system (Seitz)
14. Membrane filters (0.22-μm pore size)
15. Nutrient agar plates
16. BHI broth
17. Thioglycolate broth
18. Methylene blue solution
19. Biohazard container of disinfectant for proper disposal of tubes, slides, and pipettes
20. Biohazard container for proper disposal of patient specimens
21. ATCC 30925 (*E. histolytica* HU-1:CDC)
22. ATCC 30015 (*E. histolytica* HK-9)
23. Laredo strain (*E. histolytica*-like, ATCC 30042) (can be maintained in culture at 25°C)
24. Any strain of *Entamoeba moshkovskii* (can be maintained in culture at 25°C)

Observe standard precautions.
### C. Equipment
1. Binocular microscope with 10×, 40×, and 100× objectives; phase-contrast and/or differential interference contrast optics preferred.
2. Binocular inverted microscope with 4×, 10×, and 40× objectives; phase-contrast and/or differential interference contrast optics preferred (this microscope is recommended but not mandatory).
3. Oculars should be 10×. Some may prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
4. Tabletop centrifuge, preferably refrigerated (for centrifuging tubes containing patient specimens, e.g., watery stools, and for culture tubes).
5. Hot plate.
6. Biological safety cabinet, type II.
7. Magnetic stirrer and stir bar.
8. pH meter.
9. Drying oven or dry-heat sterilizer.
10. Incubator that can be set at 35 ± 2°C.

### III. MATERIALS (continued)

#### V. PROCEDURE

**Xenic and axenic culture**

In Greek, *xenos* means stranger. Culture amebae grown in association with an unknown microbiota are called a xenic culture. If the amebae are grown in association with a single known bacterium, the culture is monoxenic; if the culture contains several identified bacteria, then it is polyxenic. If amebae are grown as pure culture without any bacterial associate, the culture is axenic.
V. PROCEDURE (continued)

A. Wear gloves when performing this procedure.

B. Xenic culture

1. Warm several tubes of TYSGM-9 medium in the incubator (35°C for 1 to 2 h).
2. Add 0.1 ml of stock antibiotics to each tube of medium. Final concentrations of antibiotics are 100 U of penicillin and 100 μg of streptomycin per ml.
3. After vortexing or vigorously shaking the tube, use a Pasteur pipette to add 3 drops of the starch suspension to each tube of the medium.
4. Place a pea-sized portion of stool sample into the bottom of the tube, and break up the stool gently with the pipette.
5. Tightly cap the tubes, and incubate at a 45 to 50° angle at 35°C for 48 h.
6. With an inverted microscope and the 10 × objective, examine the tubes for the presence of amebae. If present, amebae will usually be seen attached to the underside of the tubes interspersed with the fecal material and rice starch. Sometimes it may be necessary to gently invert the tubes to disperse the stool material and rice starch to uncover the amebae. If you do not have an inverted microscope, proceed to step V.B.13.
7. If amebae are not seen, stand the tubes upright for about 30 min at 35°C.
8. With a Pasteur pipette, remove from the bottom of each tube 0.5 to 0.75 ml of sediment, and inoculate the sediment into fresh tubes containing rice starch and antibiotics. Centrifuge the original tubes at 250 × g for 10 min, decant the supernatant, and add 8 ml of fresh medium.
9. Incubate all tubes as described above (step V.B.5) for another 48 h.
10. Examine the tubes as before, and discard the tubes if amebae are still not seen. Report patient results as negative.
11. If amebae are present in small numbers, then chill the tube in ice water for 5 min, and centrifuge the tube for 5 min at 250 × g. Aspirate and discard the supernatant, and inoculate the sediment into a fresh tube as before.
12. If amebae are present in large numbers, then let the tube stand upright for 30 min, and remove about 0.5 ml of sediment from the bottom. Inoculate the sediment into fresh tubes as before.
13. If you do not have an inverted microscope, stand the tubes upright for about 30 min at 35°C. With a sterile pipette, remove about 0.5 ml of sediment from the bottom of the tube, and place a couple of drops onto each of two slides. Add 2 drops of methylene blue solution to one of the slides. Cover both slides with coverslips, and examine the slides under the microscope for amebae. Amebae may be rounded or have pseudopodial extrusions. The nucleus may be clearly seen in the methylene blue preparation. Proceed to steps V.B.7 through 12.

C. Axenic culture (used for research)

1. Remove tubes containing TYI-S-33 medium from 4°C and incubate at 35°C for 1 to 2 h.
2. With an inverted microscope, examine stock culture tubes of E. histolytica (HK-9 strain) for any signs of bacterial contamination (if present, tubes are no longer acceptable for use). Select one or several tubes showing good growth of amebae. Since the tubes are incubated in a slanted position, usually at an angle of 5° to 10°, a thick button of amebae will be seen at the bottom of the tube. Gently invert the tube once or twice to disperse the amebae uniformly, and examine the tubes again. A majority of the amebae should be attached to the tube walls and show pseudopodial motility. If you do not have an inverted microscope, examine organisms from the bottom of the tube (as a wet smear). If you can see pseudopodial motility, proceed to the next step (step V.C.3).
V. PROCEDURE (continued)

3. Immerse the tubes in a bucket of ice-cold water for about 5 to 10 min. This will dislodge the amebae from the tube walls. Invert the tubes several times in order to distribute the amebae.

4. With a Pasteur pipette, remove about 1.0 to 1.5 ml of culture medium; inoculate 0.5 to 1 ml into a fresh tube. Inoculate the rest of the fluid into nutrient agar, BHI broth, and thioglycolate broth for routine monitoring of bacterial contamination. Inoculate several tubes this way, and incubate the cultures slanted at a 5 to 10° angle at 35°C as before.

5. If amebic growth is not good but some amebae are attached to the tube walls, then use a serological pipette to remove about 10 ml of medium from the bottom, and add 10 ml of fresh medium.

6. If amebic growth is not good and only a few amebae are present along with a lot of debris, then centrifuge the tube at 250 \( \times \) g for 10 min, aspirate the supernatant fluid, transfer the sediment to a fresh tube, and incubate the tube as before.

VI. RESULTS

Protozoan trophozoites and/or cysts may be recovered and identified.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. If the tubes containing the fecal material are positive for amebae after 48 h of incubation, then confirm the identification with a permanent stained smear.

B. To ascertain that the amebae cultured are *E. histolytica*, prepare a permanently stained smear (trichrome or iron hematoxylin), since *Entamoeba coli* may also be isolated in culture.

Example: *Entamoeba histolytica* present.

C. If the tubes do not show any amebae, then subculture the tubes as described above and incubate for an additional 48 h. If the tubes are still negative for amebae, then report the specimen as negative and discard the tubes.

Example: No *Entamoeba histolytica* isolated.

VIII. PROCEDURE NOTES

A. When initiating xenic cultures, inoculate stool samples into at least two tubes, one with and the other without antibiotics. In some cases, some component of the natural microbiota may be helpful or even necessary for the amebae to become established and to differentiate (possibly) into cysts.

B. If the culture tubes become contaminated during routine subculture and maintenance of the axenically grown amebae, try to eliminate the contaminant by adding 1,000 U of penicillin and 1,000 \( \mu \)g of streptomycin or 50 \( \mu \)g of gentamicin per ml. If, however, the contaminant happens to be *Pseudomonas* spp., it is probably better to discard the tube and use an uncontaminated tube for subculture purposes.

C. Vitamin mixture no. 13 used in the TYI-S-33 medium may be replaced with 10 ml of NCTC-109 medium (any supplier of tissue culture media). Although the final yield of amebae may not be as good as that obtained with medium containing the vitamin mix, the procedure is simpler and saves a lot of time and effort.
IX. LIMITATIONS OF THE PROCEDURE

A. Culture of *E. histolytica* serves only as a supplemental procedure and never replaces primary diagnosis by microscopic examination. Axenic culture is used for maintaining QC strains and for research purposes. Xenic cultures may be used as a supplemental diagnostic procedure.

B. Even when the culture system is within QC guidelines, a negative culture is still not definitive in ruling out the presence of *E. histolytica* (1–5).

REFERENCES


APPENDIX 9.9.1–1

Reagents

Indicate the expiration date on the label and in the work record or on the manufacturer’s label.

A. TYSGM-9 medium

1. Nutrient broth

   potassium phosphate, dibasic (K$_2$HPO$_4$) …2.8 g
   potassium phosphate, monobasic
   (KH$_2$PO$_4$) ……………………………0.4 g
   sodium chloride (NaCl) …………………7.5 g
   casein digest peptone (BBL; catalog no.
   97023) …………………………………2.0 g
   yeast extract (BBL) ……………………..1.0 g
   glass-distilled water ……………………970.0 ml

   The nutrient broth may be stored for several months at −20°C (without sterilization), according to L. S. Diamond (1, 2). Note the expiration date on the label and in the work record.

2. 5% Tween 80 solution

   a. With a magnetic stirrer, vigorously stir 95 ml of glass-distilled water in a bottle.
   b. Add 5 g of Tween 80 (very thick solution; must be weighed), and keep stirring for a few minutes. Avoid foam formation.
   c. Filter sterilize with a 0.22-μm-pore-size membrane.
   d. Aseptically dispense into a number of sterile screw-cap test tubes, with 10 ml per tube.
   e. Label as 5% Tween 80 solution with the preparation date and an expiration date of no longer than 1 month.
   f. Store at 4°C.

3. Phosphate-buffered solution (PBS no. 8), pH 7.2

   sodium chloride (NaCl) ………………9.5 g
   potassium phosphate, dibasic (K$_2$HPO$_4$) …3.7 g
   potassium phosphate, monobasic
   (KH$_2$PO$_4$) ……………………………1.1 g
   glass-distilled water to …………………1,000.0 ml

   a. Dissolve the salts in the distilled water by using a magnetic stirrer.
   b. Autoclave for 15 min at 121°C.
   c. When cool, label as PBS no. 8 with the preparation date and an expiration date of 3 months.
APPENDIX 9.9.1–1 (continued)

4. Rice starch
For best results, use rice starch obtained from British Drug Houses Ltd. or Gailard Schlesinger, Inc. (ATCC can also be contacted for information).
   a. Dispense 500 mg of rice starch into each of several screw-cap tubes (16 by 125 mm). Do not tighten the caps.
   b. Place the tubes horizontally in a dry-heat sterilizer or an oven. Make sure that the rice is uniformly distributed loosely over the undersurface of the tubes.
   c. Heat the tubes for 2.5 h at 150°C.
   d. When cool, tighten caps and label as rice starch with the date of preparation and an expiration date of 3 months.

5. Rice starch suspension
   a. Add 9.5 ml of sterile PBS no. 8 to each tube of rice starch.
   b. Shake vigorously or use a vortex mixer to uniformly suspend the rice starch at the time of use.

6. Stock antibiotic solution
   a. Using a sterile 6-ml syringe and 20-gauge needle, add 5 ml of sterile distilled water to a vial of penicillin G sodium (10^6 U).
   b. Using a 6-ml syringe and a 20-gauge needle, add 5 ml of sterile distilled water to a vial of streptomycin sulfate (10^6 µg/ml).
   c. Shake gently, and let stand for 30 min to dissolve the antibiotics completely in the distilled water.
   d. Mix the two antibiotics in a sterile graduated flask or cylinder, and bring the volume to 125 ml with distilled water. Stock concentrations of antibiotics are 8,000 U of penicillin and 8,000 µg of streptomycin per ml.
   e. Filter sterilize the antibiotic solution through a 0.22-µm-pore-size membrane filter, dispense the filtrate into a number of sterile screw-cap vials or sterile cryovials (1 ml per vial), and label as stock antibiotic solution (Pen/Strep) with the preparation date and an expiration date of 1 year.
   f. Store at −20°C in a cryovial box.

7. Buffered methylene blue solution
   a. Solution A, 0.2 M acetic acid
      
      glacial acetic acid ....................... 11.55 ml
      distilled water ............................. 988.45 ml

      Add the acetic acid to the water, mix, and store in a glass-stoppered bottle. Label with the date of preparation and an expiration date of 1 year.
   b. Solution B, 0.2 M sodium acetate
      
      sodium acetate (NaC₂H₃O₂) ............... 16.4 g
      distilled water to ....................... 1,000.0 ml

      Dissolve the sodium acetate in 400 ml of distilled water in a volumetric flask, bring the volume to 1,000 ml, mix well, and store in a glass-stoppered bottle. Label with the date of preparation and an expiration date of 1 year.
   c. Acetate buffer, pH 3.6
      
      solution A ............................ 46.3 ml
      solution B .............................. 3.7 ml
      distilled water to ...................... 100.0 ml

      Mix solutions A and B in a volumetric flask, and bring the volume to 100.0 ml with distilled water. pH should be 3.6. Store in a glass-stoppered bottle. Label with the date of preparation and an expiration date of 1 year.
   d. Methylene blue stain
      
      methylene blue dye ................. 60.0 mg
      acetate buffer (from previous step [step A.7.c]) .................. 100.0 ml

      Dissolve the dye in the buffer, and store in a glass-stoppered bottle. Label with the date of preparation and an expiration date of 1 year.
APPENDIX 9.9.1–1 (continued)

8. Complete medium (TYSGM-9 medium)
   a. Place 200 mg of gastric mucin (U.S. Biochemical Corp.; catalog no. 16025) in
      125-ml screw-cap bottle or Erlenmeyer flask.
   b. Add 97 ml of nutrient broth (see item A.1). Using a magnetic stirrer, stir vigorously
      for at least 1 h or until the medium becomes clear.
   c. Autoclave for 15 min at 121°C; cool to room temperature.
   d. In a biological safety cabinet, aseptically add 5.0 ml of heat-inactivated bovine
      serum.
   e. Add 0.1 ml of the 5% Tween 80 solution.
   f. In a biological safety cabinet, aseptically dispense 8 ml per tube into a number of
      sterile screw-cap tubes (16 by 125 mm).
   g. After vigorously shaking the tube, add 0.25 ml of rice starch solution.
   h. Store the tubes at 4°C for not more than 1 month.
   i. The final pH of the medium should be 7.2.

B. TYI-S-33 medium

1. Nutrient broth

   potassium phosphate, dibasic (K$_2$HPO$_4$) … 1.0 g
   potassium phosphate, monobasic
      (KH$_2$PO$_4$) ....................................0.6 g
   sodium chloride (NaCl) .............................2.0 g
   casein digest peptone (BBL; catalog
      no. 97023) .........................................20.0 g
   yeast extract (BBL) ..................................10.0 g
   glucose .............................................10.0 g
   l-cysteine-HCl ...................................1.0 g
   ascorbic acid .....................................0.2 g
   ferric ammonium citrate .........................22.8 mg
   glass-distilled water to .......................870.0 ml

   a. Use a magnetic stirrer to dissolve the ingredients listed above in about 700 ml of
      glass-distilled water.
   b. Adjust pH to 6.8 with 1 N NaOH.
   c. Bring the volume to 870 ml with glass-distilled water.
   d. Filter through a no. 1 Whatman filter paper.
   e. Autoclave for 15 min at 121°C.
   f. Cool to room temperature.
   g. Store at 4°C.

2. Vitamin mixture no. 13
   a. Solution 1a
   
      niacin ............................................40 mg
      p-aminobenzoic acid .........................180 mg
   
      Dissolve in glass-distilled water, and bring the volume to 125 ml.
   b. Solution 1b
   
      nicotinamide ....................................40 mg
      pyridoxal hydrochloride .....................40 mg
      pyridoxine ......................................80 mg
      calcium pantothenate ..........................25 mg
      choline chloride ...............................830 mg
      l-inositol ......................................125 mg
      thiamine hydrochloride .......................25 mg
      vitamin B$_{12}$ .................................12 mg
   
      Dissolve in about 100 ml of glass-distilled water, and bring the volume to 125 ml.
   c. Solution 1c
   
      riboflavin ....................................25 mg

      Using a magnetic stirrer, dissolve in about 100 ml of glass-distilled water with the
      dropwise addition of 1 N NaOH, and bring the volume to 450 ml with glass-
      distilled water.
d. Solution 1d

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>folic acid</td>
<td>30 mg</td>
</tr>
</tbody>
</table>

Using a magnetic stirrer, dissolve in about 100 ml of glass-distilled water with the dropwise addition of 1 N NaOH, and bring the volume to 450 ml with glass-distilled water.

e. Solution 1e

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-biotin</td>
<td>30 mg</td>
</tr>
</tbody>
</table>

Using a magnetic stirrer, dissolve in about 100 ml of glass-distilled water with the dropwise addition of 1 N NaOH, and bring the volume to 450 ml with glass-distilled water.

f. Solution 1

Mix all 5 solutions (a, b, c, d, and e). pH should be 6.5 to 7.0.

g. Solution 2a

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-6,8-thiotic acid (oxidized form)</td>
<td>100 mg</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>50.0 ml</td>
</tr>
</tbody>
</table>

Using a magnetic stirrer, dissolve in about 100 ml of distilled water, and bring the volume to 200 ml.

h. Solution 2b

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 80 (very thick solution, must</td>
<td>5.0 g</td>
</tr>
<tr>
<td>be weighed)</td>
<td></td>
</tr>
<tr>
<td>menadione sodium bisulfite</td>
<td>30 mg</td>
</tr>
<tr>
<td>α-tocopherol acetate</td>
<td>25 mg</td>
</tr>
</tbody>
</table>

Using a magnetic stirrer, dissolve in about 100 ml of distilled water, and bring the volume to 200 ml.

i. Solution 2c

Mix solutions 2a and 2b, and bring the volume to 300 ml with sterile glass-distilled water.

j. Solution 3

Combine solution 1 and solution 2c, and bring the volume to 2,000 ml with glass-distilled water. Filter sterilize the solution through a 0.22-μm-pore-size membrane filter.

Dispense in small volumes (e.g., 10 ml), and label as vitamin mix no. 13. Give the date of preparation on the label. Store at –20 or –70°C. The vitamin mix may be stored for several years in this fashion.

3. Complete medium (TYI-S-33 medium)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>nutrient broth</td>
<td>870.0 ml</td>
</tr>
<tr>
<td>bovine serum (inactivated at 56°C)</td>
<td>100.0 ml</td>
</tr>
<tr>
<td>for 30 min</td>
<td></td>
</tr>
<tr>
<td>vitamin mix no. 13</td>
<td>20.0 ml</td>
</tr>
</tbody>
</table>

The final medium should have a pH of 6.6.

a. Aseptically dispense 13 to 14 ml per tube into a number of sterile screw-cap glass culture tubes (16 by 125 mm).

b. Label as TYI-S-33 medium with the date of preparation and an expiration date of no longer than 2 weeks.

c. Store at 4°C in a dark place, as the medium is extremely sensitive to light.

**NOTE:** Casein digest peptone varies from lot to lot in its ability to support the growth of *E. histolytica*. Because of the difficulties in obtaining a uniformly good lot that supports good growth of *E. histolytica*, Diamond et al. (3) have formulated a new medium (YI-S), which does not include casein digest peptone. YI-S medium is similar to TYI-S-33 medium. The major difference between the two media, however, is the increase in the concentration of yeast extract to 30 g (in YI-S medium) to compensate for the omission of casein digest peptone in the TYI-S-33 medium.
9.9.2 Parasite Culture: *Acanthamoeba* and *Naegleria* spp.

**PREANALYTICAL CONSIDERATIONS**

I. **PRINCIPLE**

Members of the genera *Acanthamoeba* and *Naegleria* are free-living soil and freshwater amebae known to cause human disease. Only one species of *Naegleria*, *Naegleria fowleri*, is known to cause primary amebic meningoencephalitis, which is almost always fatal, whereas several species of *Acanthamoeba* are known to cause fatal granulomatous amebic encephalitis and the nonfatal but vision-threatening *Acanthamoeba* keratitis (1–4).

Members of both genera can be easily cultivated in the laboratory either monoxenically (with a single species of bacterium included in the culture medium) or axenically (containing no organisms other than the amebae). When the etiologic agent in CSF or other body fluids cannot be identified in suspected cases of amebic encephalitis, it is imperative to try to culture the organisms. Culture should be attempted even in those cases for which a presumptive diagnosis has been made on the basis of the morphologic features of the presumed agent, since these organisms can be mistaken for host cells. Axenic cultivation of the organisms is invaluable for the following: (i) studying the biochemistry, physiology, and metabolism of the organisms to determine their nutritional requirements; (ii) producing antigens for monoclonal and polyclonal antibodies to these amebae for serological diagnosis as well as for other immunologic studies; (iii) differentiating species within the genera by using isoenzyme electrophoresis, monoclonal antibody, and/or DNA probes; (iv) in vitro screening of drugs to identify isolates susceptible and resistant to particular drugs so that advances in chemotherapy can be made; (v) infecting experimental animals so that pathologic processes involved in the disease state can be understood; and (vi) understanding the organization of the parasite at the ultrastructural level.

II. **SPECIMENS**

A. For both *Acanthamoeba* and *Naegleria* spp., the specimens usually consist of CSF, biopsy tissue, or autopsy tissue of the brain, and for *Acanthamoeba*, biopsy or autopsy tissue of the lungs, corneal scrapings or biopsy material, contact lenses and contact lens paraphernalia such as contact lens cases and solutions, skin abscess material, ear discharge, or feces can also be used.

B. Soil and water samples may also be processed for the isolation of these small free-living amebae.

C. For best results, process the specimens, especially CSF and tissue samples, for culture within 24 h, preferably sooner if possible.

D. The samples should never be frozen but may be refrigerated.

E. If the samples can be processed within 4 to 8 h, keep them at room temperature (24°C) until processed.

F. Collect all samples aseptically, and place them in sterile containers.

G. Collect at least 100 ml of water sample for the isolation of the amebae; the container should be large enough to have plenty of air space.

*Observe standard precautions.*
III. MATERIALS

A. Reagents (see Appendix 9.9.2–1)

B. Supplies
1. Nonnutrient agar plates, Nelson’s and PYG media
2. Moist chamber
3. Microscope slides (1 by 3 in. or larger)
4. Coverslips (no. 1, 22 by 22 mm or larger)
5. Sterile 1× amebae saline
6. Sterile distilled water
7. Bacteriological loop
8. Fine spatula made of nichrome wire
9. 18- to 24-h-old culture of *Escherichia coli* or *Enterobacter aerogenes*, *Acanthamoeba castellani* ATCC 30010, and *Naegleria gruberi* ATCC 30133
10. Sterile Pasteur and serological pipettes (1, 5, and 10 ml)
11. Sterile screw-cap test tubes (13 by 100 or 16 by 125 mm)
12. Volumetric flasks (1,000 ml)
13. Vaspar
14. Paraflim (American Can Co.) or equivalent

C. Equipment
1. Binocular microscope with 10×, 40×, and 100× objectives; phase-contrast and/or differential interference contrast optics preferred
2. Binocular inverted microscope with 4×, 10×, and 40× objectives; phase-contrast and/or differential interference contrast optics preferred (this microscope is recommended but not mandatory)
3. Oculars should be 10×. Some may prefer 5×; however, smaller magnification may make final organism identifications more difficult.
4. Tabletop centrifuge, preferably refrigerated (for centrifuging tubes containing patient specimens, contact lens solutions, and water samples)
5. Hot plate
6. Biological safety cabinet, type II
7. Magnetic stirrer and stir bar
8. pH meter

IV. QUALITY CONTROL

A. Check all reagents and media (ameba saline, distilled water, nonnutrient agar plates, Nelson’s and PYG media) each time they are used or periodically (once a week).
1. The media should be free of any signs of precipitation and bacterial and/or fungal contamination.
2. Page’s saline should be clear, with no visible sign of contamination.
3. Examine the nonnutrient agar plates under the 40× objective of an inverted or binocular microscope, and make sure that no fungal contamination has occurred.

B. Maintain stock cultures of *A. castellani* and *N. gruberi* at 25°C.
1. Transfer stock cultures monthly with nonnutrient agar slants and Page’s ameba saline.
2. *N. fowleri* is 10 to 35 μm long and demonstrates an eruptive locomotion by producing smooth hemispherical bulges. The cyst produces smooth walls (7 to 15 μm thick). The flagellate stage does not have a cytostome.
3. *Acanthamoeba* is 15 to 45 μm long and produces fine, tapering, hyaline projections called acanthapodia. It has no flagellate stage but produces a double-walled cyst with an outer wrinkled wall (10 to 25 μm thick).
4. Trophozoites of *Naegleria* and *Acanthamoeba* spp. are uninucleate and have a large, dense, central nucleolus.
5. Stain
   a. Run a slide prepared from a stock strain of amebae in parallel with the patient slide.
   b. Staining results are acceptable when the control amebae stain.
6. Culture
   a. Plate both stock cultures onto fresh media, and incubate at 37°C in parallel with patient culture.
   b. Culture results are acceptable when growth appears by day 7.
Parasite Culture: Acanthamoeba and Naegleria spp.  9.9.2.3

IV. QUALITY CONTROL (continued)

7. Enflagellation
   a. Run N. gruberi in parallel with patient culture being observed for enflagellation.
   b. Test is acceptable when free-swimming, pear-shaped flagellates with two flagella are observed in 2 to 24 h on the control slide.

C. The microscope(s) should be calibrated, and the original optics used for the calibration should be in place on the microscope(s). Post the calibration factors for all objectives on the microscope for easy access (see procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

D. Record all QC results.

V. PROCEDURE

A. Wear gloves when performing this procedure.

B. Remove the nonnutrient agar plates from the refrigerator, and place them in a 37°C incubator for 30 min.

C. Add 0.5 ml of ameba saline to a slant culture of E. coli or E. aerogenes. Gently scrape the surface of the slant (do not break the agar surface). Suspend the bacteria uniformly by gently pipetting with a Pasteur pipette, and add 2 or 3 drops of this suspension to the middle of the warmed agar plate. Spread the bacteria on the surface of the agar with a bacteriological loop.

D. Inoculate the specimen on the center of the agar plate as follows.
   1. CSF
      Centrifuge CSF at 250 × g for 10 min. With a sterile serological pipette, carefully transfer all but 0.5 ml of the supernatant to a sterile tube, and store at 4°C (for possible future use). Mix the sediment in the rest of the fluid, and use a Pasteur pipette to place 2 or 3 drops in the center of the nonnutrient agar plate that has been precoated with bacteria. After the fluid has been absorbed, seal the plates with a 5- to 6-in. length of 1-in.-wide Parafilm. Incubate the plate upright at 37°C.

   2. Tissue
      Triturate a small piece of the tissue (brain, lung, skin abscess, corneal biopsy, or similar specimens) in a small quantity (ca. 0.5 ml) of ameba saline. Process as described above. Corneal smear, ear discharge material, etc., may be placed directly on the agar surface and incubated as described above (step V.D.1).

   3. Water sample
      Water samples (10 to 100 ml) may be processed to isolate amebae. First, filter the water sample through three layers of sterile gauze or cheesecloth to remove leaves, dirt, etc. Next, (i) filter the sample through a sterile 5.0-μm-pore-size cellulose acetate membrane (47-mm diameter), invert the membrane over a nonnutrient agar plate precoated with bacteria, and incubate the plates as described above (step V.D.1); or (ii) centrifuge the water sample for 10 min at 250 × g, aspirate the supernatant, suspend the sediment in about 0.5 ml of ameba saline, deposit this suspension in the center of the nonnutrient agar plate precoated with bacteria, seal, and incubate the plate at 37°C as before.

   4. Soil
      Mix about 1 g of the soil sample with enough ameba saline (ca. 0.5 to 1 ml) to make a thick slurry. Inoculate this slurry in the center of the nonnutrient agar plate precoated with bacteria, and incubate as described above (step V.D.1).
V.  PROCEDURE  (continued)

5. Contact lens solutions
Small volumes (ca. 1 to 2 ml) may be inoculated directly onto the nonnutrient agar plates precoated with bacteria. Centrifuge larger volumes (2 to 50 ml) as in step V.D.3, inoculate the sediment onto the center of the nonnutrient agar plate, and incubate it as before (step V.D.1).

E. Examine the plates microscopically for amebae (cysts or trophozoites) every day for 10 days. Thin linear tracks (areas where amebae have ingested bacteria) may be seen. If amebae are seen, circle that area with a wax pencil, carefully remove the Parafilm seal under a biological safety cabinet, open the lid of the petri dish, and carefully cut out the marked area from the agar by using a spatula that has been heated to red hot and cooled before use in order to prevent contamination (a sterile disposable scalpel can also be used). Transfer the piece facedown onto the surface of a fresh agar plate coated with bacteria, seal the plate with Parafilm, and incubate as before (step V.D.1).

F. Enflagellation experiment
1. Examine the plates every day for signs of amebae. If present, amebae will feed on bacteria, multiply, and cover the entire surface of the plate within a few days. Once the food supply is exhausted, the amebae will differentiate into cysts.
2. Mark the area containing a large number of amebic trophozoites with a wax pencil.
3. Using a bacteriological loop, scrape the surface of the agar at the marked area, and transfer several loopfuls of the scraping to a sterile tube containing about 2 ml of sterile distilled water. Alternatively, flood the surface of the agar plate with about 10 ml of sterile distilled water, gently scrape the agar surface with a loop, transfer the liquid to a sterile tube, and incubate at 37°C.
4. Periodically examine the tube with an inverted microscope for the presence of flagellates.
   a. *N. fowleri*, the causal agent of primary amebic meningoencephalitis, undergoes transformation to a pear-shaped flagellate, usually with two flagella but occasionally with three or four flagella. The flagellate stage is a temporary nonfeeding stage and usually reverts to the trophozoite stage. *N. fowleri* trophozoites are typically amebalike and move in a sinuous way. They are characterized by a nucleus with a large, centrally located nucleolus. The trophozoites are also characterized by the presence of a contractile vacuole that appears once every 45 to 50 s and discharges its contents. The contractile vacuole looks like a hole or a dark depression inside the trophozoite and can easily be seen when examining the plate under the 10× or 40× objective. When the food supply is exhausted, *N. fowleri* trophozoites differentiate into spherical, smooth-walled cysts.
   b. In contrast, *Acanthamoeba* spp., which cause keratitis and granulomatous amebic encephalitis, do not transform into the flagellate stage. *Acanthamoeba* trophozoites are characterized by the presence of fine, thornlike processes that are constantly extended and retracted. The trophozoites produce double-walled cysts characterized by a wrinkled outer wall (ectocyst) and a polygonal, stellate, oval, or even round inner wall (endocyst). The trophozoites are also characterized by the presence of the contractile vacuole, which disappears and reappears at regular intervals (45 to 50 s).
   c. The cysts of both *Acanthamoeba* and *Naegleria* spp. are uninucleate.

VI.  RESULTS
Protozoan trophozoites (amebic, flagellate forms) and/or cysts may be recovered and identified.
Parasite Culture: Acanthamoeba and Naegleria spp.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Patient specimens
If a plate is positive for amebae and the amebae transform into flagellates, then the specimen should be reported as positive for *N. fowleri*. If the amebae do not transform into flagellates even after overnight incubation and if the trophozoites possess the characteristic acanthopodia, show a large centrally placed nucleolus in the nucleus on trichrome stain, and differentiate into the characteristic double-walled cysts, then report the specimen as positive for *Acanthamoeba*.

B. Contact lens solution
If the plates are positive for amebae and the amebae do not transform into flagellates but differentiate into cysts with an outer wrinkled ectocyst and an inner stellate, polygonal, oval, or round endocyst, then report the specimen as positive for *Acanthamoeba*. *Naegleria* spp. have not been isolated from contact lens solutions, but small amebae (e.g., hartmannellid or vahlkampfiiid amebae, which produce smooth-walled cysts), probably contaminants, have occasionally been isolated from these solutions.

C. Water samples
Plates inoculated with water samples are usually positive for many genera and species of small free-living amebae (fresh water is their normal habitat). Therefore, report the sample as positive for small free-living amebae.

D. Notify the physician immediately if patient specimens are positive for *Acanthamoeba* or *Naegleria*.

VIII. PROCEDURE NOTES

A. Examine most patient specimens, especially CSF, microscopically as soon as they arrive in the laboratory.
1. Remove a small drop of the CSF sediment (step V.D.1), place it on a microscope slide, cover it with a no. 1 coverslip, seal the edges of the coverslip with Vaspar, and examine it immediately with a 10× or 40× objective (phase-contrast or differential interference contrast optics are preferred). If bright-field microscopy is used, reduce the illumination by adjusting the iris diaphragm.
   a. *N. fowleri* trophozoites are highly motile and can be identified by their sinuous movement. A warmed penny applied to the bottom surface of the slide will activate the trophozoite. Occasionally a flagellate may be seen traversing the field.
   b. *Acanthamoeba* trophozoites are rarely seen in the CSF. If present, they may be recognized by their characteristic acanthopodia, which are constantly extending and retracting. Both amebae, especially *Acanthamoeba*, may be recognized by the contractile vacuole.
2. Process and examine lens care solutions (opened) like CSF.
3. If very small amounts of tissue are received, reserve them for culture.

B. An alternative method for the preparation of agar plates would be to prepare agar deeps.
1. Aliquot 20 ml of the nonnutrient agar into screw-cap tubes (20 by 150 mm).
2. Autoclave the tubes at 121°C for 15 min.
3. Store at 4°C with a 12-month expiration date.
4. Prior to use, melt the agar deeps in boiling water.
5. Pour into a petri dish (100 by 15 mm).
6. Cool. Store at 4°C with a 3-month expiration date.

C. ATCC strains of *E. coli* or *E. aerogenes* are not necessary. Any routine clinical isolate or stock organism is acceptable.
VIII. PROCEDURE NOTES
(continued)

D. Material from the surface of a positive agar plate can be removed, fixed, and stained by trichrome for microscopic examination at a higher magnification ($\times 1,000$).

IX. LIMITATIONS OF THE PROCEDURE

A. Always confirm results obtained with wet mounts by permanent stained smears (trichrome, iron hematoxylin) for nuclear characteristics in order to differentiate the amebae from host cells.

B. Organisms may not be recovered if appropriate centrifugation speeds and times are not used.

C. *Balamuthia mandrillaris* cannot be cultured using the agar plate method.

REFERENCES


SUPPLEMENTAL READING


APPENDIX 9.9.2–1

Reagents

Indicate the expiration date on the label and in the work record or on the manufacturer’s label.

A. *Page’s ameba saline (10×)*

- sodium chloride ($\text{NaCl}$) ......................... 1.20 g
- magnesium sulfate ($\text{MgSO}_4 \cdot 7\text{HOH}$) .... 0.04 g
- sodium phosphate, dibasic ($\text{Na}_2\text{HPO}_4$) .... 1.42 g
- potassium phosphate, monobasic ($\text{KH}_2\text{PO}_4$) .................................. 1.36 g
- calcium chloride ($\text{CaCl}_2 \cdot 2\text{HOH}$) ............. 0.04 g
- double-distilled water to .........................1,000 ml

1. Using a magnetic stirrer, dissolve the above ingredients in the order listed in distilled water in an appropriate glass flask or bottle.
2. Distribute 100 ml into each of 10 glass bottles.
3. Label as ameba saline (10×) with the preparation date and an expiration date of 3 months.
4. Sterilize by autoclaving at 121°C for 15 min.
5. Cool and store at 4°C.
B. Nonnutrient agar

10 × ameba saline (from step A) ........... 100.0 ml  
Difco agar ................................. 15.0 g  
double-distilled water ................. 900.0 ml

1. Mix ameba saline with distilled water by using a magnetic stirrer to make 1 × ameba saline. Add 15.0 g of agar to this solution, and dissolve with heat. Autoclave at 121°C for 15 min.
2. Cool to about 60°C, and aseptically pour into sterile plastic petri dishes (20 ml for 100- by 15-mm dish or 5 ml for 60- by 15-mm dish).
3. Label as nonnutrient agar plates with the preparation date and an expiration date of 3 months.
4. After the agar gels, store the agar plates in canisters at 4°C.

C. Nutrient agar, pH 6.8

beef extract ................................. 3.0 g  
peptone or Gelysate pancreatic digest of gelatin ......................... 5.0 g  
agar ........................................... 15.0 g  
distilled water .......................... 1,000.0 ml

1. Mix well, and autoclave at 121°C for 15 min.
2. Cool to 60°C, and dispense 5 ml per tube; slant to obtain a slant with a thick butt.
Nutrient agar slants may be obtained from BBL (catalog no. 20970/20971).

D. Modified Nelson’s medium for N. fowleri

Panmede (ox liver digest) (Difco) .... 10.0 g  
glucose ..................................... 10.0 g  
10 × ameba saline (from step A) ....... 100.0 ml  
double-distilled water ................... 900.0 ml

1. Add ameba saline to distilled water to make 1 × ameba saline.
2. Dissolve the ingredients in ameba saline by using a magnetic stirrer.
3. Dispense 10 ml per tube into screw-cap tubes (16 by 125 mm).
4. Autoclave for 15 min at 121°C.
5. Cool, and label as Nelson’s medium with the preparation date and an expiration date of 3 months.
6. Store at 4°C.
7. Add 0.2 ml of heat-inactivated fetal calf serum to each tube before inoculating with the amebae.

E. Peptone-yeast extract-glucose (PYG) medium for Acanthamoeba spp., pH 6.5 ± 0.2

Proteose Peptone (Difco) .............. 20.0 g  
yeast extract (Difco) ...................... 2.0 g  
magnesium sulfate (MgSO4 · 7H2O) .... 0.980 g  
calcium chloride (CaCl2) ............. 0.059 g  
sodium citrate (Na3C6H5O7 · 2H2O) .... 1.0 g  
ferric ammonium sulfate  
[Fe(NH4)2(SO4)2 · 6H2O] .............. 0.02 g  
potassium phosphate, monobasic  
(KH2PO4) .............................. 0.34 g  
sodium phosphate, dibasic  
(Na2HPO4 · 7H2O) ............... 0.355 g  
glucose ................................... 18.0 g  
distilled water to ...................... 1,000.0 ml

1. Dissolve all ingredients except CaCl2 in about 900 ml of distilled water in a bottle or flask by using a magnetic stirrer.
2. Add CaCl2 while stirring.
3. Bring the volume to 1,000 ml with distilled water.
4. Dispense 5 ml per tube into screw-cap tubes (16 by 125 mm).
5. Autoclave at 121°C for 15 min.
6. When tubes have cooled, label the tubes as *Acanthamoeba* medium with the preparation date and an expiration date of 3 months.
7. Store at 4°C.

F. *Vasper*

This is a 1:1 mixture of petroleum jelly and paraffin.

1. Melt paraffin in a large (400-ml) beaker, and record the volume, e.g., 100 ml.
2. Gradually add petroleum jelly while stirring with a glass rod until the volume reaches 200 ml.
3. Remove mixture from heat, dispense about 10 to 15 ml into each of several 20-ml flasks or beakers, and cover with a piece of silver foil.
4. Store at room temperature.
Parasite Culture: *Trichomonas vaginalis*

### PREANALYTICAL CONSIDERATIONS

#### I. PRINCIPLE

Cultivation is the most sensitive method for the diagnosis of trichomoniasis; however, it may take 3 to 4 days to determine culture results (1–6). Axenic cultivation of the organism is invaluable for diagnosis and for studying the biochemistry, physiology, metabolism, immunology, and ultrastructure of the organism as well as for screening drugs in vitro so that advances in chemotherapy can be achieved. Axenic cultivation of the parasite also helps us understand the pathologic processes in experimental animals and determine if it is possible to infect suitable experimental animals with organisms grown in culture and thus simulate the naturally occurring disease process. Along with culture, it is imperative that wet smears and/or stained smears be examined microscopically. If smears are positive, appropriate therapy can be instituted before culture results are available.

#### II. SPECIMENS

*Observe standard precautions.*

A. Specimens from women may consist of vaginal exudate collected from the posterior fornix on cotton-tipped applicator sticks or genital secretions collected on polyester sponges.

B. Specimens from men can include semen, prostatic fluid, urethral samples collected with polyester sponges, or urine.

C. Urine samples collected from the patient should be the first-voided specimen in the morning.

D. It is critical that clinical specimens be inoculated into culture medium as soon as possible after collection. Although collection swabs can be used, there are often problems with specimens drying prior to culture. Many laboratories are currently using the plastic envelope methods simultaneous transport and culture (*see* procedure 9.9.4).

#### III. MATERIALS

A. **Reagents** (*see* Appendix 9.9.3–1)

B. **Supplies**

1. Disposable sterile Pasteur and serological pipettes
2. Microscope slides (1 by 3 in. or larger)
3. Coverslips (no. 1, 22 by 22 mm or larger)
4. Volumetric flasks (100 ml)
5. Graduated cylinders (1,000 ml)
6. Flasks or beakers
7. Sterile and nonsterile screw-cap tubes (16 by 125 mm)
8. Büchner or other funnel
9. Filter paper, Whatman no. 1
10. Culture tube racks
11. Nutrient agar plates, BHI plates, and thioglycolate broth
12. Sterile cryovials or screw-cap vials (to hold 1 ml)
13. Box for vial storage in freezer
14. ATCC 30001 (*Trichomonas vaginalis*)

C. **Equipment**

1. Binocular microscope with 10×, 40×, and 100× objectives; phase-contrast and or differential interference contrast optics preferred
III. MATERIALS (continued)

2. Binocular inverted microscope with 10×, 40×, and 100× objectives; phase-contrast and differential interference contrast optics preferred (this microscope is recommended but not mandatory).

3. Oculars should be 10×. Some may prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.

4. Tabletop centrifuge, preferably refrigerated (for tubes containing patient specimens, e.g., urine, and for culture tubes).

5. Water bath or heating block (range up to 80°C).

6. Freezer, −20°C.

7. Magnetic stirrer and stir bar.

8. pH meter.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Check all reagents and media (at least once a week). All media, including Ringer’s solution, should be free of any signs of precipitation and bacterial and/or fungal contamination.

B. The microscope(s) should be calibrated, and the original optics used for the calibration should be in place on the microscope(s). Post the calibration factors for all objectives on the microscope for easy access (see procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

C. Maintain stock cultures of *T. vaginalis* (ATCC 30001).

   1. Transfer stock cultures weekly.
      a. Always culture stock organisms at the same time a patient specimen is inoculated into culture medium.
      b. If the stock organisms multiply and remain viable during the 96 h, report patient results.

   2. Stain
      a. Run a slide prepared from a stock strain of *T. vaginalis* in parallel with the patient slide.
      b. Staining results are acceptable when the control organisms stain well.

D. Record all QC results.

V. PROCEDURE

A. Wear gloves when performing this procedure.

B. Inoculation of culture medium

   1. Remove tubes containing culture medium from 4°C, and incubate at 37°C for 1 to 2 h.

   2. Vigorously shake the cotton-tipped portion of the applicator stick containing the patient specimen in the medium, and then break off the tip with sterile forceps and drop it into the medium.

   3. If the material is collected on polyester sponges, then drop the sponges into the medium and shake the tube.

   4. Centrifuge urine samples for 10 min at 250 × g, aspirate the supernatant, and inoculate the sediment into the medium.

   5. Examine the tubes daily for several days, and subculture if necessary. To subculture, first shake the tube to disperse the organisms uniformly, remove about 1 to 2 ml, and inoculate into a warmed, fresh tube.

   6. Incubate the tubes in a slanted position (45° angle) at 37°C.
V. PROCEDURE (continued)

7. Incubate control tubes and those containing patient material for at least 72 to 96 h.

8. Examine the entire length of the tube. If the specimen is positive, *T. vaginalis* will be found freely swimming or attached to the tube walls.

9. Do not report negative results until 96 h.

C. To maintain stock cultures

1. With an inverted microscope, examine stock culture tubes of *T. vaginalis* for any signs of bacterial contamination. Select one or more tubes showing good growth. Since the tubes are incubated in a slanted position, usually at an angle of 45º, a thick button of organisms will be seen at the bottom of the tube. Gently invert the tube once or twice to disperse the trichomonads uniformly, and examine the tubes again. A large number of the organisms should be freely swimming, and a few of the organisms will be attached to the tube walls.

2. Immerse the tubes in a bucket of ice-cold water for about 5 to 10 min. This will dislodge the trichomonads from the tube walls. Invert the tubes several times in order to distribute the organisms.

3. With a sterile Pasteur pipette, remove about 1.0 to 1.5 ml; inoculate 0.5 to 1.0 ml into a fresh tube. Inoculate the rest of the fluid into nutrient agar, BHI, and thioglycolate broth for routine monitoring of bacterial contamination. Inoculate several additional tubes this way, and incubate the cultures slanted at a 45º angle at 37ºC as before (step V.B.6).

4. If growth is poor and only few organisms are present along with a lot of debris, then centrifuge the tube at 250 × *g* for 10 min, aspirate the supernatant, transfer the sediment to a fresh tube, and incubate as before (step V.B.6).

VI. RESULTS

*T. vaginalis* trophozoites may be recovered and identified.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. If organisms are found prior to or at the end of 96 h, report the specimen as positive.

Example: Positive for *Trichomonas vaginalis*

B. If no trophozoites are seen after 4 days of incubation, then discard the tubes and report as negative.

Example: Negative for *Trichomonas vaginalis*

VIII. PROCEDURE NOTES

A. Cultivation is the most sensitive method for the diagnosis of trichomoniasis. Every effort, therefore, must be made to inoculate patient materials into culture medium. However, since this method may take 3 to 4 days and the patient materials may occasionally contain nonviable organisms, it is imperative that microscopic examination of wet smears and/or stained smears (Giemsa) also be performed.

B. Culture control organisms each time a patient specimen is inoculated into the culture medium.

C. Use the same medium for controls and patient specimen.
A. Even though cultivation is the most sensitive method for the diagnosis of trichomoniasis, it may take 3 to 4 days to arrive at a diagnosis. Every effort, therefore, must be made to microscopically examine wet smears and or stained smears so that appropriate therapy can be instituted without delay in case of positive findings.

B. Do not report results of patient specimens as positive unless control cultures are positive.

REFERENCES


APPENDIX 9.9.3–1

Reagents

Indicate the expiration date on the label and in the work record or on the manufacturer’s label.

A. Ringer’s solution

- sodium chloride (NaCl) .........................0.6 g
- sodium bicarbonate (NaHCO3) ............. 0.01 g
- potassium chloride (KCl) ..................... 0.01 g
- calcium chloride (CaCl2) .................... 0.01 g
- double-distilled water to ....................100.0 ml

Dissolve the ingredients in the order listed, and bring the volume to 100.0 ml with distilled water.

B. Liver infusion

- double-distilled water .........................330.0 ml
- Bacto liver infusion powder (Difco) ....... 20.0 g

1. Place the distilled water in a large beaker.
2. Add the liver infusion powder.
3. Infuse for 1 h at 50°C.
4. Raise the temperature to 80°C for 5 min to coagulate the protein.
5. Filter through a Whatman no. 1 filter paper with a Büchner funnel.

C. Methylene blue solution

- methylene blue ..............................0.5 g
- glass-distilled water .......................100.0 ml

Mix well until dissolved.

D. Cysteine-peptone-liver-maltose (CPLM) complete medium

- Bacto Peptone (Difco) ...................... 32.0 g
- Bacto Agar (Difco) .........................1.6 g
- cysteine HCl ................................2.4 g
- maltose .................................... 1.6 g
- Bacto liver infusion (Difco) ...........320.0 ml
- Ringer’s solution .........................960.0 ml
APPENDIX 9.9.3–1 (continued)

1. Mix Ringer’s solution and liver infusion in a large beaker by using a magnetic stirrer.
2. Add peptone, maltose, cysteine HCl, and agar in that order, and heat the mixture until dissolved.
3. Add 0.7 ml of aqueous methylene blue.
4. Adjust pH to 5.8 to 6.0 with 1 N NaOH or 1 N HCl.
5. Dispense 8-ml volumes into culture tubes.
6. Autoclave at 121°C for 15 min.
7. Aseptically add 2 ml of human serum (heat inactivated at 56°C for 30 min and cooled)
   per tube. Horse serum is recommended as a replacement for human serum, particularly
   when considering safety issues such as handling human blood and blood products.
8. Label as CPLM medium with the preparation date.
9. Store at room temperature. Use as long as the amber zone, indicating an anaerobic
   condition, persists.

E. Diamond’s Trypticase-yeast extract-maltose (TYM) complete medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypticase (BBL)</td>
<td>20.0</td>
</tr>
<tr>
<td>yeast extract (BBL)</td>
<td>10.0</td>
</tr>
<tr>
<td>maltose</td>
<td>5.0</td>
</tr>
<tr>
<td>L-cysteine HCl</td>
<td>1.0</td>
</tr>
<tr>
<td>L-ascorbic acid</td>
<td>0.2</td>
</tr>
<tr>
<td>potassium phosphate, dibasic</td>
<td>0.8</td>
</tr>
<tr>
<td>potassium phosphate, monobasic</td>
<td>0.8</td>
</tr>
<tr>
<td>Bacto Agar (Difco)</td>
<td>0.5</td>
</tr>
<tr>
<td>double-distilled water</td>
<td>900.0 ml</td>
</tr>
</tbody>
</table>

1. Dissolve the buffer salts in the distilled water by using a magnetic stirrer.
2. Add the remaining ingredients except the agar in the order given, one at a time, until dissolved.
3. Adjust pH to 6.0 with 1 N HCl.
4. Add agar, and heat to dissolve.
5. Autoclave at 121°C for 15 min.
6. Cool to 45°C and add 100 ml of sterile bovine, sheep, or horse serum that has been
   heat inactivated for 30 min at 56°C.
7. Aseptically dispense 10-ml volumes into screw-cap tubes (16 by 125 mm).
8. Label as TYM medium with the preparation date and an expiration date of 10 days.
9. Store at 4°C.

F. Diamond’s complete medium (modified by Klass)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypticase (BBL)</td>
<td>24.0</td>
</tr>
<tr>
<td>yeast extract (BBL)</td>
<td>12.0</td>
</tr>
<tr>
<td>maltose</td>
<td>6.0</td>
</tr>
<tr>
<td>cysteine HCl</td>
<td>1.2</td>
</tr>
<tr>
<td>ascorbic acid</td>
<td>0.24</td>
</tr>
<tr>
<td>double-distilled water</td>
<td>900.0 ml</td>
</tr>
</tbody>
</table>

1. Dissolve the ingredients one at a time in the order given.
2. Adjust pH to 6.0 with 1 N HCl or 1 N NaOH.
3. Dispense in 12.5-ml aliquots into screw-cap tubes (16 by 125 mm).
4. Autoclave at 121°C for 15 min.
5. When cool (50°C), add 1 ml of sterile inactivated horse serum and 0.5 ml of antibiotic
   mixture to each tube.
6. Label as modified TYM medium with the date of preparation and an expiration date
   of 3 weeks.
G. Antibiotics mixture

- sodium penicillin G .................. 1,000,000 U
- streptomycin sulfate .................. 1,000,000 µg
- amphotericin B (Fungizone) .......... 2,000 µg
- sterile double-distilled water .......... 50.0 ml

1. Mix thoroughly. Concentration of stock solution is
   - penicillin ............................... 20,000 U/ml
   - streptomycin ........................... 20,000 µg/ml
   - amphotericin B .......................... 40 µg/ml

2. Dispense 1 ml of the antibiotic mixture into sterile screw-cap vials or sterile cryovials.
3. Label as antibiotic solution with the date of preparation and an expiration date of 1 year.
4. Store at −20°C in cryoboxes.

H. Serum substitutions

Bovine, sheep, or horse serum can be substituted in step D.7.
Parasite Culture: InPouch TV System for *Trichomonas vaginalis*

**PREANALYTICAL CONSIDERATIONS**

**I. PRINCIPLE**

Trichomoniasis is a sexually transmitted infection caused by the flagellated protozoan *Trichomonas vaginalis*. It is recognized as one of the most prevalent forms of sexually transmitted disease (STD) worldwide, with over 180 million cases occurring annually. The CDC estimates that 3 million cases occur in the United States on a yearly basis. *T. vaginalis* can produce premature rupture of membranes in pregnancy and can also cause nongonococcal urethritis in males.

Cultivation is the most sensitive method for the diagnosis of trichomoniasis; however, it may take 3 to 4 days to determine culture results. The InPouch TV system has demonstrated a greater sensitivity than either the saline wet mount or Hollander’s, Trichosel, or modified Diamond’s media (1–5). The InPouch TV serves first as a specimen transport container and growth chamber during incubation, then as a viewing chamber during microscopy (using a plastic clip that fits onto the microscope stage). It consists of a clear, gas-impermeable, plastic pouch that is double chambered. The medium contains Trypticase, Proteose Peptone, yeast extract, maltose and other sugars, amino acids, salts, and antifungal and antimicrobial agents in normal saline phosphate buffer. An inoculum containing 1 to 10 organisms is sufficient to cause a positive test if the specimen is inoculated immediately after collection.

**II. SPECIMENS**

*Observe standard precautions.*

A. Specimens from women may consist of vaginal exudate collected from the posterior fornix using sterile cotton or Dacron swabs.

B. Specimens from men can include semen, prostatic fluid, and urethral samples collected with a cotton swab on a wire handle.

C. Although urine specimens are not recommended as the most likely to yield positive culture results, urine specimens collected from the patient should be the first-voided specimen in the morning.

D. Patient specimens that are contaminated with birth control foams or jellies will result in decreased recovery of trichomonads.

**III. MATERIALS**

**A. Reagents**

1. InPouch kits [BioMed Diagnostics, Inc.; (800) 964-6466] stored vertically at 15 to 25°C up to 1 year from date of manufacture.

2. **Warning:** This product contains a chemical known to cause cancer, birth defects, or other reproductive harm.

**B. Supplies**

1. Viewing slide holder (provided in the kit)

2. Disposable protective gloves

**C. Equipment**

Microscope, binocular with mechanical stage; low-power (10×), high dry power (40×), and oil immersion (100×) objectives; 10× oculars; calibrated ocular micrometer; light source equivalent to a 20-W halogen or 100-W tungsten bulb; blue and white ground-glass diffuser filters
A. Inoculate a pouch from each new lot with one drop from a culture of actively growing *T. vaginalis* and incubate at 37°C. Observe for actively motile organisms at 3 days.

B. An InPouch containing cloudy medium should not be used.

C. The organism is maintained in subculture medium, purchased separately, where it will remain viable for 7 days.

D. If product does not meet standards, inform the parasitologist or supervisor, record all QC results.

E. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (see procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

V. PROCEDURE

A. Inoculation of InPouch TV: *wear gloves when performing this procedure.*

1. Remove InPouch TV from box.
2. Fold pouch back over itself to reduce the folded crease. Make sure that the liquid in the upper chamber is below the closure tape to prevent fluid from leaking upon opening.
3. Tear open the pouch at the notch just above the white closure. Open the pouch sufficiently to admit the swab, by pulling apart the (white) tape’s middle tabs of the closure tape.
4. Insert swab containing the specimen into the liquid of the pouch’s upper chamber (Fig. 9.9.4–1 and 9.9.4–2).
5. Squeeze the swab to express specimen by gently pressing it between the walls of the upper chamber. Discard swab in a biohazard waste container.

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**Figure 9.9.4–1** InPouch TV diagnostic system for culturing *Trichomonas vaginalis* (BioMed Diagnostics). The swab containing a specimen from the patient is inserted into the liquid medium within the plastic pouch.
V. PROCEDURE (continued)

6. Squeeze pouch closed, fold the top edge down, and roll three times. Fold the wire tape’s end-tabs behind the pouch to lock the roll.
7. Label with patient’s name. Do not obstruct clear plastic with label.
8. Hold specimen pouch upright at room temperature until prompt delivery to laboratory.

B. Specimen not received in InPouch TV
1. Notify submitter that laboratory procedure requires submission in InPouch TV.
2. If a specimen is received in saline within 30 min of collection, do a wet mount.
3. If a swab is received within 30 min of collection, inoculate an InPouch TV.

C. Microscopic evaluation immediately upon receipt
1. Concentrate the cellular material by standing the pouch vertically for at least 15 min prior to microscopic evaluation. The trichomonads will concentrate at the bottom of the chamber.
2. Place the upper chamber of the pouch on the raised platform of the open plastic microscope clip, positioning the pouch so that the lower portion of the upper chamber is a few millimeters above the lower border of the clip. Close and lock the clip over the pouch.
3. View the pouch from the upper (open window) side of the microscope clip. Observe with a microscope under low power (10×); use high dry power (40×) if necessary for confirmation. Focus on the liquid and not on the textured plastic of the pouch. Clue cells and yeast can also be observed.
4. When no trichomonads can be found in the direct preparation in the upper pouch chamber, the inoculum has fewer than 100 organisms or may be negative. Remove microscope clip after observation.

D. Culture and microscopic evaluation
1. Express the contents of the upper pouch chamber into the lower chamber.
2. Roll down the pouch until the tape is at the top of the label. Fold the wire tape’s end tabs to lock the roll. This action helps maintain partial anaerobiosis.
3. Incubate the pouch vertically at 37°C for 18 to 24 h.
4. Prior to reading, distribute the trichomonads by gently rubbing your thumb across the viewing area or pull the pouch upward (five times) across the edge of a table for mixing.
5. Place the bottom of the lower chamber on the raised platform of the open microscope slip, and then close and lock the clip over the pouch.
6. Observe microscopically under low power (10×). The best location in the pouch to find trichomonads is slightly above the bottom edge of the pouch. Focus on the liquid and not on the textured plastic of the pouch.
7. Repeat evaluations daily for the presence of motile trichomonads for up to 5 days. Do not mistake Brownian motion of small debris particles for evidence of Trichomonas activity.

Figure 9.9.4–2 Illustration of the InPouch TV culture system for Trichomonas vaginalis (BioMed Diagnostics). From top to bottom: (1) introduction of the specimen into the upper chamber containing a small amount of medium; (2) application of a plastic holder for microscope viewing prior to expressing medium into the lower chamber (optional); (3) transfer of a small amount of medium in the upper chamber to the lower chamber; (4) rolling down the upper chamber and sealing it with tape; (5) plastic viewing frame used to immobilize the medium in the pouch for examination under the microscope. (Diagram courtesy of BioMed Diagnostics; reprinted from L. S. Garcia, Diagnostic Medical Parasitology, 4th ed., 2001, ASM Press, Washington, D.C.)
VI. RESULTS

*T. vaginalis* trophozoites may be recovered and identified.

**POSTANALYTICAL CONSIDERATIONS**

VII. REPORTING RESULTS

A. If organisms are found prior to or at the end of 96 h, report the specimen as positive.
   
   **Example:** Positive for *Trichomonas vaginalis*

B. If no trophozoites are seen after 5 days of incubation, then discard the pouch and report as negative.
   
   **Example:** Negative for *Trichomonas vaginalis*

**REFERENCES**


**IX. LIMITATIONS OF THE PROCEDURE**

A. Even though cultivation is the most sensitive method for the diagnosis of trichomoniasis, it may take up to 5 days to arrive at a diagnosis. Every effort, therefore, must be made to carefully review contents of the pouch on a daily basis.

B. Do not report results of patient specimens as positive unless control cultures are positive.

C. InPouch TV medium suppresses but does not entirely eliminate the growth of yeast.

VIII. PROCEDURE NOTES

A. Specificity: for cultivation of *T. vaginalis* only. Other *Trichomonas* species will not survive and replicate at the pH and medium composition found in the InPouch TV test kit.

B. Culture: *T. vaginalis* present in low numbers will replicate within 5 h after inoculation into the InPouch system. An inoculum containing 1 to 10 organisms is sufficient to cause a positive test (1, 3).

C. Patient materials may occasionally contain nonviable organisms; it is recommended that if suspicious nonmotile objects are seen, stained smears can be prepared (Giemsa stain).

D. Use the same lot number of medium for controls and patient specimens.
Leishmania spp. produce in humans a wide variety of diseases ranging from a mild, self-curing cutaneous form (Oriental sore) to the relatively severe mucocutaneous disease (espundia) to severe, fatal visceral leishmaniasis (kala-azar). Leishmania spp. have two stages in their life cycle: the extracellular promastigote, which occurs in the gut of the sand fly vector, and an intracellular amastigote stage, which multiplies in the macrophage of the vertebrate host. The culture form is usually the promastigote stage; however, differentiation of promastigotes into the amastigote form in cell cultures can also be achieved (1–4). In vitro cultivation of Leishmania greatly facilitates diagnosis in clinical infections and studies of the taxonomy, biochemistry, physiology, metabolism, immunology, and ultrastructure of the organism. Cultivation is also valuable for in vitro screening of drugs that might lead to advances in chemotherapy. Axenic cultivation of the parasite also helps in understanding the pathologic process in experimental animals, which can be infected with organisms grown in culture, thereby inducing disease processes that simulate the naturally occurring disease.

Trypanosoma cruzi, which causes Chagas’ disease, is a major public health problem in Latin America. In addition to the promastigote and amastigote forms, T. cruzi also has epimastigote and trypomastigote stages. The culture form is usually the epimastigote stage; however, differentiation of the epimastigotes into metacyclic trypomastigotes in cell-free media and amastigotes in cell cultures can also be achieved. Cultivation of the organisms from patient specimens is an important diagnostic tool. In vitro cultivation of T. cruzi also greatly facilitates study of the organism, provides a mechanism for drug screening, and provides a source of organisms for animal studies.

Observe standard precautions.

Specimens for culturing Leishmania spp. may consist of aspirates, scrapings, or biopsy material from skin lesions of patients with cutaneous leishmaniasis; bone marrow aspirates or, more rarely, splenic aspirates from visceral-leishmaniasis patients; or normal skin biopsy specimens, lymph node aspirates, or pieces of liver and spleen from suspected or potential wild- or domestic-animal reservoirs. Specimens for culturing T. cruzi may consist of the patient’s blood or the gut contents of the triatomid bug.

A. Reagents (see Appendix 9.9.5–1)
B. Supplies
1. Disposable sterile Pasteur and serological pipettes
2. Microscope slides (1 by 3 in. or larger)
3. Coverslips (no. 1, 22 by 22 mm or larger)
4. Volumetric flasks (1,000 ml)
5. Erlenmeyer flasks (500 ml)
6. Graduated cylinders (100 and 500 ml)
7. Sterile and nonsterile screw-cap tubes (16 by 125 mm)
8. Culture tube racks
9. Sterile cryovials or screw-cap vials (to hold 1 ml)
10. Box for vial storage in freezer
11. Büchner funnel
12. Filter paper, Whatman no. 42
13. 0.23-µm-pore-size membrane filters
III. MATERIALS (continued)

14. ATCC 30883 (*Leishmania mexicana*)
15. ATCC 30160 (*T. cruzi*)

C. Equipment

1. Binocular microscope with 10×, 40×, and 100× objectives; phase-contrast and/or differential interference contrast optics preferred
2. Binocular inverted microscope with 10×, 40×, and 100× objectives; phase-contrast and differential interference contrast optics are preferred (this microscope is recommended but not mandatory)
3. Oculars should be 10×. Some may prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
4. Tabletop centrifuge, preferably refrigerated (for tubes containing patient and animal specimens, e.g., blood and aspirates, or for culture tubes)
5. Water bath (range up to 80°C)
6. Freezer, −20°C
7. Magnetic stirrer and stir bar
8. pH meter

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Check all reagents and media at least once a week. The media should be free of any signs of precipitation and bacterial and/or fungal contamination.

B. The microscope(s) should be calibrated, and the original optics used for the calibration should be in place on the microscope(s). Post the calibration factors for all objectives on the microscope for easy access (see procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

C. Maintain stock cultures of *Leishmania* spp.
   1. Transfer stock cultures weekly.
      a. Always culture stock organisms at the same time a patient specimen is inoculated into culture medium.
      b. If the stock organisms multiply and remain viable during the 96 h, then report patient results.
   2. Stain
      a. Stain a slide prepared from stock culture in parallel with the patient slide.
      b. Staining results are acceptable when the control organisms stain well.

D. Record all QC results.

V. PROCEDURE

A. **Wear gloves when performing this procedure.**

B. Inoculation of culture medium

1. Remove tubes containing culture medium (one of NNN medium, modified NNN medium, or Tobie’s medium and one of Schneider’s medium for leishmaniasis and one of NNN medium or Tobie’s medium and one of LIT medium for *T. cruzi*) from 4°C, add fetal bovine serum and antibiotics if required, and incubate at 20 to 23°C for 1 to 2 h.
2. Inoculate the specimen (aspirate, scraping, or biopsy material from skin lesions from cutaneous-leishmaniasis patients; bone marrow aspirates or splenic aspirates from visceral-leishmaniasis patients; or normal skin biopsy specimens, lymph node aspirates, or pieces of liver and spleen from suspected or potential wild- or domestic-animal reservoirs) into the culture tubes. For Chagas’ disease, inoculate a few drops of buffy coat into the culture tubes.
3. Add 0.5 ml of overlay (either saline or other overlay, depending on medium).
4. Incubate the tubes at 20 to 24°C.
V. PROCEDURE (continued)

5. Once every 2 to 3 days, remove a drop of medium and examine it under the low power (100×) of a microscope, preferably one equipped with phase-contrast optics.

6. If promastigotes are seen, then inoculate a couple of drops of the medium into fresh culture tubes. Add a couple of drops of 0.85% NaCl or the overlay solution (depending on the culture medium used) to the old tube.

7. If visible contamination occurs, add antibiotics to the overlay (to contain 200 U of penicillin and 200 μg of streptomycin per ml).

8. Incubate the tubes containing the patient’s specimen for at least 2 weeks.

9. If no organisms are seen even after 2 weeks of incubation, then examine several drops of fluid under the microscope for promastigotes.

VI. RESULTS

A. Leishmania spp. may be recovered and identified.

B. T. cruzi may be recovered and identified.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. If organisms are found prior to or at the end of 2 weeks, report as positive.
   Examples: Positive for Leishmania spp., positive for Trypanosoma cruzi

B. If no organisms are seen even after 2 weeks of incubation, then discard the tubes and report as negative.
   Examples: Negative for Leishmania spp., negative for Trypanosoma cruzi

C. Identification of the species is generally made on the basis of the clinical symptoms, geographic origin, culture characteristics, isoenzyme profile, and DNA buoyant density.

VIII. PROCEDURE NOTES

A. Ensure that the skin surrounding the ulcer is thoroughly cleaned and swabbed with 70% alcohol (sterile saline is not acceptable as a cleansing agent) and allowed to dry before the sample is removed. Also ensure that alcohol does not get into the ulcerated area or broken skin.

B. Patient skin may be simply touched to the wall of the tube to release the amastigotes. If skin from an animal reservoir is used, the specimen must be macerated or triturated.

C. A punch biopsy taken from the advancing margin of the lesion is often recommended.

D. It is imperative that only a few drops of bone marrow juice or spleen aspirate be inoculated into tubes. Inoculate several tubes with a few drops each rather than a single tube with a large volume (1 to 2 ml), as the serum in the specimen may contain leishmanicidal or inhibitory factors that will prevent the growth of organisms.

E. Alternatively, bone marrow juice may be centrifuged for 10 min at 250 × g, and the sediment may be washed in 0.85% NaCl by centrifugation and then inoculated into culture tubes.

F. Buffy coat from the blood sample rather than whole blood should be inoculated.

G. Because leishmaniae are fastidious organisms and all isolates may not grow in any one medium, it is imperative that at least two media be used; for example, use NNN or modified Tobie’s medium and Schneider’s Drosophila medium.

H. It is advisable to use two different media such as LIT and NNN media for the initial isolation of T. cruzi. Once growth is established, use the medium in which best growth is obtained for subculture. According to James Sullivan, LIT medium used as an overlay on Tobie’s slants is excellent for isolation and diagnosis. The major culture form is the epimastigote; occasionally, however, try-
pomastigotes and amastigotes may also be seen. Make sure that the defibrinated rabbit blood is fresh. In any case, it should not be more than 10 days old. It should be aseptically collected and stored at 4°C until used.

I. Although blood collected using EDTA anticoagulant can be used for routine stock culture subcultures, it may not be quite as effective as defibrinated blood in isolating organisms from patient specimens. However, if defibrinated blood is not available, blood collected using EDTA anticoagulant can be used.

IX. LIMITATIONS OF THE PROCEDURE

Cultivating the organism from suspected materials provides a definitive diagnosis, but it may take 3 to 7 days. Every effort, therefore, must be made to microscopically examine wet smears and/or stained smears so that appropriate therapy can be instituted without delay if findings are positive.

REFERENCES


APPENDIX 9.9.5–1

Reagents

Include QC information on reagent container and in QC records.

A. NNN medium (leishmaniasis or Chagas’ disease)

- Bacto Agar (Difco) .........................1.4 g
- sodium chloride (NaCl) ......................0.6 g
- double-distilled water ..................... 90.0 ml

1. Mix the NaCl and agar in the distilled water in a 500-ml flask.
2. Heat the mixture until the agar melts.
3. Autoclave at 121°C for 15 min.
4. Cool to about 50°C.
5. Add 10 ml of aseptically collected defibrinated rabbit blood.
6. Dispense 4 ml into sterile screw-cap culture tubes (16 by 125 mm).
7. Place the tubes at a 10° angle (shallow slant position) until the agar sets.
8. Immediately transfer the tubes into test tube stands, and let stand in an upright position at 4°C so that the bottom portion of the slants will be covered with the water of condensation. Rapid cooling increases the water of condensation.
9. Label as NNN medium with the preparation date and an expiration date of 3 weeks from the date of preparation.
10. Store at 4°C.

B. NNN medium, Offutt’s modification (leishmaniasis)

- blood agar base (Difco) .....................8.0 g
- double-distilled water ...................200.0 ml

1. Heat until the agar is dissolved in the distilled water in a 500-ml flask.
2. Autoclave at 121°C for 15 min.
3. Cool to about 50°C.
4. Add 15 ml of aseptically collected defibrinated rabbit blood.
5. Dispense 4 ml into sterile screw-cap culture tubes (16 by 125 mm).
6. Place the tubes at a 10° angle (shallow slant position) until the agar sets.
APPENDIX 9.9.5–1 (continued)

7. Immediately transfer the tubes into test tube stands, and let stand in an upright position at 4°C so that the bottom portion of the slants is covered with the water of condensation. Rapid cooling increases the water of condensation.

8. Label as Offutt’s medium with the preparation date and an expiration date of 3 weeks from the date of preparation.

9. Store at 4°C.

C. Overlay solution (to be used with NNN or NNN modified medium)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>sodium chloride (NaCl)</td>
<td>4.5 g</td>
</tr>
<tr>
<td>double-distilled water</td>
<td>500.0 ml</td>
</tr>
</tbody>
</table>

1. Autoclave at 121°C.
2. Dispense 4 ml aseptically into sterile culture tubes (16 by 125 mm).
3. Label as 0.9% NaCl with the preparation date and an expiration date 3 weeks from the date of preparation.
4. Store at 4°C.

D. Evan’s modified Tobie’s medium (leishmaniasis or Chagas’ disease)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>beef extract (Oxoid Lab-Lemco L29)</td>
<td>0.3 g</td>
</tr>
<tr>
<td>bacteriological peptone (Oxoid L37)</td>
<td>0.5 g</td>
</tr>
<tr>
<td>sodium chloride (NaCl)</td>
<td>0.8 g</td>
</tr>
<tr>
<td>agar (Oxoid purified)</td>
<td>2.0 g</td>
</tr>
<tr>
<td>double-distilled water</td>
<td>100.0 ml</td>
</tr>
</tbody>
</table>

1. Mix all the ingredients in the distilled water in a large beaker by using a magnetic stirrer.
2. Heat the mixture until the agar melts.
3. Dispense 5 ml into screw-cap culture tubes (16 by 125 mm).
4. Autoclave at 121°C for 15 min.
5. Cool to about 50°C.
6. Add 1.2 ml of aseptically collected defibrinated horse blood.
7. Hold the tubes in an upright position in the palms of your hands, and roll the tubes gently to mix the blood and agar well.
8. Place the tubes at a 10° angle (shallow slant position) until the agar sets.
9. Immediately transfer the tubes into test tube stands, and let stand in an upright position at 4°C so that the bottom portion of the slants will be covered with the water of condensation. Rapid cooling increases the water of condensation.
10. Label as Evan’s modified Tobie’s medium with the preparation date and an expiration date 3 weeks from the date of preparation.
11. Store at 4°C.

E. Overlay solution (to be used with Tobie’s medium)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>potassium chloride (KCl)</td>
<td>0.4 g</td>
</tr>
<tr>
<td>sodium phosphate, dibasic</td>
<td>0.06 g</td>
</tr>
<tr>
<td>(Na2HPO4 · 12H2O)</td>
<td></td>
</tr>
<tr>
<td>potassium phosphate, monobasic</td>
<td>0.06 g</td>
</tr>
<tr>
<td>(KH2PO4)</td>
<td></td>
</tr>
<tr>
<td>calcium chloride (CaCl2 · 2H2O)</td>
<td>0.185 g</td>
</tr>
<tr>
<td>magnesium sulfate (MgSO4 · 7H2O)</td>
<td>0.1 g</td>
</tr>
<tr>
<td>magnesium chloride (MgCl2 · 6H2O)</td>
<td>0.1 g</td>
</tr>
<tr>
<td>sodium chloride (NaCl)</td>
<td>8.0 g</td>
</tr>
<tr>
<td>L-proline</td>
<td>1.0 g</td>
</tr>
<tr>
<td>phenol red</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>double-distilled water</td>
<td>1,000.0 ml</td>
</tr>
</tbody>
</table>

1. Place 750 ml of distilled water in a 1-liter beaker, and add the above ingredients one at a time in the order given until dissolved. Use a magnetic stirrer.
2. Adjust the pH to 7.2 by adding slowly, while stirring, solid Tris.
3. Bring the volume to 1,000 ml with distilled water.
4. Dispense 100 ml into a number of screw-cap flasks or bottles.
5. Autoclave at 121°C for 15 min.
6. Label as overlay solution with the preparation date and an expiration date of 1 month.
7. Store at 4°C.

**F. Yaeger’s liver infusion tryptose (LIT) medium (for Chagas’ disease)**

- liver infusion (Difco) .................................. 35.0 g
- tryptose (Difco) .......................................... 5.0 g
- sodium chloride (NaCl) .................................. 4.0 g
- potassium chloride (KCl) .................................. 0.4 g
- sodium phosphate, dibasic (Na₂HPO₄ • 12H₂O) ................. 8.0 g
- glucose .................................................... 2.0 g
- hemin (stock solution) .................................... 4.0 ml
- double-distilled water to ....................... 1,000.0 ml

1. Add all the ingredients to the distilled water, and mix well by using a magnetic stirrer until dissolved. Heat if necessary to dissolve all the ingredients.
2. Using a Whatman no. 42 filter paper in a Büchner funnel, filter with suction. Do this filtration once more, using new filter paper.
3. Adjust pH to 7.2 with 1 N NaOH or 1 N HCl.
4. Sterilize by filtration using a 0.22-μm-pore-size membrane filter.
5. Dispense 4.5 ml into each tube.
6. Label as LIT medium with the date of preparation and an expiration date of 1 month.

**G. Hemin stock solution**

- hemin ........................................ 100.0 mg
- triethanolamine .............................. 10.0 ml
- sterile double-distilled water .............. 10.0 ml

1. Mix triethanolamine with water, add the mixture to tube containing hemin, shake well, and let dissolve.
2. Complete medium
   Just before inoculation, add 0.5 ml of inactivated fetal bovine serum and 0.25 ml of antibiotic solution. Final concentrations of the antibiotics are 100 U of penicillin, 100 μg of streptomycin, and 0.2 μg of amphotericin B per ml.

**H. Schneider’s *Drosophila* medium (leishmaniasis)**

This medium was originally designed for cultivation of insect tissue culture cells. Hendricks used this medium containing 30% inactivated fetal bovine serum to isolate and grow etiologic agents of cutaneous leishmaniasis. This medium can be purchased from GIBCO, New York, N.Y.

**I. Stock antibiotic solution (to be used with all media)**

- sodium penicillin G ................. 1,000,000 U
- streptomycin sulfate ................. 1,000,000 μg
- amphotericin B ......................... 2,000 μg
- sterile double-distilled water ........... 50.0 ml

1. Mix the above thoroughly. Concentrations of stock solution are
   - penicillin .......................................... 20,000 U/ml
   - streptomycin .................................... 20,000 μg/ml
   - amphotericin B .................................. .40 μg/ml
2. Dispense 1 ml of the antibiotic mixture into sterile screw-cap vials or sterile cryovials.
3. Label as antibiotic solution with the date of preparation and an expiration date of 1 year.
4. Store at −20°C in cryoboxes.

**J. Fetal bovine serum**

**K. Rabbit blood, fresh, defibrinated**

**L. Horse blood**

**M. 1 N NaOH**

**N. 1 N HCl**
I. PRINCIPLE

Laboratorians must differentiate extraneous materials present in a specimen from actual parasites.

II. STOOL ARTIFACTS

A. Sources

Gross and microscopic examination of stool may be complicated by the presence of artifacts resembling parasitic trophoites, cysts, eggs, larvae, and adult worms (Table 9.10.1–A1). Many such artifacts arise from the large array of vegetable and meat products ingested every day by humans. Cells of human enteric origin may also mimic pathogenic or commensal protozoa in their appearance. Spurious infections with human or nonhuman parasites are known to occur following ingestion of contaminated or infected meats. The use of improper collection techniques offers another mechanism by which specimens are contaminated with extraneous organisms (3, 4).

B. Protozoa

1. Amebae

Improper collection and preservation of feces may result in contamination with free-living amebae from soil or water. Most species have large cytoplasmic contractile vacuoles, thick cyst walls, and nuclei with large karyosomes. Inflammatory cells are often present in a variety of infectious and noninfectious enteric syndromes and include PMNs, eosinophils, lymphocytes, and macrophages. Careful evaluation should be made to prevent these cells being mistaken as intestinal parasites, especially Entamoeba histolytica or the E. histolytica/E. dispar group. Report any occurrence of inflammatory cells, and make a quantitative assessment. Amebae also must be differentiated from intestinal epithelial cells (squamous or columnar), yeasts, plant cells, and another protozoan, Blastocystis hominis.

2. Flagellates

Free-living aquatic flagellates may be recovered from feces contaminated with water or saline and are difficult to differentiate from enteric flagellates. Stool samples contaminated with urine may also contain Trichomonas vaginalis and urine samples contaminated with stool may contain Pentatrichomonas hominis.

3. Ciliates

As with amebae and flagellates, free-living ciliates are also commonly found in standing water and may contaminate improperly collected stool specimens. Some species appear similar to Balantidium coli and may require differentiation by an expert.
4. Coccidia

The identification of Cryptosporidium spp. poses significant challenges because of the smallness of the organism and the lack of specificity of the commonly used acid-fast stains (certain other organisms, including some yeasts, may stain positive). Use of an immunofluorescence method increases specificity significantly and may be used as either a primary or confirmatory method. Certain pollen grains are known to mimic Isospora belli or Sarcocystis sp. oocysts and should be differentiated on the basis of size.

5. Microsporidia

The identification of microsporidia also presents challenges because of the small size of the spores and the lack of specificity of the commonly used modified trichrome stains (other organisms, including bacteria and small yeasts, often stain pink/reddish pink, as do the microsporidal spores). Optical brightening agents (calcofluor white) are helpful but also nonspecific; microsporidal spores, as well as small yeasts, will fluoresce. Unfortunately, no specific immunoassay reagents are commercially available for the identification of the microsporidia.
II. STOOL ARTIFACTS (continued)

C. Helminths

1. Adult worms

Many types of partially digested vegetable or fruit fibers are similar in appearance to adult nematodes or tapeworm proglottids. This is especially common with individuals whose enteric transit time has been decreased following administration of a cathartic. Adult free-living nematodes may be recovered from stool specimens contaminated with soil or water. Specimens in question should be preserved in formalin and forwarded for identification.

2. Helminth eggs

A large variety of plant cells, algae, pollen grains, and fungal conidia are routinely seen in feces and may resemble *Ascaris lumbricoides*, *Taenia* spp., *Clonorchis sinensis*, and other helminth eggs. Vegetables contaminated with mites or infected with plant nematodes are a ready source of eggs similar in size and shape to pathogenic forms. With the ingestion of meat products from mammals, fish, birds, or other hosts, coincidental ingestion of a wide variety of helminths and their eggs may occur and result in spurious infections. Organisms with which such infections have been reported have included *Fasciola hepatica*, *Dicrocoelium dendriticum*, and *Capillaria hepatica*, among others (1). True infections may be ruled out with subsequent stool examinations.

3. Helminth larvae

Many kinds of plant or root hairs show a superficial resemblance in size and shape to nematode larvae. However, most appear as clear, refractile structures that lack both symmetry and identifiable internal organs. Unlike plant hairs, true larvae retain iodine when stained.

III. BLOOD ARTIFACTS

Artifacts present in thick and thin blood films may be mistaken for parasites and result in inappropriate or unnecessary treatment of the patient. These artifacts are usually of two types: (i) normal cellular elements, such as platelets, which may mimic malarial parasites when superimposed on erythrocytes, and (ii) contaminants from the staining process, such as yeast cells, bacteria, stain precipitate, or cellulose fibers, which may mimic true fungemia, bacteremia, or parasitemia with malaria or microfilariae.

IV. BODY FLUID ARTIFACTS

The appearance of detached ciliary tufts (ciliocytophthoria) in a variety of body fluids (especially peritoneal and amniotic fluids) has been recognized for many years. Such tufts are the luminal remnants of ciliated epithelium that occur as part of the normal cellular turnover in a variety of organs (respiratory tract and sinuses, ventricles of the brain, central canal of the spinal cord, and epithelia of the male and female reproductive tracts). In wet preparations, these ciliated tufts are 10 to 15 μm in diameter, exhibit rhythmic motion, and may easily be confused with ciliated or flagellated protozoa, including *Trichomonas*, *B. coli*, *Giardia lamblia*, and *Chilomastix mesnili*. However, on close examination of direct wet or permanent stained preparations, there is little internal structure reminiscent of such organisms (2).
V. CORRECTIVE ACTION

Misidentification of artifacts as parasites may be minimized by providing appropriate training of the parasitologist, performing preservation and staining procedures according to established protocols, stressing the importance of correct collection procedures to patients or staff, and strictly adhering to established morphologic criteria when performing the microscopic examinations.

REFERENCES

### Table 9.10.2-A1 Body sites and specimen collection

<table>
<thead>
<tr>
<th>Site</th>
<th>Specimen option</th>
<th>Collection method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>Smears of whole blood</td>
<td>Fresh thick and thin films (1st choice)</td>
</tr>
<tr>
<td></td>
<td>Anticoagulated blood</td>
<td>Anticoagulant (2nd choice)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EDTA (1st choice)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heparin (2nd choice)</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>Aspirate</td>
<td>Sterile</td>
</tr>
<tr>
<td>Central nervous system</td>
<td>Spinal fluid</td>
<td>Sterile</td>
</tr>
<tr>
<td>Cutaneous ulcers</td>
<td>Aspirates from below surface</td>
<td>Sterile plus air-dried smears</td>
</tr>
<tr>
<td></td>
<td>Biopsy specimen</td>
<td>Sterile, nonsterile to histopathology (formalin acceptable)</td>
</tr>
<tr>
<td>Eye</td>
<td>Biopsy specimen</td>
<td>Sterile (in saline)</td>
</tr>
<tr>
<td></td>
<td>Scrapings</td>
<td>Sterile (in saline)</td>
</tr>
<tr>
<td></td>
<td>Contact lens</td>
<td>Sterile (in saline)</td>
</tr>
<tr>
<td></td>
<td>Lens solution</td>
<td>Sterile</td>
</tr>
<tr>
<td>Intestinal tract</td>
<td>Fresh stool</td>
<td>0.5-pt (ca. 0.237-liter) waxed container</td>
</tr>
<tr>
<td></td>
<td>Preserved stool</td>
<td>5 or 10% formalin, MIF, SAF, Schaudinn’s, PVA</td>
</tr>
<tr>
<td></td>
<td>Sigmoidoscopy material</td>
<td>Fresh, PVA or Schaudinn’s smears</td>
</tr>
<tr>
<td></td>
<td>Duodenal contents</td>
<td>Entero-Test or aspirates</td>
</tr>
<tr>
<td></td>
<td>Anal impression smear</td>
<td>Cellulose tape (pinworm examination)</td>
</tr>
<tr>
<td></td>
<td>Adult worm or worm segments</td>
<td>Saline, 70% alcohol</td>
</tr>
<tr>
<td>Liver, spleen</td>
<td>Aspirates</td>
<td>Sterile, collected in four separate aliquots (liver)</td>
</tr>
<tr>
<td></td>
<td>Biopsy specimen</td>
<td>Sterile, nonsterile to histopathology (formalin acceptable)</td>
</tr>
</tbody>
</table>

(continued)
Table 9.10.2–A1  Body sites and specimen collection (continued)

<table>
<thead>
<tr>
<th>Site</th>
<th>Specimen option</th>
<th>Collection methoda</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>Sputum</td>
<td>True sputum (not saliva)</td>
</tr>
<tr>
<td></td>
<td>Induced sputum</td>
<td>No preservative (10% formalin if time delay)</td>
</tr>
<tr>
<td></td>
<td>Bronchoalveolar lavage fluid</td>
<td>Sterile</td>
</tr>
<tr>
<td></td>
<td>Transbronchial aspirate</td>
<td>Air-dried smears</td>
</tr>
<tr>
<td></td>
<td>Tracheobronchial aspirate</td>
<td>Air-dried smears</td>
</tr>
<tr>
<td></td>
<td>Brush biopsy specimen</td>
<td>Air-dried smears</td>
</tr>
<tr>
<td></td>
<td>Open lung biopsy specimen</td>
<td>Air-dried smears</td>
</tr>
<tr>
<td></td>
<td>Aspirate</td>
<td>Sterile</td>
</tr>
<tr>
<td>Muscle</td>
<td>Biopsy specimen</td>
<td>Fresh, squash preparation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nonsterile to histopathology (formalin acceptable)</td>
</tr>
<tr>
<td>Skin</td>
<td>Scrapings</td>
<td>Aseptic, smear or vial</td>
</tr>
<tr>
<td></td>
<td>Skin snip</td>
<td>No preservative</td>
</tr>
<tr>
<td></td>
<td>Biopsy specimen</td>
<td>Sterile (in saline)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nonsterile to histopathology</td>
</tr>
<tr>
<td>Urogenital system</td>
<td>Vaginal discharge</td>
<td>Saline swab, transport swab (no charcoal), culture medium</td>
</tr>
<tr>
<td></td>
<td>Urethral discharge</td>
<td>Air-dried smear for FA</td>
</tr>
<tr>
<td></td>
<td>Prostatic secretions</td>
<td>Air-dried smear for FA</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>Unpreserved spot specimen or 24-h unpreserved specimen</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Midday urine</td>
</tr>
</tbody>
</table>

*a* MIF, merthiolate-iodine-formalin; SAF, sodium acetate-acetic acid-formalin; PVA, polyvinyl alcohol; FA, fluorescent-antibody assay.
### Table 9.10.2–A2 Body sites and possible parasites recovered (diagnostic stage)

<table>
<thead>
<tr>
<th>Site and specimen</th>
<th>Parasite(s)</th>
<th>Site and specimen</th>
<th>Parasite(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood</strong></td>
<td></td>
<td><strong>Intestinal tract</strong></td>
<td></td>
</tr>
<tr>
<td>RBCs</td>
<td><em>Plasmodium</em> spp.</td>
<td>(continued)</td>
<td><em>Hookworm</em></td>
</tr>
<tr>
<td></td>
<td><em>Babesia</em> spp.</td>
<td></td>
<td><em>Strongyloides stercoralis</em></td>
</tr>
<tr>
<td>WBCs</td>
<td><em>Leishmania</em> donovani</td>
<td></td>
<td><em>Trichuris trichiura</em></td>
</tr>
<tr>
<td></td>
<td><em>Toxoplasma</em> gondii</td>
<td></td>
<td><em>Hymenolepis nana</em></td>
</tr>
<tr>
<td>Whole blood, plasma</td>
<td><em>Trypanosoma</em> spp.</td>
<td></td>
<td><em>Taenia saginata</em></td>
</tr>
<tr>
<td>Bone marrow</td>
<td>Microfilariae</td>
<td></td>
<td><em>Taenia solium</em></td>
</tr>
<tr>
<td>Central nervous system</td>
<td><em>Leishmania</em> donovani</td>
<td></td>
<td><em>Diphyllobothrium latum</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Opisthorchis (Clonorchis) sinensis</em></td>
</tr>
<tr>
<td><strong>Cutaneous ulcers</strong></td>
<td><em>Leishmania</em> spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Eye</strong></td>
<td><em>Acanthamoeba</em> spp.</td>
<td></td>
<td><em>Paragonimus westermani</em></td>
</tr>
<tr>
<td></td>
<td><em>Toxoplasma</em> gondii</td>
<td></td>
<td><em>Schistosoma</em> spp.</td>
</tr>
<tr>
<td></td>
<td><em>Taenia</em> solium (cysticerci)</td>
<td></td>
<td><em>Heterophyes</em> sp.</td>
</tr>
<tr>
<td></td>
<td><em>Loa</em> loa</td>
<td></td>
<td><em>Metagonimus</em> sp.</td>
</tr>
<tr>
<td></td>
<td>Microsporidia &amp;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intestinal tract</td>
<td><em>Entamoeba</em> dispar</td>
<td></td>
<td><em>Liver, spleen</em></td>
</tr>
<tr>
<td></td>
<td><em>Entamoeba</em> histolytica</td>
<td></td>
<td><em>Echinococcus</em> spp.</td>
</tr>
<tr>
<td></td>
<td><em>Entamoeba</em> hartmanni</td>
<td></td>
<td><em>Entamoeba</em> histolytica</td>
</tr>
<tr>
<td></td>
<td><em>Endolimax</em> nana</td>
<td></td>
<td><em>Leishmania</em> donovani</td>
</tr>
<tr>
<td></td>
<td><em>Iodamoeba</em> bütschlii</td>
<td></td>
<td><em>Opisthorchis sinensis</em></td>
</tr>
<tr>
<td></td>
<td><em>Blastocystis</em> hominis</td>
<td></td>
<td><em>Fasciola hepatica</em></td>
</tr>
<tr>
<td></td>
<td><em>Giardia</em> lamblia</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Chilomastix</em> mesnili</td>
<td></td>
<td><em>Lung</em></td>
</tr>
</tbody>
</table>
|                   | *Dientamoeba* fragilis |             | *Pneumocystis carinii*
|                   | *Pentatrichomonas hominis* |             |                                                          |
|                   | *Balantium* coli |             | *Echinococcus* spp. |
|                   | *Cryptosporidium* parvum |             | *Paragonimus westermani* |
|                   | *Cyclospora* cayetanensis |             | *Cryptosporidium parvum* |
| Muscle            | *Taenia* solium (cysticerci) |             | *Ascaris lumbricoides* larvae |
|                   | *Trichinella* spiralis |             | *Hookworm* larvae |
|                   | *Onchocerca* volvulus (nODULES) |             | *Strongyloides stercoralis* larvae |
| Skin              | *Leishmania* spp. |             | *Muscle* |
|                   | *Onchocerca* volvulus |             | *Taenia* solium (cysticerci) |
|                   | Microfilariae |             | *Trichinella spiralis* |
| Urogenital system | *Acanthamoeba* spp. |             | *Onchocerca* volvulus |
|                   | *Microsporidia* |             | *Microsporidia* |
|                   | *Enterobius* vermicularis |             |             |

* This table does not include every possible parasite that could be found in a particular body site. Only the most likely organisms are listed. Diagnostic stages include trophozoites, cysts, oocysts, spores, adults, larvae, eggs, amastigotes, trypanastigotes.

* Disseminated in severely immunocompromised individuals.
Table 9.10.2–A3 Body site, specimen, and recommended stain(s)∗

<table>
<thead>
<tr>
<th>Body site</th>
<th>Specimen(s)</th>
<th>Recommended stain for suspect organism</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>Whole or anticoagulated blood</td>
<td>Giemsa for all blood parasites Hematoxylin-based stain for microfilariae (sheathed)</td>
<td>Most drawings and organism descriptions of blood parasites were originally based on Giemsa-stained blood films. Although Wright’s stain (or Wright-Giemsa combination stain) will work, stippling in malaria will normally not be visible and organism colors will not match descriptions. However, if other stains (those listed above) are used in addition to some of the “quick” blood stains, organisms should be visible on blood films.</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>Aspirate</td>
<td>Giemsa for all blood parasites</td>
<td>See comments for blood</td>
</tr>
</tbody>
</table>
| Central nervous system   | Spinal fluid, brain biopsy specimen | Giemsa for trypanosomes, *Toxoplasma gondii*  
Giemsa, trichrome, or calcofluor for amebae (*Naegleria, Acanthamoeba, and Balamuthia spp.*) (cysts only)  
Giemsa, acid-fast PAS, modified trichrome, calcofluor, and silver methenamine for microsporidia  
Hematoxylin and eosin (routine histology) for larval cestodes | If CSF is received (with no suspect organism suggested), Giemsa is the best choice, but calcofluor is recommended as a second stain. If brain biopsy material is received (particularly from an immunocompromised patient), EM studies may be required to identify microsporidia to the genus or species level.                                                                                     |
| Cutaneous ulcer          | Aspirate, biopsy specimen    | Giemsa for leishmaniae  
Hematoxylin and eosin (routine histology) for *Acanthamoeba spp.*, *Entamoeba histolytica*         | Most likely causative agents would be leishmaniae, all of which would stain with Giemsa. Hematoxylin and eosin (routine histology) could also be used to identify these organisms.                                                                                                                                                                                                                               |
| Eye                      | Biopsy specimen, scrapings, contact lens, lens solution | Calcofluor for amebae (*Acanthamoeba spp.*) (cysts only)  
Giemsa for amebae (trophozoites, cysts)  
Hematoxylin and eosin (routine histology) for cysticerci, *Loa loa*, *Toxoplasma gondii*  
Silver methenamine, PAS, acid-fast stains, calcofluor, and EM studies for microsporidia | Some free-living amebae (most commonly *Acanthamoeba spp.*) have been implicated as a cause of keratitis. Although calcofluor will stain cyst walls, it will not stain trophozoites. Therefore, in suspected cases of amebic keratitis, use both stains. Hematoxylin and eosin (routine histology) can be used to detect and confirm cysticercosis. Adult *Loa loa* worm, when removed from the eye, can be stained with hematoxylin-based stain (Delafield’s) or can be stained and examined via routine histology. *Toxoplasma* infection could be diagnosed by using routine histology and/or serology results. Confirmation of microsporidia to the genus and species levels may require EM studies. |
| Intestinal tract         | Stool, sigmoidoscopy material, duodenal contents | Trichrome or iron hematoxylin for intestinal protozoa  
Modified trichrome and calcofluor for microsporidia  
Modified acid-fast stain for *Cryptosporidium parvum* and *Cyclospora cayetanensis*  
Immunooassay reagents (EIA, FA, cartridge format) for *Entamoeba histolytica/E. dispar*, *Entamoeba histolytica*, *Giardia lambia*, *Cryptosporidium parvum*, and microsporidia (experimental) | Although trichrome or iron hematoxylin stains can be used on almost all specimens from the intestinal tract, actual worm segments (tape-worm proglottids) can be stained with special stains. However, after routine dehydration with alcohols and xylenes (or xylene substitutes), the branched uterine structure will be visible, allowing identification of proglottids to the species level. Immunooassay detection kits are also available for the identification of *Giardia lambia*, *Entamoeba histolytica/E. dispar*, *Entamoeba histolytica*, and *Cryptosporidium parvum*. Confirmation of microsporidia to the genus or species level may require EM studies. |
Table 9.10.2–A3 (continued)

<table>
<thead>
<tr>
<th>Body site</th>
<th>Specimen(s)</th>
<th>Recommended stain for suspect organism</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intestinal tract</strong></td>
<td>Anal impression smear</td>
<td>No stain, cellulose tape, or egg collection device</td>
<td>Four to six consecutive negative tapes are required to rule out infection</td>
</tr>
<tr>
<td></td>
<td>Adult worm or worm segments</td>
<td>Carmin stains (rarely used)</td>
<td>Proglottids can usually be identified to the species level without using tissue stains</td>
</tr>
<tr>
<td></td>
<td>Biopsy specimen</td>
<td>Hematoxylin and eosin (routine histology) for <em>Entamoeba histolytica</em> (also PAS), <em>Cryptosporidium parvum</em>, <em>Cyclospora cayetanensis</em>, <em>Isospora belli</em>, <em>Giardia lamblia</em>, and microsporidia</td>
<td>Special stains may be helpful in the identification of microsporidia: tissue Gram stains, silver stains, PAS, and Giemsa stain</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver, spleen</td>
<td>Aspirates</td>
<td>Giemsa for leishmaniae</td>
<td>Aspirates and/or touch preparations from biopsy material can be routinely stained with Giemsa stain. This will allow identification of leishmaniae. Definite risks are associated with spleen aspirates and/or biopsy material. Other parasites, such as larval cestodes, trematodes, amebae, or microsporidia can be seen and identified by routine histological staining.</td>
</tr>
<tr>
<td></td>
<td>Biopsy specimen</td>
<td>Hematoxylin and eosin (routine histology)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>Sputum, induced sputum, bronchoalveolar lavage fluid, transbronchial aspirate, tracheobronchial aspirate, brush biopsy specimen, open lung biopsy specimen</td>
<td>Silver methenamine stain, calcofluor for <em>Pneumocystis carinii</em> (cysts only)</td>
<td><em>Pneumocystis carinii</em> is the most common parasite recovered from the lung and identified by using silver or Giemsa stains or monoclonal reagents (FA). Monoclonal reagents (FA) for the diagnosis of pulmonary cryptosporidiosis are also available. Routine histology procedures would allow identification of any helminths or helminth eggs in the lung.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Giemsa for <em>Pneumocystis carinii</em> (trophozoites only)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Modified acid-fast stains for <em>Cryptosporidium parvum</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hematoxylin and eosin (routine histology) for <em>Strongyloides stercoralis</em>, <em>Paragonimus spp.</em>, amebae</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Silver methenamine stain, PAS, acid-fast, modified trichrome, tissue Gram stains, and EM studies for microsporidia</td>
<td>Pneumocystis carinii is the most common parasite recovered from the lung and identified by using silver or Giemsa stains or monoclonal reagents (FA). Monoclonal reagents (FA) for the diagnosis of pulmonary cryptosporidiosis are also available. Routine histology procedures would allow identification of any helminths or helminth eggs in the lung.</td>
</tr>
<tr>
<td>Muscle</td>
<td>Biopsy specimen</td>
<td>Hematoxylin and eosin (routine histology) for <em>Trichinella spiralis</em>, cysticerci</td>
<td>If <em>Trypanosoma cruzi</em> is present in the striated muscle, it can be identified from routine histological preparations. Confirmation of microsporidia to the genus or species level may require EM studies.</td>
</tr>
<tr>
<td>Skin</td>
<td>Aspirates</td>
<td>See Cutaneous Ulcer above.</td>
<td>Any of these parasites can be identified by using routine histological procedures and stains.</td>
</tr>
<tr>
<td></td>
<td>Skin snip, scrapings, biopsy specimen</td>
<td>Hematoxylin and eosin (routine histology) for <em>Onchocerca volvulus</em>, <em>Dipetalonema streptocerca</em></td>
<td>Any of these parasites can be identified by using routine histological procedures and stains.</td>
</tr>
<tr>
<td>Urogenital system</td>
<td>Vaginal discharge</td>
<td>Giemsa</td>
<td>Although <em>Trichomonas vaginalis</em> is probably the most common parasite identified, there are others to consider, the most recently implicated organisms being in the microsporidia. Microfilariae can also be recovered and stained.</td>
</tr>
<tr>
<td></td>
<td>Urethral discharge, prostatic secretions</td>
<td>Immunoassay reagents (FA) for <em>Trichomonas vaginalis</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>Delafield’s hematoxylin for microfilariae</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Biopsy specimen</td>
<td>Hematoxylin and eosin (routine histology) for <em>Schistosoma haematobium</em>, microfilariae</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Silver methenamine stain, PAS, acid-fast, modified trichrome, tissue Gram stains, and EM studies for microsporidia</td>
<td></td>
</tr>
</tbody>
</table>

*PAS, periodic acid-Schiff; EM, electron microscopy; FA, fluorescent antibody.
<table>
<thead>
<tr>
<th>Suspect causative agent(s)</th>
<th>Disease(s)</th>
<th>Appropriate test(s)</th>
<th>Positive result</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Naegleria fowleri</em></td>
<td>Primary amebic meningoencephalitis</td>
<td>1. Wet examination of CSF (not in counting chamber)</td>
<td>Trophozoites present and identified</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Stained preparation of CSF sediment</td>
<td></td>
</tr>
<tr>
<td><em>Acanthamoeba</em> spp.</td>
<td>Amebic keratitis, chronic meningoencephalitis</td>
<td>1. Culture or stained smears</td>
<td>Trophozoites and/or cysts present and identified</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Calcofluor (cysts only)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. Biopsy or routine histology</td>
<td></td>
</tr>
<tr>
<td><em>Balamuthia mandrillaris</em></td>
<td>Chronic meningoencephalitis (granulomatous amebic encephalitis)</td>
<td>1. Calcofluor (cysts only)</td>
<td>Trophozoites and/or cysts present and identified</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Biopsy for routine histology</td>
<td></td>
</tr>
<tr>
<td><em>Entamoeba histolytica</em></td>
<td>Amebiasis</td>
<td>Biopsy or routine histology</td>
<td>Trophozoites present and identified</td>
</tr>
<tr>
<td><em>Giardia lamblia</em></td>
<td>Giardiasis</td>
<td>1. Duodenal aspirate</td>
<td>Trophozoites and/or cysts present and identified</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Duodenal biopsy or routine histology</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. Entero-Test capsule</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4. Immunoassays</td>
<td></td>
</tr>
<tr>
<td><em>Leishmania</em> spp. (cutaneous lesions)</td>
<td>Cutaneous leishmaniasis</td>
<td>1. Material from under bed of ulcer</td>
<td>Amastigotes recovered in macrophages of skin or from animal inoculation; other stages recovered in culture</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a. Smear</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>b. Culture</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>c. Animal inoculation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Punch biopsy</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>a. Routine histology</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>b. Squash preparation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>c. Culture</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>d. Animal inoculation</td>
<td></td>
</tr>
<tr>
<td><em>Leishmania</em> spp. (mucocutaneous lesions)</td>
<td>Mucocutaneous leishmaniasis</td>
<td>As for cutaneous leishmaniasis</td>
<td>Amastigotes recovered in macrophages of skin and mucous membranes or from animal inoculation; other stages recovered in culture</td>
</tr>
<tr>
<td><em>Leishmania</em> spp. (visceral)</td>
<td>Visceral leishmaniasis (kala-azar)</td>
<td>1. Buffy coat</td>
<td>Amastigotes recovered in cells of reticuloendothelial system</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a. Stain</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>b. Culture</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>c. Animal inoculation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Bone marrow</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>a. Stain</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>b. Culture</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>c. Animal inoculation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. Liver or spleen biopsy with routine histology</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>a. Stain</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>b. Culture</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>c. Animal inoculation</td>
<td></td>
</tr>
<tr>
<td><em>Pneumocystis carinii</em></td>
<td>Pneumocystis</td>
<td>1. Open lung biopsy for histology</td>
<td>Trophozoites or cysts present and identified</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Lung needle aspirate</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. Bronchial brush</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4. Transtracheal aspirate</td>
<td>Monoclonal antibody fluorescent detection of cysts and trophozoites</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5. Bronchoulveolar lavage</td>
<td>Cysts present and identified</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6. Induced sputum (AIDS patients)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>7. Calcofluor</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>8. Immunoassays</td>
<td></td>
</tr>
<tr>
<td><em>Toxoplasma gondii</em></td>
<td>Toxoplasmosis</td>
<td>1. Lymph node biopsy</td>
<td>Identification of organisms plus appropriate serological test results</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a. Routine histology</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>b. Tissue culture isolation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>c. Animal inoculation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Serology</td>
<td></td>
</tr>
<tr>
<td>Suspect causative agent(s)</td>
<td>Disease(s)</td>
<td>Appropriate test(s)</td>
<td>Positive result</td>
</tr>
<tr>
<td>---------------------------</td>
<td>------------</td>
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<td>-----------------</td>
</tr>
<tr>
<td><strong>Protozoa (continued)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cryptosporidium parvum</em></td>
<td>Cryptosporidiosis</td>
<td>1. Duodenal scraping</td>
<td>Identification of organisms in microvillus border or other tissues (lung and gall bladder have also been involved); routine stains or monoclonal antibody reagents to identify oocysts in stool</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Duodenal biopsy</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>a. Stain</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>b. Routine histology</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. Punch biopsy</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>a. Routine histology</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>b. Squash preparation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4. Sputum</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5. Immunoassays</td>
<td></td>
</tr>
<tr>
<td><strong>Microsporidia</strong></td>
<td>Microsporidiosis</td>
<td>Routine histology; modified trichrome, tissue Gram stains, silver, periodic acid-Schiff, and Giemsa stains recommended (spores); animal inoculation not recommended/latent infections</td>
<td>These organisms (spores) have been found as insect or other animal parasites; route of infection is probably ingestion. Human cases involve muscle, CSF (AIDS); other body sites have also been documented.</td>
</tr>
<tr>
<td><em>Nosema</em> spp.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Encephalitozoon</em> spp.</td>
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<td></td>
</tr>
<tr>
<td><em>Enterocytozoon</em> spp.</td>
<td></td>
<td></td>
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</tr>
<tr>
<td><em>Septata</em> sp.</td>
<td></td>
<td></td>
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</tr>
<tr>
<td><em>Pleistophora</em> spp.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Trachipleistophora</em> spp.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Brachiola</em> sp.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Microsporidium</em> spp.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Vittaforma cornea</em></td>
<td>Microsporidiosis</td>
<td>Routine histology; modified trichrome, tissue Gram stains, silver, periodic acid-Schiff, and Giemsa stains recommended (spores); animal inoculation not recommended/latent infections</td>
<td>These organisms (spores) have been found as insect or other animal parasites; route of infection is probably ingestion. Human cases involve muscle, CSF (AIDS); other body sites have also been documented.</td>
</tr>
<tr>
<td><strong>Helminths</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larvae (<em>Ascaris</em> and <em>Strongyloides</em> spp.)</td>
<td>“Pneumonia”</td>
<td>Sputum, wet preparation</td>
<td>This is an incidental finding but has been reported in severe infections. Eggs will be coughed up and appear as “iron filings”; eggs could also be found in stool.</td>
</tr>
<tr>
<td>Eggs (<em>Paragonimus</em> spp.)</td>
<td>Paragonimiasis</td>
<td>Sputum, wet preparation</td>
<td>Rare finding, but hooklets can be found when the hydatid cyst is in the lung.</td>
</tr>
<tr>
<td>Hooklets (<em>Echinococcus</em> spp.)</td>
<td>Hydatid disease</td>
<td>Sputum, wet preparation</td>
<td>Rare finding, but hooklets can be found when the hydatid cyst is in the lung.</td>
</tr>
<tr>
<td><em>Onchocerca volvulus</em></td>
<td>Onchocerciasis</td>
<td>Skin</td>
<td>Skin snips examined in saline; microfilariae may be present.</td>
</tr>
<tr>
<td><em>Mansonella streptocerca</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Schistosoma</em> spp.</td>
<td>Schistosomiasis</td>
<td>1. Rectal valve biopsy</td>
<td>Eggs present and identified</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Bladder biopsy</td>
<td></td>
</tr>
</tbody>
</table>

* Now classified with the fungi.
### Table 9.10.2–A5 Protozoa of the intestinal tract and urogenital system: key characteristics

<table>
<thead>
<tr>
<th>Organism</th>
<th>Trophozoite or tissue stage</th>
<th>Cyst or other stage in specimen</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amebae</strong>&lt;br&gt;Entamoeba histolytica (pathogenic)</td>
<td>Cytoplasm clean; presence of RBCs is diagnostic, but cytoplasm may also contain some ingested bacteria; peripheral nuclear chromatin evenly distributed, with central, compact karyosome</td>
<td>Mature cyst contains four nuclei; chromatoidal bars have smooth, rounded ends; immature cyst usually contains one enlarged nucleus (precyst)</td>
<td>Considered pathogenic; report to Public Health; trophozoites can be confused with macrophages and cysts can be confused with WBCs in the stool</td>
</tr>
<tr>
<td><strong>Entamoeba dispar</strong> (nonpathogenic)</td>
<td>Morphology identical to that of <em>E. histolytica</em> (confirmed by presence of RBCs in cytoplasm). If no RBCs, zymodeme analysis or immunodiagnostic is necessary to confirm species designation.</td>
<td>Mature cyst has morphology identical to that of <em>E. histolytica</em>.</td>
<td>Nonpathogenic: morphology resembles that of <em>E. histolytica</em>; these organisms will continue to be signed out as <em>E. histolytica/E. dispar</em> and reported to Public Health. Immunodiagnostic reagents are now available to differentiate pathogenic <em>E. histolytica</em> and nonpathogenic <em>E. dispar</em>; some laboratories may decide to use these reagents on a routine basis, depending on positivity rate and cost.</td>
</tr>
<tr>
<td><strong>Entamoeba histolytica/E. dispar</strong></td>
<td>Looks identical to <em>E. histolytica</em> but smaller (&lt;12 μm); RBCs will not be ingested</td>
<td>Shrinkage occurs on permanent stain (especially in cyst form). <em>E. histolytica</em> may actually be below the 12- and 10-μm cutoff limits; it could be as much as 1.5 μm below the limits quoted for wet preparation measurements. Entire space (including shrinkage) must be measured.</td>
<td>Correct way to report, unless immunodiagnostic is used to identify <em>E. histolytica</em> or trophozoites are seen with ingested RBCs (<em>E. histolytica</em>)</td>
</tr>
<tr>
<td><strong>Entamoeba hartmanni</strong> (nonpathogenic)</td>
<td>Cytoplasm dirty, may contain ingested bacteria or debris; peripheral nuclear chromatin unevenly distributed, with a large, eccentric karyosome</td>
<td>Mature cyst contains eight nuclei, may see more; chromatoidal bars (if present) tend to have sharp, pointed ends</td>
<td>If a smear is too thick or thin and if stain is too dark or light, then <em>E. histolytica/E. dispar</em> and <em>E. coli</em> are often confused; much overlap in morphology. Assess smear thickness before identifying organisms to the species level.</td>
</tr>
<tr>
<td><strong>Entamoeba coli</strong> (nonpathogenic)</td>
<td>Cytoplasm clean, not diagnostic, great deal of nuclear variation, may even be some peripheral nuclear chromatin. Normally, only karyosomes are visible.</td>
<td>Cyst is round to oval, with four nuclear karyosomes visible</td>
<td>More nuclear variation in this ameba than in any others; can be confused with <em>Dientamoeba fragilis</em> and/or <em>E. hartmanni</em></td>
</tr>
<tr>
<td><strong>Endolimax nana</strong> (nonpathogenic)</td>
<td>Cytoplasm contains much debris; organisms usually larger than <em>E. nana</em> but may look similar; large karyosome</td>
<td>Cyst contains single nucleus (may be “basket nucleus”) with bits of nuclear chromatin arranged on nuclear membrane (karyosome is the basket, bits of chromatin are the handle); large glycogen vacuole</td>
<td>Glycogen vacuole stains brown with addition of iodine in wet preparation; “basket nucleus” more common in cyst but can be seen in trophozoite; vacuole may be so large that the cyst collapses on itself</td>
</tr>
<tr>
<td><strong>Iodamoeba bütschlii</strong> (nonpathogenic)</td>
<td></td>
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</tr>
</tbody>
</table>
Table 9.10.2–A5 (continued)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Trophozoite or tissue stage</th>
<th>Cyst or other stage in specimen</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amebae (continued)</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>Giardia lamblia</em> (pathogenic)</td>
<td>Trophozoites teardrop shaped from front, like a curved spoon from side; contain nuclei, linear axonemes, and curved median bodies</td>
<td>Cyst is round to oval; contains multiple nuclei, axonemes, and median bodies</td>
<td>Organisms live in duodenum, and multiple stools may be negative; may have to use additional techniques (immunoassay, aspirate, Entero-Test). Organism numbers may have little relevance to presence of symptoms.</td>
</tr>
<tr>
<td><strong>Chilomastix mesnili</strong> (nonpathogenic)</td>
<td>Trophozoites teardrop shaped; cytostome must be visible for identification</td>
<td>Cyst is lemon shaped, with one nucleus and curved fibril called shepherd’s crook</td>
<td>Cyst can be identified much easier than trophozoite form. Trophozoite will look like other small flagellates.</td>
</tr>
<tr>
<td><strong>Dientamoeba fragilis</strong> (pathogenic)</td>
<td>Cytoplasm contains debris; may contain one or two nuclei (chromatin often fragmented into four dots)</td>
<td>No known cyst form</td>
<td>Tremendous size and shape range on a single smear; trophozoites with one nucleus can resemble <em>E. nana</em></td>
</tr>
<tr>
<td><strong>Trichomonas vaginalis</strong> (pathogenic)</td>
<td>Supporting rod (axostyle) present; undulating membrane comes halfway down the organism; small dots may be seen in cytoplasm along axostyle</td>
<td>No known cyst form</td>
<td>Recovered from genitourinary system; often diagnosed at bedside with wet preparation (motility)</td>
</tr>
<tr>
<td><strong>Pentatrichomonas hominis</strong> (nonpathogenic)</td>
<td>Supporting rod (axostyle) present; undulating membrane comes all the way down the organism; small dots may be seen in cytoplasm along axostyle</td>
<td>No known cyst form</td>
<td>Recovered in stool; trophozoites may resemble other small flagellate trophozoites</td>
</tr>
<tr>
<td><strong>Ciliate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Balantidium coli</em> (pathogenic)</td>
<td>Very large trophozoites (50–100 μm long) covered with cilia; large bean-shaped nucleus</td>
<td>Morphology not significant except for large, bean-shaped nucleus</td>
<td>Rarely seen in United States; causes severe diarrhea with large fluid loss; will be seen in proficiency testing specimens</td>
</tr>
<tr>
<td><strong>Coccidia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cryptosporidium parvum</em> (pathogenic)</td>
<td>Seen in intestinal mucosa (edge of brush border), gall bladder, and lung biopsy specimens</td>
<td>Oocysts seen in stool and/or sputum; organisms acid fast, 4–6 μm long; hard to find if few in number</td>
<td>Chronic infection in compromised host (internal autoinfective cycle), self-cure in immunocompetent host; numbers of oocysts correlate with stool consistency; can cause severe, watery diarrhea; oocysts immediately infective when passed</td>
</tr>
<tr>
<td><em>Cyclospora cayetanensis</em> (pathogenic)</td>
<td>Experience with this organism is not extensive; organism may be difficult to identify in tissue; since patients are immunocompetent, biopsy specimens will probably rarely be required or requested</td>
<td>Oocysts seen in stool; approximately 8–10 μm in size; are unsporulated and thus difficult to recognize as coccidia; will mimic <em>C. parvum</em> (4–6 μm) on modified acid-fast-stained smears.</td>
<td>Oocysts have been seen in stool but were not recognized as coccidia until recently. To date, most infections are associated with immunocompetent individuals, but they may also be seen in immunosuppressed patients; organism may be associated with traveler’s diarrhea and has been linked to the ingestion of imported food (raspberries, mesclun, basil)</td>
</tr>
</tbody>
</table>

(continued)
### Table 9.10.2–A5 Protozoa of the intestinal tract and urogenital system: key characteristics (continued)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Trophozoite or tissue stage</th>
<th>Cyst or other stage in specimen</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Coccidia (continued)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Isospora belli</em> (pathogenic)</td>
<td>Seen in intestinal mucosal cells and in biopsy specimens; less common than <em>C. parvum</em></td>
<td>Oocysts seen in stool; organisms acid fast; best technique is concentration, not permanent stained smear</td>
<td>Thought to be the only <em>Isospora</em> sp. that infects humans; oocysts not immediately infective when passed; oocysts will be missed if PVA-preserved specimens are concentrated</td>
</tr>
<tr>
<td><strong>Microsporidia</strong></td>
<td>Developing stages sometimes difficult to identify; spores can be identified by size, shape, and presence of polar tubules</td>
<td>Depending on the genus involved, spores could be identified in stool or urine using the modified trichrome stain, calcofluor, or immunoassay reagents (experimental).</td>
<td>Spores are generally quite small (1–2.0 μm for <em>Enterocytozoon</em> spp.) and can easily be confused with other organisms or artifacts (particularly in stool). Infections tend to be present in immunosuppressed patients. It is unclear whether all genera listed here can disseminate to all parts of the body (gastrointestinal tract, urogenital system); however, the list is complete in terms of all body sites.</td>
</tr>
</tbody>
</table>

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*a* PVA, polyvinyl alcohol; EM, electron microscopy.

*b* Zymodeme analysis is based on the identification of various isoenzymes found in cultured isolates of *Entamoeba histolytica*/*E. dispar*. These zymodemes (given separate numbers) tend to separate into two groups: those from symptomatic patients, some of whom have extraintestinal amebiasis, tend to group with the zymodeme isolates characteristic of the pathogen *E. histolytica*, while those from asymptomatic carriers tend to group with the zymodeme isolates characteristic of the nonpathogen, *E. dispar*. 

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### Table 9.10.2–A6  Tissue protozoa: characteristics

<table>
<thead>
<tr>
<th>Species</th>
<th>Shape and size</th>
<th>Other features</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Toxoplasma gondii</strong></td>
<td><strong>Trophozoites (tachyzoites)</strong> Crescent shaped; 4–6 μm long by 2–3 μm wide</td>
<td>Found in peritoneal fluid of experimentally infected mice; intracellular forms somewhat smaller and not usually seen in humans. May be isolated in tissue culture, particularly from CSF. Diagnosis is most frequently based on clinical history and serological evidence (acute- and convalescent-phase sera).</td>
</tr>
<tr>
<td><strong>Cysts (bradyzoites)</strong></td>
<td>Generally spherical; 200 μm to 1 mm in diam</td>
<td>Occur in many body tissues (approx 30–50% of the U.S. population have these organisms in tissues, indicating past infection). Many infections are asymptomatic. Infections in the compromised host are very serious and involve the CNS. In these patients, particularly those with AIDS, diagnostic serological titers may be very difficult to demonstrate.</td>
</tr>
<tr>
<td><strong>Pneumocystis carinii</strong></td>
<td><strong>Trophozoites</strong> Ameboid in shape; about 5 μm; nucleus visible with Giemsa or hematoxylin stain</td>
<td>AIDS patients may have longer incubation period (avg, approx 40 days, but can be up to 1 yr). As many as 28% of these patients show normal chest X rays and no or ill-defined physical signs in chest. Rales may or may not be detected. Serological studies indicate that by age 4 yr, approx 80% of those tested are positive. Diagnosis is based on actual demonstration of the organism.</td>
</tr>
<tr>
<td></td>
<td><strong>Cysts</strong> Usually round; when mature, contain eight trophozoites; often measure 5 μm and contain very small trophozoites (1 μm)</td>
<td>Before AIDS epidemic, procedure of choice was open lung biopsy. Currently, BAL, transbronchial biopsy, and collection of induced sputum specimens are more widely used. No commercial reagents are available for serological diagnosis. Monoclonal reagents for direct organism detection are commercially available.</td>
</tr>
<tr>
<td><strong>Cryptosporidium parvum</strong></td>
<td><strong>Oocyst usually round, 4–6 μm, each mature oocyst containing sporozoites (infective on passage)</strong></td>
<td>Oocyst usually diagnostic stage in stool, sputum, and possibly other body specimens. Various other stages in life cycle can be seen in biopsy specimens taken from GI tract (brush border of epithelial cell-intestinal tract) and other tissues (lung, gall bladder). Several modified acid-fast stains have been used successfully. Direct detection methods using immunoassay reagents are also available.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Note: Infection in the immunocompetent host is self-limiting; however, in immunodeficient patients (AIDS), infection is chronic because of an autoinfective capability in the life cycle. The number of oocysts usually correlates with symptoms (watery diarrhea = many oocysts in specimen). The more normal the stool, the more difficult it is to find oocysts. Risk groups include animal handlers, travelers, immunocompromised individuals, children in day care centers, and those who come in contact with these individuals. Since oocysts are immediately infective, nosocomial transmission has been documented.</td>
</tr>
<tr>
<td><strong>Cyclospora cayetanensis</strong></td>
<td><strong>Oocyst usually round, 8–10 μm; each oocyst is immature on passage; no internal morphology visible; oocysts appear as “wrinkled” cellophane</strong></td>
<td>Oocyst is the diagnostic stage in stool. Various other stages in the life cycle can be seen in biopsy specimens taken from the GI tract (within epithelial cells and intestinal tract). The morphology is similar to that of <em>Isospora belli</em>. A number of modified acid-fast stains have been used successfully to demonstrate the oocysts (quite acid-fast variable). Detection methods involving immunoassay reagents are under development.</td>
</tr>
</tbody>
</table>

(continued)
<table>
<thead>
<tr>
<th>Species</th>
<th>Shape and size</th>
<th>Other features</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Isospora belli</em></td>
<td>Ellipsoidal oocyst; usual range, 20–30 μm long by 10–19 μm wide; sporocysts rarely seen broken out of oocysts but measure 9–11 μm</td>
<td>Mature oocyst contains two sporocysts with four sporozoites each; usual diagnostic stage in feces is immature oocyst containing spherical mass of protoplasm (diarrhetic stool). Developing stages can be recovered from intestinal biopsy specimens. Oocysts are also acid fast and can be detected during acid-fast staining of stool for <em>Cryptosporidium</em> spp. Oocysts are often detected in concentration sediment (wet preparation).</td>
</tr>
<tr>
<td>Microsporidia</td>
<td>Spores are extremely small and have been recovered from all body sites, including the eye.</td>
<td>These organisms have been found as insect or other animal parasites; the route of infection may be ingestion, inhalation, or direct inoculation (eye). Histology results vary (spores are acid fast); PAS, silver, tissue Gram, and Giemsa stains are recommended for spores. Animal inoculation is not recommended; laboratory animals may carry occult infection; electron microscopy may be necessary for confirmation and identification to the genus and species levels. Although difficult to diagnose, infections have been found in a large number of AIDS patients (<em>Enterocytozoon bieneusi, Encephalitozoon</em> [Septata]* intestinalis* in the intestinal tract, <em>Pleistophora</em> spp. in muscle, and various other microsporidia in other tissues, including the CNS). To date, it is still somewhat difficult to diagnose this infection by examining stool specimens prepared with optical brightening agents (calcofluor) or routine stains (modified trichrome, acid-fast trichrome stains). Diagnostic immunosassay reagents are under development but are not yet commercially available.</td>
</tr>
<tr>
<td><em>Sarcocystis hominis,</em></td>
<td>Thin-walled oocyst contains two mature sporocysts, each containing four sporozoites; frequently thin oocyst wall ruptures; ovoidal sporocysts are 9–16 μm long by 7.5–12 μm wide</td>
<td>Thin-walled oocyst or ovoidal sporocysts occur in stool. Compromised host may show fever, severe diarrhea, abdominal pain, and weight loss, although the number of patients has been small. Infections occur from ingestion of uncooked pork or beef. Life cycle occurs within intestinal cells, with eventual production of sporocysts in stool.</td>
</tr>
<tr>
<td><em>S. suihominis,</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. bovihominis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Sarcocystis “lindemanni”</em></td>
<td>Shapes and sizes of skeletal and cardiac muscle sarcocysts vary considerably.</td>
<td>When humans accidentally ingest oocysts from other animal stool sources, sarcocysts that develop in human muscle apparently do little if any harm. These can be identified by routine histological methods.</td>
</tr>
</tbody>
</table>

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*a* CNS, central nervous system; BAL, bronchoalveolar lavage; GI, gastrointestinal; FA, fluorescent antibody; PAS, periodic acid-Schiff.
### Table 9.10.2–A7 Helminths: Key Characteristics

<table>
<thead>
<tr>
<th>Helminth</th>
<th>Diagnostic Stage</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nematodes (roundworms)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascaris lumbricoides (pathogenic)</td>
<td>Egg: both fertilized (oval to round with thick, mammillated-tuberculated shell) and unfertilized (tends to be more oval-elongate, with bumpy shell exaggerated) can be found in stool. Adult worms: 10–12 in., found in stool. Rarely (in severe infections), migrating larvae can be found in sputum.</td>
<td>Unfertilized eggs will not float in flotation concentration method; adult worms tend to migrate when irritated (anesthesia, high fever), so check patients from areas of endemicity for infection prior to elective surgery.</td>
</tr>
<tr>
<td>Trichuris trichiura (whipworm) (pathogenic)</td>
<td>Egg: barrel shaped, with two clear, polar plugs; adult worm rarely seen; quantify eggs (rare, few, etc.), since light infections may not be treated.</td>
<td>Dual infections with Ascaris may be seen (both infections acquired from egg ingestion in contaminated soil); in severe infections, rectal prolapse may occur in children or bloody diarrhea can be mistaken for amebiasis (bloody diarrhea usually not seen in United States).</td>
</tr>
<tr>
<td>Enterobius vermicularis (pinworm)</td>
<td>Egg: football shaped, with one flattened side</td>
<td>May cause symptoms (itching) in some patients; test of choice is tape preparation; six consecutive tapes necessary to rule out infection; symptomatic patient often treated without actual confirmation of infection; eggs become infective within a few hours.</td>
</tr>
<tr>
<td>Ancylostoma duodenale (Old World hookworm), Necator americanus (New World hookworm) (pathogenic)</td>
<td>Egg: eggs of both identical; oval, with broadly rounded ends, thin shell, clear space between shell and developing embryo (8–16 ball stage)</td>
<td>May cause symptoms in some patients (blood loss anemia on differential smear in heavy infections). If stool remains unpreserved for several hours or days, eggs may continue to develop and hatch; rhabditiform larvae may resemble those of Strongyloides stercoralis.</td>
</tr>
<tr>
<td>Strongyloides stercoralis (pathogenic)</td>
<td>Rhabditiform larvae (noninfective) usually found in stool (short buccal cavity or capsule with large, genital primordial packet of cells [“short and sexy”]); in very heavy infections, larvae occasionally found in sputum and/or filariform (infective) larvae found in stool (slit in tail)</td>
<td>May see unexplained eosinophilia, abdominal pain, unexplained episodes of sepsis and/or meningitis, pneumonia (migrating larvae) in compromised patient. Potential for internal autoinfection can maintain low-level infections for many years (patient will be asymptomatic, with elevated eosinophilia); hyperinfection can occur in compromised patient (leading to disseminated stronglyoidiasis and death); agar plate culture is the most sensitive diagnostic method; many infections are low level, and larvae are difficult to recover.</td>
</tr>
<tr>
<td>Ankylostoma braziliensis (dog-cat hookworm) (pathogenic)</td>
<td>Humans are accidental hosts; larvae wander through outer layer of skin, creating tracks (severe itching, eosinophilia); no practical microbiological diagnostic tests</td>
<td>Cause of cutaneous larva migrans; typical setup for infection: dogs and cats defecate in sandbox, and hookworm eggs hatch and penetrate human skin in contact with infected sand or soil (children playing in sandbox).</td>
</tr>
<tr>
<td>Toxocara cati or Toxocara caninum (dog-cat ascarid) (pathogenic)</td>
<td>Humans are accidental hosts; ingestion of dog or cat ascarid eggs from contaminated soil; larvae wander through deep tissues (including eye); proglottids can be mistaken for cancer of the eye; serology helpful for confirmation; eosinophilia</td>
<td>Cause of visceral larva migrans and ocular larva migrans; requests for laboratory services often originate in ophthalmology clinic.</td>
</tr>
<tr>
<td>Cestodes (tapeworms)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taenia saginata (beef tapeworm)</td>
<td>Scolex (four suckers, no hooklets), gravid proglottids (&gt;12 branches on single side) are diagnostic; eggs indicate Taenia spp. only (thick, striated shell containing six-hooked embryo or oncosphere); worm usually approx 12 ft long</td>
<td>Adult worm can cause symptoms in some individuals; acquired from ingestion of raw or poorly cooked beef; usually only single worm/patient; individual proglottids may crawl from anus; proglottids can be injected with India ink to show uterine branches for identification.</td>
</tr>
</tbody>
</table>
Table 9.10.2–A7 Helminths: key characteristics (continued)

<table>
<thead>
<tr>
<th>Helminth</th>
<th>Diagnostic stage</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cestodes (tapeworms)</strong> (continued)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Taenia solium</em> (pork tapeworm)</td>
<td>Scolex (four suckers with hooklets), gravid proglottids (&lt;12 branches on single side) are diagnostic; eggs indicate <em>Taenia</em> spp. only (thick, striated shell, containing six-hooked embryo or oncosphere), worm usually approx 12 ft long</td>
<td>Adult worm can cause GI complaints in some individuals; cysticercosis (accidental ingestion of eggs) can cause severe symptoms in the CNS; acquired from ingestion of raw or poorly cooked pork; usually only single worm/patient; occasionally two to three proglottids (hooked together) are passed; proglottids can be injected with India ink to show uterine branches for identification; cysticerci are normally small and contained within enclosing membrane; occasionally they may develop as racemose type, where worm tissue grows in the body like a metastatic cancer.</td>
</tr>
<tr>
<td><em>Diphyllobothrium latum</em> (broad fish tapeworm)</td>
<td>Scolex (lateral sucking grooves), gravid proglottid (wider than long, reproductive structures in center “rosette”); eggs operculated</td>
<td>Can cause GI complaints in some individuals; acquired from ingestion of raw or poorly cooked freshwater fish; life cycle has two intermediate hosts (copepod, fish); worm may be 30 ft long; associated with vitamin B₁₂ deficiency in genetically susceptible groups (Scandinavians)</td>
</tr>
<tr>
<td><em>Hymenolepis nana</em> (dwarf tapeworm)</td>
<td>Adult worm not normally seen; egg round to oval, thin shell, containing six-hooked embryo or oncosphere with polar filaments lying between embryo and egg shell</td>
<td>Can cause GI complaints in some individuals; acquired from ingestion of eggs (only life cycle where the intermediate host, the grain beetle, can be bypassed); life cycle of egg to larval form to adult can be completed in human; most common tapeworm in the world</td>
</tr>
<tr>
<td><em>Hymenolepis diminuta</em> (rat tapeworm)</td>
<td>Adult worm not normally seen; egg round to oval, thin shell, containing six-hooked embryo or oncosphere with no polar filaments lying between embryo and egg shell</td>
<td>Uncommon; egg can be confused with that of <em>H. nana</em>; eggs will be submitted in proficiency testing specimens and must be differentiated from those of <em>H. nana</em>.</td>
</tr>
<tr>
<td><em>Echinococcus granulosus</em> (pathogenic)</td>
<td>Adult worm found only in the carnivore (dog); hydatid cysts develop (primarily in liver) when humans accidentally ingest eggs from dog tapeworms; cyst contains daughter cysts and many scolices; examine fluid aspirated from cyst at surgery.</td>
<td>Humans are accidental intermediate hosts; normal life cycle is in sheep and dog, with hydatid cysts developing in liver, lung, etc., of sheep. Human may be unaware of infection unless fluid leaks from cyst (can trigger an anaphylactic reaction) or pain is felt from cyst location.</td>
</tr>
<tr>
<td><em>Echinococcus multilocularis</em> (pathogenic)</td>
<td>Adult worm found only in the carnivore (fox, wolf); hydatid cysts develop (primarily in liver) when humans accidentally ingest eggs from carnivore tapeworms; cyst grows like a metastatic cancer with no limiting membrane.</td>
<td>Humans are accidental intermediate hosts; prognosis poor; surgical removal of tapeworm tissue very difficult; found in Canada, Alaska, and, less frequently, in the northern United States, although it is becoming more common in the United States, where the geographic range is moving further south</td>
</tr>
<tr>
<td><strong>Trematodes (flukes)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Fasciolopsis buski</em> (giant intestinal fluke)</td>
<td>Eggs found in stool; very large and operculated (morphology like that of <em>Fasciola hepatica</em> eggs)</td>
<td>Symptoms depend on worm burden; acquired from ingestion of plant material (water chestnuts) on which metacercariae have encysted; worms hermaphroditic</td>
</tr>
<tr>
<td><em>Fasciola hepatica</em> (sheep liver fluke)</td>
<td>Eggs found in stool; cannot be differentiated from those of <em>F. buski</em></td>
<td>Symptoms depend on worm burden; acquired from ingestion of plant material (watercress) on which metacercariae have encysted; worms hermaphroditic</td>
</tr>
<tr>
<td><em>Opisthorchis (Clonorchis) sinensis</em> (Chinese liver fluke)</td>
<td>Eggs found in stool; very small (&lt;35 μm); operculated, with shoulders into which operculum fits</td>
<td>Symptoms depend on worm burden; acquired from ingestion of raw fish; eggs can be missed unless 400× power is used for examination; eggs can resemble those of <em>Metagonimus yokogawai</em> and <em>Heterophyes heterophyes</em> (small intestinal flukes); worms hermaphroditic</td>
</tr>
<tr>
<td><em>Paragonimus westernmani</em> (lung flukes)</td>
<td>Eggs coughed up in sputum (brownish “iron filings” = egg packets); can be recovered in sputum or stool (if swallowed); operculated, with shoulders into which operculum fits</td>
<td>Symptoms depend on worm burden and egg deposition; acquired from ingestion of raw crabs; eggs can be confused with those of <em>D. latum</em>; infections seen in Orient; infections with <em>Paragonimus mexicanus</em> found in Central and South America; worms hermaphroditic but often cross-fertilize with another worm if present</td>
</tr>
</tbody>
</table>
Table 9.10.2–A7 (continued)

<table>
<thead>
<tr>
<th>Helminth</th>
<th>Diagnostic stage</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trematodes (flukes)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(continued)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Schistosoma mansoni (blood fluke) (pathogenic)</td>
<td>Eggs recovered in stool (large lateral spine); collect specimens with no preservatives (to indicate egg viability); worms in veins of large intestine</td>
<td>Acquired from skin penetration of single cercariae from freshwater snail; pathology caused by body’s immune response to eggs in tissues; adult worms in veins cause no problems; adult worms are separate sexes.</td>
</tr>
<tr>
<td>Schistosoma haematobium (blood fluke) (pathogenic)</td>
<td>Eggs recovered in urine (large terminal spine); collect specimens with no preservatives (to indicate egg viability); worms in veins of bladder</td>
<td>Acquired from skin penetration of single cercariae from freshwater snail; pathology as with S. mansoni; collect 24-h and spot urine samples; chronic infection associated bladder cancer; adult worms are separate sexes.</td>
</tr>
<tr>
<td>Schistosoma japonicum (blood fluke) (pathogenic)</td>
<td>Eggs recovered in stool (very small lateral spine); collect specimens with no preservatives (to indicate egg viability); worms in veins of small intestine</td>
<td>Acquired from skin penetration of multiple cercariae from freshwater snail; pathology as with S. mansoni; infection usually most severe of the three because of original-loading infective dose of cercariae from freshwater snail (multiple cercariae stick together); pathology associated with egg production, which is greatest in S. japonicum infections</td>
</tr>
</tbody>
</table>

* GI, gastrointestinal; CNS, central nervous system.
### Table 9.10.2–A8 Parasites found in blood: characteristics

<table>
<thead>
<tr>
<th>Protozoa</th>
<th>Diagnostic stage</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Malaria</strong></td>
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<tr>
<td><em>Plasmodium vivax</em> (benign tertian malaria)</td>
<td>Ameboid rings; Schüffner’s dots, beginning in older rings (appear later than those in <em>P. ovale</em>); all stages seen in peripheral blood; mature schizont contains 16–18 merozoites.</td>
<td>Infects young cells; 48-h cycle; large geographic range; tends to have true relapse from residual liver stages, enlarged RBCs</td>
</tr>
<tr>
<td><em>Plasmodium ovale</em> (ovale malaria)</td>
<td>Nonameboid rings; Schüffner’s dots, beginning in young rings (appear earlier than those in <em>P. vivax</em>); all stages seen in peripheral blood; mature schizont contains 8–10 merozoites; RBCs may be oval and have fimbriated edges.</td>
<td>Infects young cells; 48-h cycle; narrow geographic range; tends to have true relapse from residual liver stages, enlarged RBCs</td>
</tr>
<tr>
<td><em>Plasmodium malariae</em> (quartan malaria)</td>
<td>Thick rings; no stippling; all stages seen in peripheral blood; “band forms” and “rosette”-shaped mature schizont; lots of malarial pigment</td>
<td>Infects old cells; 72-h cycle; narrow geographic range; associated with recrudescence and nephrotic syndrome, no true relapse, normal or small RBCs</td>
</tr>
<tr>
<td><em>Plasmodium falciparum</em> (malignant tertian malaria)</td>
<td>Multiple rings; appliqué-accolé forms; no stippling (rare Mauer’s clefts); rings and crescent-shaped gametocytes seen in peripheral blood (no other developing stages; rare exception, mature schizont)</td>
<td>Tick-borne infection associated with Nantucket Island; infection mimics malaria; ring forms more pleomorphic than those in malaria; more rings/cell (usually) than in malaria; endemic in several areas within the United States; organisms occasionally seen outside RBCs (unlike malaria merozoites)</td>
</tr>
<tr>
<td><strong>Babesia spp.</strong></td>
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<td></td>
<td>Ring forms only (resemble <em>P. falciparum</em> rings); seen in splenectomized patients; endemic in United States (no travel history necessary); if present, “Maltese cross” configuration diagnostic, but it is not always seen</td>
<td></td>
</tr>
<tr>
<td><strong>Trypanosomes</strong></td>
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<tr>
<td><em>Trypanosoma brucei gambiense</em> (West African sleeping sickness)</td>
<td>Trypomastigotes long and slender, with typical undulating membrane; lymph nodes and blood can be sampled; microhematocrit tube concentration helpful; examine spinal fluid in later stages of infection.</td>
<td>Tsetse fly vector; tends to be chronic infection, exhibiting real symptoms of sleeping sickness</td>
</tr>
<tr>
<td><em>Trypanosoma brucei rhodesiense</em> (East African sleeping sickness)</td>
<td>Trypomastigotes long and slender, with typical undulating membrane; lymph nodes or blood can be sampled; microhematocrit tube concentration helpful; examine spinal fluid in later stages of infection.</td>
<td>Tsetse fly vector; tends to be more severe, short-lived infection (particularly in children); patient may expire before progressive symptoms of sleeping sickness appear.</td>
</tr>
<tr>
<td><em>Trypanosoma cruzi</em> (Chagas’ disease; American trypanosomiasis)</td>
<td>Trypomastigotes short, stumpy, often curved in C shape; sample blood early in infection; trypomastigotes enter striated muscle (heart, GI tract) and transform into amastigote form.</td>
<td>Reduviid bug (“kissing bug”) vector; chronic in adults, severe in young children; great morbidity associated with cardiac failure and loss of muscle contractility in heart and GI tract</td>
</tr>
<tr>
<td><em>Leishmania</em> spp. (cutaneous) (not actually a blood parasite but presented for comparison with <em>L. donovani</em>)</td>
<td>Amastigotes found in skin macrophages; intracellular forms containing nucleus and kinetoplast are diagnostic.</td>
<td>Not actually blood parasite but presented for comparison with <em>Leishmania donovani</em>; sand fly vector; organisms recovered from site of lesion only; stain specimens or culture in NNN and/or Schneider’s medium; animal inoculation (hamster) rarely used</td>
</tr>
<tr>
<td><em>Leishmania braziliensis</em> (mucocutaneous) (not actually a blood parasite but presented for comparison with <em>L. donovani</em>)</td>
<td>Amastigotes found in macrophages of skin and mucous membranes; intracellular forms containing nucleus and kinetoplast are diagnostic.</td>
<td>Not actually blood parasite but presented for comparison with <em>L. donovani</em>; sand fly vector; organisms recovered from site of lesion only; stain specimens or culture in NNN and/or Schneider’s medium; animal inoculation (hamster) rarely used</td>
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<tr>
<td>Protozoa</td>
<td>Diagnostic stage</td>
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<td><strong>Trypanosomes (continued)</strong></td>
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<tr>
<td><em>Leishmania donovani</em> (visceral)</td>
<td>Amastigotes found throughout reticuloendothelial system; spleen, liver, bone marrow, etc.; intracellular forms containing nucleus and kinetoplast are diagnostic.</td>
<td>Sand fly vector; organisms recovered from buffy coat (rarely found), bone marrow aspirate, spleen or liver puncture (rarely performed); stain specimens or culture in NNN and/or Schneider’s medium; animal inoculation (hamster) rarely used; cause of kala-azar</td>
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<td><strong>Helminths</strong></td>
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<tr>
<td><em>Wuchereria bancrofti</em></td>
<td>Microfilaria sheathed, clear space at end of tail; nocturnal periodicity; elephantiasis seen in chronic infections</td>
<td>Pathogenicity due to adult worms; mosquito vector; microfilariae recovered in blood (membrane filtration, Knott concentrate, thick films); hematoxylin stains sheath; sheath does not stain well with Giemsa, but the Innerkörper (inner body) stains a pink color</td>
</tr>
<tr>
<td><em>Brugia malayi</em></td>
<td>Microfilaria sheathed, subterminal and terminal nuclei at end of tail; nocturnal periodicity; elephantiasis seen in chronic infections</td>
<td>Pathogenicity due to adult worms; mosquito vector; microfilariae recovered in blood (membrane filtration, Knott concentrate, thick films); hematoxylin stains sheath; sheath tends to stain pink with Giemsa</td>
</tr>
<tr>
<td><em>Loa loa</em> (African eye worm)</td>
<td>Microfilaria sheathed, nuclei continuous to tip of tail; diurnal periodicity; adult worm may cross conjunctiva of the eye.</td>
<td>Pathogenicity due to adult worms; mango fly vector; history of Calabar swellings, worms in the eye; microfilariae difficult to recover from blood; hematoxylin stains sheath</td>
</tr>
<tr>
<td><em>Mansonella</em> spp.</td>
<td>Microfilaria unsheathed, nuclei may or may not extend to tip of tail (depending on species); nonperiodic; symptoms usually absent or mild</td>
<td>Pathogenicity mild and due to adult worms; midge or blackfly vector; microfilariae recovered in blood (membrane filtration, Knott concentrate, thick films)</td>
</tr>
<tr>
<td><em>Mansonella streptocerca</em></td>
<td>Microfilaria unsheathed, nuclei extend to tip of tail; when immobile, curved like shepherd’s crook; adults in dermal tissues</td>
<td>Pathogenicity mild and due to adult worms and/or microfilariae; midge vector; microfilariae found in skin snips; microfilarial tails split rather than blunt</td>
</tr>
<tr>
<td><em>Onchocerca volvulus</em></td>
<td>Microfilaria unsheathed, nuclei do not extend to tip of tail; adults in nodules</td>
<td>Pathogenicity due to microfilariae; blackfly vector; microfilariae found in skin snips; microfilariae migrate to optic nerve; cause of river blindness</td>
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</table>

* GI, gastrointestinal.
### Table 9.10.2–A9 Parasitic infections: clinical findings in healthy and compromised hosts

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<th>Organism(s)</th>
<th>Clinical findings in:</th>
<th>Compromised host*</th>
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<tr>
<td><strong>Entamoeba histolytica</strong></td>
<td>Asymptomatic to chronic-acute colitis; extraintestinal disease may occur (primary site: right upper lobe of liver).</td>
<td>Diminished immune capacity may lead to extraintestinal disease.</td>
</tr>
<tr>
<td>Free-living amebae</td>
<td>Patients tend to have eye infections with <em>Acanthamoeba</em> spp.; linked to poor eye care</td>
<td>Primary amebic meningoencephalitis caused by <em>Naegleria fowleri</em>, granulomatous amebic encephalitis caused by <em>Acanthamoeba</em> spp. and <em>Balamuthia</em> sp.; severe cutaneous infections in compromised patients (<em>Acanthamoeba</em> spp.)</td>
</tr>
<tr>
<td><em>Giardia lamblia</em></td>
<td>Asymptomatic to malabsorption syndrome</td>
<td>Certain immunodeficiencies tend to predispose an individual to infection.</td>
</tr>
<tr>
<td><em>Blastocystis hominis</em></td>
<td>Asymptomatic to symptomatic, often depending on number of organisms present</td>
<td>Not enough information to tell whether actual differences occur in compromised patients; infections may be more difficult to eradicate in AIDS patients</td>
</tr>
<tr>
<td><em>Toxoplasma gondii</em></td>
<td>Approximately 50% of individuals have anti-body and organisms in tissue but are asymptomatic.</td>
<td>Disease in compromised host tends to involve CNS, with various neurological symptoms.</td>
</tr>
<tr>
<td><em>Pneumocystis carinii</em></td>
<td>Most individuals are probably carriers but asymptomatic.</td>
<td>Disease state develops as pneumonia.</td>
</tr>
<tr>
<td><em>Cryptosporidium parvum</em></td>
<td>Self-limiting infection with diarrhea and abdominal pain</td>
<td>Due to autoinfective nature of life cycle, will not be self-limiting, may produce fluid loss of over 10 liters/day, and may show multisystem involvement; no known totally effective therapy</td>
</tr>
<tr>
<td><em>Cyclospora cayetanensis</em></td>
<td>Self-limiting infection with diarrhea (3–4 days), with relapses common</td>
<td>Diarrhea may persist for 12 wk or more; biliary disease has also been reported for this group, particularly for those with AIDS.</td>
</tr>
<tr>
<td><em>Isospora belli</em></td>
<td>Self-limiting infection with mild diarrhea or no symptoms</td>
<td>May lead to severe diarrhea, abdominal pain, and possible death (rare case reports); diagnosis may occasionally be missed due to nonrecognition of oocyst stage; will not be seen when concentrated from PVA fixative</td>
</tr>
<tr>
<td><em>Sarcocystis</em> spp.</td>
<td>Self-limiting infection with diarrhea or mild symptoms</td>
<td>Symptoms may be more severe and last longer.</td>
</tr>
<tr>
<td>Microsporidia (<em>Nosema, Brachiola, Vittaforma, Encephalitozoon, Septata, Enterocytozoon, Pleistophora, Trachipleistophora, “Microsporidium”</em> spp.)</td>
<td>Less known about these infections in the healthy host; serologic evidence suggests that infections may be more common than recognized.</td>
<td>Can infect various parts of the body; diagnosis often depends on histologic examination of tissues; routine examination of clinical specimens (stool, urine, etc.) is becoming more common; organisms can cause death.</td>
</tr>
<tr>
<td><em>Leishmania</em> spp.</td>
<td>Asymptomatic to mild disease</td>
<td>More serious manifestations of visceral leishmaniasis; some cutaneous species will manifest visceral disease; difficult to treat and manage; definite coinfection with AIDS</td>
</tr>
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Table 9.10.2–A9 (continued)

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<th>Organism(s)</th>
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</thead>
<tbody>
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<td></td>
<td>Healthy host</td>
</tr>
<tr>
<td>Strongyloides stercoralis</td>
<td>Asymptomatic to mild abdominal complaints; can remain latent for many years due to low-level infection maintained by internal autoinfective life cycle</td>
</tr>
<tr>
<td>Crusted (Norwegian) scabies (Sarcoptes scabiei)</td>
<td>Infections can range from asymptomatic to causing moderate itching.</td>
</tr>
<tr>
<td></td>
<td>Compromised host*</td>
</tr>
<tr>
<td></td>
<td>Can result in disseminated disease (hyperinfection syndrome due to autoinfective nature of life cycle); abdominal pain, pneumonitis, sepsis-meningitis with gram-negative bacilli, eosinophilia</td>
</tr>
<tr>
<td></td>
<td>Severe infection with reduced itching response; hundreds of thousands of mites on body; infection very easily transferred to others; secondary infection very common</td>
</tr>
</tbody>
</table>

* CNS, central nervous system; PVA, polyvinyl alcohol; EM, electron microscopy.
Appendix 9.10.3–1—Common Problems in Organism Identification

Figure 9.10.3–A1  (a) Entamoeba histolytica/E. dispar trophozoite. Note the evenly arranged nuclear chromatin, central compact karyosome, and relatively “clean” cytoplasm. (b) Entamoeba coli trophozoite. Note the unevenly arranged nuclear chromatin, eccentric karyosome, and “messy” cytoplasm. These characteristics are very representative of the two organisms. (Illustration by Sharon Belkin.)

Figure 9.10.3–A2  (a) Entamoeba histolytica/E. dispar trophozoite. Note the evenly arranged nuclear chromatin, central compact karyosome and “clean” cytoplasm. (b) Entamoeba coli trophozoite. Note that the nuclear chromatin appears to be evenly arranged, the karyosome is central (but more diffuse), and the cytoplasm is “messy,” with numerous vacuoles and ingested debris. The nuclei of these two organisms tend to resemble one another (very common finding in routine clinical specimens). (Illustration by Sharon Belkin.)
Appendix 9.10.3–1—Common Problems in Organism Identification

9.10.3.2

Figure 9.10.3–A3 (a) Entamoeba histolytica/E. dispar trophozoite. Again, note the typical morphology (evenly arranged nuclear chromatin, central compact karyosome, and relatively “clean” cytoplasm). (b) Entamoeba coli trophozoite. Although the nuclear chromatin is eccentric, note that the karyosome seems to be compact and central. However, note the various vacuoles containing ingested debris. These organisms show some characteristics that are very similar (very typical in clinical specimens). (Illustration by Sharon Belkin).

Figure 9.10.3–A4 (a) Entamoeba histolytica trophozoite. Note the evenly arranged nuclear chromatin, central compact karyosome, and RBCs in the cytoplasm. (b) Human macrophage. The key difference between the macrophage nucleus and that of E. histolytica is the size. Usually, the ratio of nucleus to cytoplasm in a macrophage is approximately 1:6 or 1:8, while the true organism has a nucleus/cytoplasm ratio of approximately 1:10 or 1:12. The macrophage also contains ingested RBCs. In cases of diarrhea or dysentery, trophozoites of E. histolytica and macrophages can often be confused, occasionally leading to a false-positive diagnosis of amebiasis when no parasites are present. Both the actual trophozoite and the macrophage may also be seen without ingested RBCs, and they can mimic one another. (Illustration by Sharon Belkin.)
9.10.3.3 Parasitology

Figure 9.10.3–A5 (a) *Entamoeba histolytica/E. dispar* precyst. Note the enlarged nucleus (prior to division) with evenly arranged nuclear chromatin and central compact karyosome. Chromatoidal bars (rounded ends, with smooth edges) are also present in the cytoplasm. (b) PMN. The nucleus is somewhat lobed (normal morphology) and represents a PMN that has not been in the gut very long. Occasionally, the positioning of the chromatoidal bars and the lobed nucleus of the PMN can mimic one another. The chromatoidal bars will stain more intensely, but shapes can overlap, as seen here. (Illustration by Sharon Belkin.)

Figure 9.10.3–A6 (a) *Entamoeba histolytica/E. dispar* cyst. Note that the four nuclei are very consistent in size and shape. (b) PMN. Note that the normal lobed nucleus has now broken into four fragments, which mimic four nuclei with peripheral chromatin and central karyosomes. When PMNs have been in the gut for some time and have begun to disintegrate, the nuclear morphology can mimic that seen in an *E. histolytica/E. dispar* cyst. However, human cells are often seen in the stool in cases of diarrhea; with rapid passage of the gastrointestinal tract contents, there will not be time for amebic cysts to form. Therefore, in cases of diarrhea and/or dysentery, if “organisms” are seen that resemble the cell in panel b, think first of PMNs, not *E. histolytica/E. dispar* cysts. (Illustration by Sharon Belkin.)

Figure 9.10.3–A7 (a) *Endolimax nana* trophozoite. This organism is characterized by a large karyosome with no peripheral chromatin, although there are normally many nuclear variations seen in any positive specimen. (b) *Dientamoeba fragilis* trophozoite. Normally, the nuclear chromatin is fragmented into several dots (often a “tetrad” arrangement). The cytoplasm is normally more “junky” than that seen in *E. nana*. If the morphology is typical, as in these two illustrations, then differentiating between these two organisms is not that difficult. However, the morphologies of the two will often be very similar. (Illustration by Sharon Belkin.)
Appendix 9.10.3–1—Common Problems in Organism Identification

9.10.3.4

Figure 9.10.3–A8 (a) Endolimax nana trophozoite. Notice that the karyosome is large and surrounded by a “halo,” with very little, if any, chromatin on the nuclear membrane. (b) Dientamoeba fragilis trophozoite. In this organism, the karyosome is beginning to fragment, and there is a slight clearing in the center of the nuclear chromatin. If the nuclear chromatin has not become fragmented, D. fragilis trophozoites can very easily mimic E. nana trophozoites. This could lead to a report indicating that no pathogens were present, when, in fact, D. fragilis is considered a definite cause of symptoms. (Illustration by Sharon Belkin.)

Figure 9.10.3–A9 (a) Endolimax nana trophozoite. Note the large karyosome surrounded by a clear space. The cytoplasm is relatively “clean.” (b) Iodamoeba bütschlii. Although the karyosome is similar to that of E. nana, note that the cytoplasm in I. bütschlii is much more heavily vacuolated and contains ingested debris. Often, these two trophozoites cannot be differentiated. However, the differences in the cytoplasm are often helpful. There will be a definite size overlap between the two genera. (Illustration by Sharon Belkin.)
Figure 9.10.3–A10 (a) RBCs on a stained fecal smear. Note that the cells are very pleomorphic but tend to be positioned in the direction the stool was spread onto the slide. (b) Yeast cells on a stained fecal smear. These cells tend to remain oval and are not aligned in any particular way on the smear. These differences are important when the differential identification is between Entamoeba histolytica containing RBCs and Entamoeba coli containing ingested yeast cells. If RBCs or yeast cells are identified in the cytoplasm of an organism, they must also be visible in the background of the stained fecal smear. (Illustration by Sharon Belkin.)

Figure 9.10.3–A11 (a) Entamoeba histolytica/E. dispar cyst. Note the shrinkage due to dehydrating agents in the staining process. (b) E. histolytica/E. dispar cyst. In this case, the cyst exhibits no shrinkage. Only three of the four nuclei are in focus. Normally, this type of shrinkage is seen with protozoan cysts and is particularly important when a species is measured and identified as either E. histolytica/E. dispar or Entamoeba hartmanni. The whole area, including the halo, must be measured prior to species identification. If just the cyst is measured, the organism would be identified as E. hartmanni (nonpathogenic) rather than E. histolytica/E. dispar (possibly pathogenic). (Illustration by Sharon Belkin.)
Figure 9.10.3–A12 (a) *Plasmodium falciparum* rings. Note the two rings in the RBC. Multiple rings per cell are more typical of *P. falciparum* than of the other species of human malaria. (b) *Babesia* rings. In one of the RBCs are four small *Babesia* rings. This particular arrangement is called the Maltese cross and is diagnostic for *Babesia* spp. However, the Maltese cross configuration is not always present. *Babesia* infections can be confused with cases of *P. falciparum* malaria, primarily because multiple rings can be seen in the RBCs. Another difference involves ring morphology. *Babesia* rings are often of various sizes and tend to be very pleomorphic, while those of *P. falciparum* tend to be more consistent in size and shape. (Illustration by Sharon Belkin.)

Figure 9.10.3–A13 (a) *Strongyloides stercoralis* rhabditiform larva. Note the short buccal capsule (mouth opening) and the internal structure, including the genital primordial packet of cells. (b) Root hair (plant material). Note that there is no specific internal structure and the end is ragged (where it was broken off from the main plant). Often plant material mimics some of the human parasites. This comparison is one of the best examples. These artifacts are occasionally submitted as proficiency testing specimens. (Illustration by Sharon Belkin.)
Figure 9.10.3–A14  (a) *Taenia* egg. This egg has been described as having a thick, radially striated shell containing a six-hooked embryo (oncosphere). (b) Pollen grain. Note that this trilobed pollen grain has a similar type of “shell” and, if turned the right way, could resemble a *Taenia* egg. This represents another confusion between a helminth egg and a plant material artifact. When examining fecal specimens in a wet preparation, tap on the coverslip to get objects to move around. As they move, you can see more morphological detail. (Illustration by Sharon Belkin.)

Figure 9.10.3–A15  (a) *Trichuris trichiura* egg. This egg is typical and is characterized by the barrel shape with thick shell and two polar plugs. (b) Bee pollen. This artifact certainly mimics the actual *T. trichiura* egg. However, note that the actual shape is somewhat distorted. This is an excellent example of a parasite “look-alike” and could be confusing. (Illustration by Sharon Belkin.)
Appendix 9.10.4–1—Quality Control Recording Sheets

Forms on pages 9.10.4.2 to 9.10.4.5 are for use in QC testing of cultures and reagents.
### DIAGNOSTIC PARASITOLOGY QC (REAGENTS)—EXAMPLE

1. **Reagent Name:**

2. **QC Requirements (frequency):**

3. **Acceptable Criteria:**
   - **Negative Control:**
   - **Positive Control:**

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**Corrective Action**

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**Corrective Action**

**Comments:**

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1\(^{A}A = \text{Acceptable, NA = Not Acceptable}\)
## DIAGNOSTIC PARASITOLOGY QC (REAGENTS)

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Manufacturer/Product:____________________

QC Frequency:____________________

Acceptable (A):____________________

Not Acceptable (NA):____________________
1. Patient’s Name/Organism trying to isolate: ________________________________

2. Medium Number 1: ____________________________________________________
   Medium Number 2: _____________________________________________________

3. ATCC Control Strain (Indicate Organism Name and ATCC Number): _________

4. Frequency: QC strain must be set up every time patient specimen is cultured (there are no exceptions to this requirement):

   **Medium 1:**

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<th>Date</th>
<th>Medium Lot No.</th>
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<th>A (^1)</th>
<th>NA (^2)</th>
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<th>Comments (^3)</th>
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   Comments: ________________________________________________________________

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   **Medium 2:**

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</table>

   Comments: ________________________________________________________________

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\(^1\) Acceptable: QC organism growth or motile trophozoites can be detected microscopically in sample of QC medium (inoculated with ATCC strain).

\(^2\) Not Acceptable: QC organism growth or motile trophozoites cannot be detected microscopically in sample of QC medium (inoculated with ATCC strain).

\(^3\) Follow-up: in the event the QC culture is negative, the control strain should be resubbed to fresh media.
EQUIPMENT MAINTENANCE

Equipment Description: ____________________________________________
(Include Inventory Number)

Serial Number: ___________ Model Number: ___________ Purchase Date: ___________

Equipment Location: ____________________________________________

Maintenance Requirements and Time Frames:
(List specific requirements, time frames below - use table for recording actual maintenance.)

1. ____________________________________________
2. ____________________________________________
3. ____________________________________________
4. ____________________________________________

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Remember to attach all relevant paperwork to this sheet (repair invoices, replacement part invoices, etc.).
Appendix 9.10.5–1—Flowcharts for Diagnostic Procedures

Figure 9.10.5–A1 Procedure for processing fresh stool for the ova and parasite examination. Special stains will be necessary for Cryptosporidium and Cyclospora (modified acid-fast) and the microsporidia (modified trichrome, calcofluor). Immunoassay kits are now available for some of these organisms. If the permanent staining method (iron hematoxylin) contains a carbol fuchsin step, the coccidia will stain pink. Symbols: †, some protozoa may not be identified using the wet examination only; ‡, protozoa (primarily trophozoites) can be identified and cysts can be confirmed.

Figure 9.10.5–A2 Procedure for processing liquid specimens for the ova and parasite examination. Polyvinyl alcohol (PVA) and specimen will be mixed together on the slide, allowed to air dry, and then stained (fixation is sufficient for liquid specimen but not for formed stool). Symbols: †, some protozoa may not be identified from the concentration procedure; ‡, protozoa (trophozoites can be identified).
Appendix 9.10.5–1—Flowcharts for Diagnostic Procedures

9.10.5.2

**Figure 9.10.5–A3** Procedure for processing preserved stool for the ova and parasite examination. Fixatives and effects: mercuric chloride, best polyvinyl alcohol (PVA) (trichrome, iron hematoxylin); zinc, current best substitute (trichrome, hematoxylin probably okay); copper sulfate, fair substitute (trichrome, iron hematoxylin: both fair to poor); sodium acetate-acetic acid-formalin, good substitute for PVA fixative (iron hematoxylin is best, and trichrome is okay).

**Figure 9.10.5–A4** Procedure for processing sodium acetate-acetic acid-formalin (SAF)-preserved stool for the ova and parasite examination. SAF can also be used with EIA, fluorescent-antibody, and cartridge immunoassay kits and the modified trichrome stain for microsporidia.
Figure 9.10.5–A5 Use of various fixatives and their recommended stains. PVA, polyvinyl alcohol; SAF, sodium acetate-acetic acid-formalin. Five or 10% formalin, SAF, and some of the single-vial systems can also be used with immunoassay kits and the modified trichrome stains for microsporidia.
9.10.6 Appendix 9.10.6–1—Commercial Supplies and Suppliers

[Updated March 2007]

See Tables 9.10.6–A1 to 9.10.6–A7 on the following pages. (Company names may not reflect recent mergers and subsequent name changes.)
Table 9.10.6–A1 Sources of commercial reagents and supplies

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<td>PVA fixative solution</td>
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<td>Zinc sulfate (sp.gr. 1.18 or 1.20)</td>
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<td>Giemsa, solution</td>
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<td>Giemsa, powder</td>
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<td>Modified acid fast with DMSO</td>
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<td>Lugol’s iodine, diluted 1:5</td>
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<td>Dobell &amp; O’Connor’s iodine</td>
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* PVA, polyvinyl alcohol; MIF, merthiolate-iodine-formalin; SAF, sodium acetate-acetic acid-formalin; W, Wheatley; G, Gomori; DMSO, dimethyl sulfoxide; +, with acetic acid; −, without acetic acid.

a Use grade with high hydrolysis and low viscosity for parasite studies.

b Used for the identification of microsporidial spores in stool or other specimens.
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### Table 9.10.6–A2
Addresses of suppliers listed in Table 9.10.6–A1

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<td><strong>AJP Scientific, Inc.</strong></td>
<td>P.O. Box 1589, Clifton, NJ 07015</td>
<td>(800) 922-0223, (201) 472-7200</td>
<td></td>
<td></td>
<td>Products also available through various distributors</td>
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<tr>
<td><strong>ALPHA-TEC Systems, Inc.</strong></td>
<td>P.O. Box 5435, Vancouver, WA 98668</td>
<td>(800) 221-6058, (360) 260-2779</td>
<td>Fax: (360) 260-3277</td>
<td><a href="http://www.corptech.com">http://www.corptech.com</a></td>
<td>Products also available through various distributors</td>
</tr>
<tr>
<td><strong>J. T. Baker</strong></td>
<td>222 Red School Ln., Phillipsburg, NJ 08865</td>
<td>(908) 859-2151, Fax: (908) 859-9385</td>
<td></td>
<td><a href="http://www.jtbaker.com">http://www.jtbaker.com</a></td>
<td>Products also available through American Scientific Products or VWR</td>
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<tr>
<td><strong>A. B. Becker</strong></td>
<td>21124 Malmö, Sweden</td>
<td></td>
<td></td>
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<td>Products available through American Scientific Products</td>
</tr>
<tr>
<td><strong>Bio-Spec, Inc.</strong></td>
<td>179 Mason Circle, Suite A, Concord, CA 94520-1213</td>
<td>(415) 689-0771</td>
<td></td>
<td><a href="http://www.jtbaker.com">http://www.jtbaker.com</a></td>
<td>Products also available through American Scientific Products or VWR</td>
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<tr>
<td><strong>City Chemical Corp.</strong></td>
<td>32 West 22nd St., New York, NY 10011</td>
<td>(212) 929-2723</td>
<td></td>
<td></td>
<td>Products also available through VWR Scientific</td>
</tr>
<tr>
<td><strong>Evergreen Scientific</strong></td>
<td>2300 East 49th St., P.O. Box 58248, Los Angeles, CA 90058-0248</td>
<td>(800) 421-6261, (800) 372-7300 in California</td>
<td>(213) 583-1331</td>
<td><a href="http://www.evergreensci.com">http://www.evergreensci.com</a></td>
<td>Products available through Amfak Drug or Bergen-Brunswick, Products also available through various distributors</td>
</tr>
<tr>
<td><strong>Fisher Scientific</strong></td>
<td>2761 Walnut Ave., Tustin, CA 92681</td>
<td>(800) 766-7000, Fax: (800) 926-1166</td>
<td></td>
<td><a href="http://www.fisherhamilton.com">http://www.fisherhamilton.com</a></td>
<td>Products also available through regional sales offices</td>
</tr>
<tr>
<td><strong>Hardy Diagnostics</strong></td>
<td>1430 West McCoy Ln., Santa Maria, CA 93455</td>
<td></td>
<td></td>
<td></td>
<td>Products also available through various distributors</td>
</tr>
<tr>
<td><strong>Harleco (New owner: E. Merck Co.)</strong></td>
<td>Darmstadt, West Germany</td>
<td></td>
<td></td>
<td></td>
<td>Products available through American Scientific Products; product numbers remain unchanged</td>
</tr>
<tr>
<td><strong>Medical Chemical Corp.</strong></td>
<td>19430 Van Ness Ave., Torrance, CA 90501</td>
<td>(800) 424-9394</td>
<td>Fax: (310) 787-4464</td>
<td><a href="http://www.med-chem.com">http://www.med-chem.com</a></td>
<td>Products also available through various distributors</td>
</tr>
<tr>
<td><strong>Meridian Bioscience, Inc.</strong></td>
<td>3741 River Hills Dr., Cincinnati, OH 45244</td>
<td>(800) 543-1980, (513) 271-3700</td>
<td>Fax: (513) 271-0124</td>
<td><a href="http://www.meridianbioscience.com">http://www.meridianbioscience.com</a></td>
<td>Products available only through Baxter MicroScan and Scientific Products Division, CMA, and various other regional distributors</td>
</tr>
<tr>
<td><strong>MML Diagnostic Packaging</strong></td>
<td>P.O. Box 458, Troutdale, OR 97060</td>
<td>(503) 666-8398</td>
<td>(800) 826-7186</td>
<td></td>
<td>Products available through various distributors</td>
</tr>
<tr>
<td><strong>PML Microbiologicals</strong></td>
<td>P.O. Box 459, Tualatin, OR 97062</td>
<td>(800) 547-0659, (503) 639-1500 in Oregon</td>
<td>Fax: (800) 765-4415</td>
<td><a href="http://www.pmlmicro.com">http://www.pmlmicro.com</a></td>
<td>Products also available through other distributors</td>
</tr>
<tr>
<td>Company</td>
<td>Contact Information</td>
<td>Notes</td>
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<tr>
<td>Polysciences, Inc.</td>
<td>400 Valley Rd., Warrington, PA 18976</td>
<td>Products available only through company</td>
<td></td>
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<tr>
<td></td>
<td>(800) 523-2575, (215) 343-6484</td>
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<tr>
<td></td>
<td>Fax: (215) 343-0214, <a href="http://www.polysciences.com">http://www.polysciences.com</a></td>
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<tr>
<td>Remel</td>
<td>12076 Santa Fe Dr., Lenexa, KS 66215</td>
<td>Products available through Rupp &amp; Bowman and certain other distributors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(800) 255-6730, Fax: (800) 477-5781, <a href="http://www.remelinc.com">http://www.remelinc.com</a></td>
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<tr>
<td>Rohm &amp; Haas</td>
<td>Philadelphia, PA 19105</td>
<td>Products also available through various distributors</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>(508) 948-2067, Fax: (508) 948-2206, <a href="http://www.scientificdevice.com">http://www.scientificdevice.com</a></td>
<td></td>
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<tr>
<td>Rowley Biochemical Institute</td>
<td>U.S. Route 1, Rowley, MA 01969</td>
<td>Products available only through company</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(508) 948-2067, Fax: (508) 948-2206</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Scientific Device Laboratory, Inc.</td>
<td>411 E. Jarvis Ave., Des Plaines, IL 60018</td>
<td>Products also available through various distributors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(847) 803-9495, Fax: (847) 803-8251, <a href="http://www.scientificdevice.com">http://www.scientificdevice.com</a></td>
<td></td>
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</tr>
<tr>
<td>Volu-Sol, Inc. (a division of Biomune, Inc.)</td>
<td>5095 West 2100 South, Salt Lake City, UT 84120</td>
<td>Products also available through various distributors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(800) 821-2495, (801) 974-9474, Fax: (800) 860-4317</td>
<td>(Laboratory Specialists International, <a href="http://www.labspec.com">http://www.labspec.com</a>)</td>
<td></td>
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</table>

Table 9.10.6-A2 (continued)
**Table 9.10.6–A3** Sources of available reagents for immunodetection of parasitic organisms or antigens

<table>
<thead>
<tr>
<th>Company</th>
<th>Cryptosporidium parvum EIA</th>
<th>C. parvum-Giardia lamblia combination DFA or EIA</th>
<th>Entamoeba histolytica EIA</th>
<th>E. histolytica/E. dispar group EIA</th>
<th>Giardia lamblia EIA</th>
<th>Cryptosporidium-Giardia cartridge</th>
<th>Cryptosporidium-Giardia–E. histolytica/E. dispar group cartridge</th>
<th>Pneumocystis carinii</th>
<th>Trichomonas vaginalis (various methods)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibodies Inc.</td>
<td></td>
<td>×</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>× (Triage parasite panel)</td>
<td>× (DFA)</td>
<td>× (DFA)</td>
</tr>
<tr>
<td>Biosite Diagnostics, Inc.</td>
<td></td>
<td>×</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>× (IFA), control slides also available</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemicon International, Inc.</td>
<td></td>
<td>×</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>× (IFA), control slides also available</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dako</td>
<td></td>
<td>×</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>× (IFA), control slides also available</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genzyme Diagnostics (Becton Dickinson)</td>
<td></td>
<td>×</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>× (IFA), control slides also available</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Integrated Diagnostics</td>
<td></td>
<td>×</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>× (IFA), control slides also available</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meridian Bioscience, Inc.</td>
<td></td>
<td>×</td>
<td>× (DFA)</td>
<td>×</td>
<td>×</td>
<td></td>
<td>× (DFA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medical Chemical Corp.</td>
<td></td>
<td>×</td>
<td>× (DFA)</td>
<td>×</td>
<td>×</td>
<td></td>
<td>× (DFA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MicroProbe Corp. (Becton Dickson)</td>
<td></td>
<td>×</td>
<td>× (DFA)</td>
<td>×</td>
<td>×</td>
<td></td>
<td>× (DNA probe; Afirm VP III)</td>
<td></td>
<td>(Trichomonas, Gardnerella, Candida)</td>
</tr>
<tr>
<td>Polysciences, Inc.</td>
<td></td>
<td>×</td>
<td>× (DFA)</td>
<td>×</td>
<td>×</td>
<td></td>
<td>× (DNA probe; Afirm VP III)</td>
<td></td>
<td>(Trichomonas, Gardnerella, Candida)</td>
</tr>
<tr>
<td>TechLab, Inc.</td>
<td></td>
<td>×</td>
<td>× (DFA)</td>
<td>×</td>
<td>×</td>
<td></td>
<td>× (DNA probe; Afirm VP III)</td>
<td></td>
<td>(Trichomonas, Gardnerella, Candida)</td>
</tr>
<tr>
<td>Wampole</td>
<td></td>
<td>×</td>
<td>× (DFA)</td>
<td>×</td>
<td>×</td>
<td></td>
<td>× (DNA probe; Afirm VP III)</td>
<td></td>
<td>(Trichomonas, Gardnerella, Candida)</td>
</tr>
</tbody>
</table>

*a* Any procedure for the *Entamoeba histolytica/E. dispar* group or *E. histolytica* requires the use of fresh or fresh, frozen stools. All other tests (with the exception of direct fluorescent antibody [DFA] for the Cryptosporidium-Giardia combination) can be used with fresh, frozen, or preserved stools (formalized base, not PVA [some single-vial systems without PVA may be acceptable; consult the manufacturer]). Since the combination Cryptosporidium-Giardia tests (DFA) are based on visual recognition of the fluorescing oocysts and/or cysts, the specimens must not be frozen if fresh stools are used for testing.
Table 9.10.6–A4  Addresses of suppliers listed in Table 9.10.6–A3

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Address</th>
<th>Contact Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibodies, Inc.</td>
<td>P.O. Box 1560, Davis, CA 95617-1560</td>
<td>(800) 824-8540, Fax: (530) 758-6307, E-mail: <a href="mailto:antiinc@aol.com">antiinc@aol.com</a></td>
</tr>
<tr>
<td>Biosite Diagnostics, Inc.</td>
<td>11030 Roselle St., San Diego, CA 92121</td>
<td>(858) 455-4808, Fax: (858) 455-4815, E-mail: <a href="mailto:npadilla@biosite.com">npadilla@biosite.com</a></td>
</tr>
<tr>
<td>Chemicon International, Inc.</td>
<td>28835 Single Oak Dr., Temecula, CA 92590</td>
<td>(800) 437-7500, Fax: (800) 437-7502, E-mail: <a href="mailto:custserv@chemicon.com">custserv@chemicon.com</a>, <a href="mailto:techserv@chemicon.com">techserv@chemicon.com</a></td>
</tr>
<tr>
<td>Dako</td>
<td>6392 Via Real, Carpinteria, CA 93013</td>
<td>(800) 235-5743, Fax: (800) 566-3256, E-mail: <a href="mailto:general@dakousa.com">general@dakousa.com</a>, <a href="http://www.dakousa.com">http://www.dakousa.com</a></td>
</tr>
<tr>
<td>Integrated Diagnostics (PanBio InDx, Inc.)</td>
<td>1756 Sulphur Springs Rd., Baltimore, MD 21227</td>
<td>(410) 737-8500, Fax: (410) 336-1212, E-mail: <a href="mailto:idx2@erols.com">idx2@erols.com</a></td>
</tr>
<tr>
<td>Medical Chemical Corp.</td>
<td>19430 Van Ness Ave., Torrance, CA. 90501</td>
<td>(800) 424-9394, Fax: (310) 787-4464, <a href="http://www.med-chem.com">http://www.med-chem.com</a></td>
</tr>
<tr>
<td>Meridian Bioscience, Inc.</td>
<td>3471 River Hills Dr., Cincinnati, OH 45244</td>
<td>(513) 271-3700, Fax: (513) 271-0124, <a href="http://www.meridianbioscience.com">http://www.meridianbioscience.com</a></td>
</tr>
<tr>
<td>MicroProbe Corp.</td>
<td>(Becton Dickinson Affirm VPIII) 1725 220th St. NE, Bothell, WA 98021</td>
<td>(Trichomonas, Gardnerella, Candida) BD (201) 847-6800, <a href="http://www.bd.com">http://www.bd.com</a></td>
</tr>
<tr>
<td>Polysciences, Inc.</td>
<td>400 Valley Rd., Warrington, PA 18976</td>
<td>(800) 523-2575, Fax: (215) 343-0214, <a href="http://www.polysciences.com">http://www.polysciences.com</a></td>
</tr>
<tr>
<td>Remel</td>
<td>12076 Santa Fe Dr., Lenexa, KS 66215</td>
<td>(800) 255-6730, Fax: (800) 477-5781, <a href="http://remelinc.com">http://remelinc.com</a></td>
</tr>
<tr>
<td>TechLab, Inc.</td>
<td>1861 Pratt Dr., Suite 1030, Corporate Research Center, Blacksburg, VA 24060-6364</td>
<td>(800) 832-4522, Fax: (540) 231-3942, <a href="http://www.techlabinc.com">http://www.techlabinc.com</a></td>
</tr>
<tr>
<td>Wampole Lab</td>
<td>P.O. Box 1001, Cranbury, NJ 08512</td>
<td>(800) 257-9525, Fax: (800) 532-0295, <a href="http://www.wampolelabs.com">http://www.wampolelabs.com</a></td>
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</table>
Table 9.10.6–A5 Commercial suppliers of diagnostic parasitology products

<table>
<thead>
<tr>
<th>Company</th>
<th>Address</th>
<th>Contact Information</th>
<th>Website</th>
<th>Test</th>
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<tbody>
<tr>
<td>Abbott Laboratories</td>
<td>Diagnostics Division</td>
<td>North Chicago, IL 60064</td>
<td>(800) 323-9100</td>
<td>Trypanosoma cruzi (Chagas' disease) EIA, Toxoplasma (EIA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fax: (847) 938-6255</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><a href="http://www.abbott.com">http://www.abbott.com</a></td>
<td></td>
</tr>
<tr>
<td>Acon Laboratories</td>
<td>115 Research Dr.</td>
<td>Bethlehem, PA 18015</td>
<td>(610) 861-6903</td>
<td>Leishmania (EIA)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Fax: (610) 861-6905</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><a href="http://www.aconlab.com">http://www.aconlab.com</a></td>
<td></td>
</tr>
<tr>
<td>ALPHA-TEC Systems, Inc.</td>
<td>P.O. Box 5435</td>
<td>Vancouver, WA 98668</td>
<td>(800) 221-6058</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(360) 260-2779</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Fax: (360) 260-3277</td>
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<td></td>
<td><a href="http://www.corptech.com">http://www.corptech.com</a></td>
<td></td>
</tr>
<tr>
<td>Amrad ICT</td>
<td>13 Rodborough Rd.</td>
<td>French Forest, NSW</td>
<td>(612) 9453-4411</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fax: (612) 9453-4411</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><a href="http://www.amrad.com.au">http://www.amrad.com.au</a></td>
<td></td>
</tr>
<tr>
<td>Antibodies Inc.</td>
<td>P.O. Box 1560</td>
<td>Davis, CA 95617</td>
<td>(800) 824-8540</td>
<td>Antigen detection (rapid or dipstick) for filariasis, malaria, and P. falciparum malaria</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(916) 758-4400 in California</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Fax: (530) 758-6307</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>E-mail: <a href="mailto:antoine@aol.com">antoine@aol.com</a></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Giardia (EIA)</td>
<td></td>
</tr>
<tr>
<td>Bayer</td>
<td>511 Benedict Ave.</td>
<td>Tarrytown, NE</td>
<td>(800) 242-2787</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><a href="http://www.bayer.com">http://www.bayer.com</a></td>
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</table>

Beckman Coulter
200 S. Kranes Blvd.
Brea, CA 92822
Fax: (800) 643-4366
http://www.beckman.com
Toxoplasma (EIA)

Becton Dickinson Advanced Diagnostics
2350 Qume Dr.
San Jose, CA 95131-1087
Fax: (800) 954-2347
http://www.bdfacs.com
Malaria P.f. Parasight F dipstick, QBC acridine orange tube

Bayer
511 Benedict Ave.
Tarrytown, NE
(800) 242-2787
http://www.bayer.com
Toxoplasma (EIA)
<table>
<thead>
<tr>
<th>Company Name</th>
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<th>Phone Numbers</th>
<th>Fax Numbers</th>
<th>Email Addresses</th>
<th>Products</th>
<th>Notes</th>
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<tr>
<td>Biotecx Labs</td>
<td>6023 S. Loop East, Houston, TX 77033</td>
<td>(800) 535-6286</td>
<td>(713) 643-3743</td>
<td></td>
<td>Toxoplasma (EIA)</td>
<td></td>
</tr>
<tr>
<td>Biotools</td>
<td>Av. General Peron, 2 E-28020 Madrid, Spain</td>
<td>(341) 571-1660</td>
<td>(341) 571-1232</td>
<td></td>
<td>Malaria (PCR, DNA)</td>
<td></td>
</tr>
<tr>
<td>Chemicon International, Inc.</td>
<td>28835 Single Oak Dr., Temecula, CA 92590</td>
<td>(800) 437-7500</td>
<td>(800) 437-7502</td>
<td><a href="mailto:custserv@chemicon.com">custserv@chemicon.com</a>, <a href="mailto:techserv@chemicon.com">techserv@chemicon.com</a></td>
<td>Trichomonas vaginalis (DFA) antigen detection</td>
<td></td>
</tr>
<tr>
<td>Dade Behring, Inc.</td>
<td>20400 Mariani Ave., Cupertino, CA 95014</td>
<td>(408) 239-2000</td>
<td></td>
<td></td>
<td></td>
<td>(continued)</td>
</tr>
<tr>
<td>DiaSorin</td>
<td>1990 Industrial Blvd., Stillwater, MN 55082</td>
<td>(800) 328-1482</td>
<td>(612) 779-7847</td>
<td></td>
<td>Toxoplasma (EIA)</td>
<td></td>
</tr>
<tr>
<td>DiaSys Corporation</td>
<td>49 Leavenworth St., Waterbury, CT 06702</td>
<td>(716) 458-4014</td>
<td></td>
<td></td>
<td>Semiautomated stool concentrate examination system</td>
<td></td>
</tr>
<tr>
<td>Empyrean Diagnostics, Inc.</td>
<td>2761 Marine Way, Mountain View, CA 94043</td>
<td>(415) 960-0516</td>
<td>(415) 960-0515</td>
<td></td>
<td>Visual identification and culture system for Trichomonas vaginalis</td>
<td></td>
</tr>
<tr>
<td>Evergreen Scientific</td>
<td>2300 East 49th St., Los Angeles, CA 90058</td>
<td>(800) 421-6261</td>
<td>(213) 583-1331</td>
<td></td>
<td>Collection system, concentration system</td>
<td></td>
</tr>
<tr>
<td>DiaSorin</td>
<td>1990 Industrial Blvd., Stillwater, MN 55082</td>
<td>(800) 328-1482</td>
<td>(612) 779-7847</td>
<td></td>
<td>Toxoplasma (EIA)</td>
<td></td>
</tr>
<tr>
<td>DiaSys Corporation</td>
<td>49 Leavenworth St., Waterbury, CT 06702</td>
<td>(716) 458-4014</td>
<td></td>
<td></td>
<td>Semiautomated stool concentrate examination system</td>
<td></td>
</tr>
<tr>
<td>Empyrean Diagnostics, Inc.</td>
<td>2761 Marine Way, Mountain View, CA 94043</td>
<td>(415) 960-0516</td>
<td>(415) 960-0515</td>
<td></td>
<td>Visual identification and culture system for Trichomonas vaginalis</td>
<td></td>
</tr>
<tr>
<td>Evergreen Scientific</td>
<td>2300 East 49th St., Los Angeles, CA 90058</td>
<td>(800) 421-6261</td>
<td>(213) 583-1331</td>
<td></td>
<td>Collection system, concentration system</td>
<td></td>
</tr>
<tr>
<td>DiaSorin</td>
<td>1990 Industrial Blvd., Stillwater, MN 55082</td>
<td>(800) 328-1482</td>
<td>(612) 779-7847</td>
<td></td>
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<td>(800) 421-6261</td>
<td>(213) 583-1331</td>
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<td>Collection system, concentration system</td>
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(continued)
### Table 9.10.6–A5 Commercial suppliers of diagnostic parasitology productsa (continued)

<table>
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<tr>
<th>Company</th>
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<th>Phone Number</th>
<th>Fax Number</th>
<th>Website</th>
<th>Products</th>
<th>Email Address</th>
<th>Notes</th>
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</thead>
<tbody>
<tr>
<td>Gull Laboratories, Inc.</td>
<td>1011 East Murray Holladay Rd.</td>
<td>(801) 448-4855</td>
<td>(801) 265-9268</td>
<td><a href="http://www.gullabs.com">http://www.gullabs.com</a></td>
<td>Toxoplasma (EIA, IFA), Trypanosoma cruzi (EIA), formalin suspensions (parasites), parasite slides (stained and unstained)</td>
<td>E-mail: <a href="mailto:indx2@erols.com">indx2@erols.com</a></td>
<td></td>
</tr>
<tr>
<td>Hardy Diagnostics</td>
<td>1430 West McCoy Ln.</td>
<td>(800) 266-2222</td>
<td>(801) 346-2760</td>
<td><a href="http://www.hardydiagnostics.com">http://www.hardydiagnostics.com</a></td>
<td>Stains, reagents, collection system, concentration systems, control slides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDC Corp.</td>
<td>2109 O’Toole Ave., Suite M</td>
<td>(408) 954-1909</td>
<td>(408) 954-0340</td>
<td><a href="http://www.hdcorp.com">http://www.hdcorp.com</a></td>
<td>Entero-Test capsules (adult and pediatric) (method of sampling upper gastrointestinal tract)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemagen Diagnostics</td>
<td>34-40 Bear Hill Rd.</td>
<td>(800) 436-2436</td>
<td>(781) 890-3748</td>
<td><a href="http://www.hemagen.com">http://www.hemagen.com</a></td>
<td>Chagas’ disease (EIA), Toxoplasma (EIA, IFA)</td>
<td></td>
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</tr>
<tr>
<td>Immunetics</td>
<td>63 Rogers St.</td>
<td>(617) 492-5416</td>
<td>(617) 868-7879</td>
<td><a href="http://www.immunetics.com">http://www.immunetics.com</a></td>
<td>Babesia (IB),a Chagas’ disease (IB),a cysticercosis (IB),a echinococcosis (IB),a Leishmania (IB)a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>INOVA</td>
<td>10180 Scripps Ranch Rd.</td>
<td>(800) 545-9495</td>
<td>(619) 586-9911</td>
<td><a href="http://www.inovadx.com">http://www.inovadx.com</a></td>
<td>Toxoplasma (EIA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Integrated Diagnostics Inc.</td>
<td>(PanBio InDx, Inc.)</td>
<td>1756 Sulphur Springs Rd.</td>
<td>(410) 737-8500</td>
<td><a href="http://www.indxdi.com">http://www.indxdi.com</a></td>
<td>Trichomonas (LA) antigen detection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interfacial Dynamics Corp.</td>
<td>17300 SW Upper Boones Ferry Rd., Suite 120</td>
<td>(503) 256-0076</td>
<td>(503) 255-0989</td>
<td>E-mail: <a href="mailto:idlatex@teleport.com">idlatex@teleport.com</a></td>
<td>Uniform-sized polystyrene microspheres (can be used to check microscope calibrations)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVD Research Inc.</td>
<td>5909 Sea Lion Place, Suite D</td>
<td>(760) 929-7744</td>
<td>(760) 431-7759</td>
<td><a href="http://www.ivdresearch.com">http://www.ivdresearch.com</a>, <a href="http://www.safepath.com">http://www.safepath.com</a></td>
<td>Amebiasis (EIA), cysticercosis (EIA), echinococcosis (EIA), Toxocara (EIA), trichinosis (EIA), Toxoplasma (EIA), additional antigen and antibody reagents (microbiology and parasitology)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KMI Diagnostics Inc.</td>
<td>818 51st Ave., NE, Suite 101</td>
<td>(612) 572-9354</td>
<td>(612) 586-0748</td>
<td><a href="http://www.kmidiagnostics.com">http://www.kmidiagnostics.com</a></td>
<td>Toxoplasma (IFA)</td>
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</tr>
</tbody>
</table>

a. Additional products may be available from these companies.
<table>
<thead>
<tr>
<th>Company Name</th>
<th>Address</th>
<th>Phone</th>
<th>Fax</th>
<th>Website</th>
<th>Product Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medical Chemical Corp.</td>
<td>19430 Van Ness Ave. Torrance, CA 90501 (800) 424-9394 Fax: (310) 787-4464 <a href="http://www.med-chem.com">http://www.med-chem.com</a></td>
<td>Stains, reagents, collection system, concentration system, <em>Giardia</em>, <em>Cryptosporidium</em> immunoassays (EIA, DFA, rapid cartridge), parasitology website (current information, photographs, commonly asked questions, etc.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meridian Bioscience, Inc.</td>
<td>3741 River Hills Dr. Cincinnati, OH 45244 (800) 543-1980 (513) 271-3700 Fax: (513) 271-0124 <a href="http://www.meridianbioscience.com">http://www.meridianbioscience.com</a></td>
<td>Stains, reagents, collection system, concentration system, <em>Cryptosporidium</em> spp. (EIA, FA), <em>Giardia</em> sp. (EIA, FA), <em>Cryptosporidium/Giardia</em> rapid cartridge</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MicroProbe Corporation</td>
<td>(Becton Dickinson Affirm VP IIII) 1725 220th St. NE Bothell, WA 98021 (201) 847-6800 <a href="http://www.bertec.com.tw">http://www.bertec.com.tw</a></td>
<td>(Trichomonas, Gardnerella, Candida) ColorPAC Rapid (<em>Cryptosporidium/Giardia</em>)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>MML Diagnostic Packaging</td>
<td>P.O. Box 458 Troutdale, OR 97060 (503) 666-8398 (800) 826-7186</td>
<td>Collection system</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PML Microbiologicals</td>
<td>P.O. Box 459 Tualatin, OR 97062 (800) 547-0659 (503) 639-1500 in Oregon Fax: (800) 765-4415 <a href="http://www.pmlmicro.com">http://www.pmlmicro.com</a></td>
<td>Stains, reagents, collection system, concentration system, control slides</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Polysciences, Inc.</td>
<td>400 Valley Rd. Warrington, PA 18976 (800) 523-2575 Fax: (215) 343-0214 <a href="http://www.polysciences.com">http://www.polysciences.com</a></td>
<td><em>Pneumocystis</em> (DFA) antigen detection</td>
<td></td>
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</tr>
<tr>
<td>Remel</td>
<td>12076 Santa Fe Dr. Lenexa, KS 66215 (800) 255-6730 Fax: (800) 477-5781 <a href="http://www.remelinc.com">http://www.remelinc.com</a></td>
<td>Stains, reagents, collection system, concentration system, control slides</td>
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</tr>
<tr>
<td>Sanofi Diagnostics Pasteur</td>
<td>1000 Lake Hazeltine Dr. Chaska, MN 55318 (800) 666-5111 Fax: (612) 368-1110 <em>Toxoplasma</em> (EIA, IgG, IgM) <em>Pneumocystis</em> (IFA)</td>
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</tr>
<tr>
<td>Scientific Device Laboratory, Inc.</td>
<td>411 E. Jarvis Ave. Des Plaines, IL 60018 (847) 803-9495 Fax: (847) 803-8251 <a href="http://www.scientificdevice.com">http://www.scientificdevice.com</a></td>
<td>Stains, stain kit, modified trichrome stain for microsporidia, fixative (formalin free), formalin-fixed protozoa and helminth eggs and larvae, control slides (<em>Cryptosporidium, Isospora, microsporidia, and Pneumocystis</em>), stained slides</td>
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</tr>
<tr>
<td>Shield Diagnostics</td>
<td>The Technology Park Dundee DD2 ISW, United Kingdom 44-1382-561000 Fax: 44-1382-561056 <em>Pneumocystis</em> (DFA) antigen detection, <em>Cryptosporidium</em> (DFA)*</td>
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</tr>
<tr>
<td>Sigma</td>
<td>545 South Ewing Ave. St. Louis, MO 63013 (800) 325-3424 (314) 286-7813 <a href="http://www.sigma.sial.com">http://www.sigma.sial.com</a></td>
<td><em>Amebiasis</em> (EIA), <em>Toxoplasma</em> (EIA)</td>
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<tr>
<td>Stellar Bio Systems</td>
<td>9075 Guilford Rd. Columbia, MD 21046 (301) 381-8550 <em>Toxoplasma</em> (IFA)</td>
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(continued)
### Table 9.10.6–A5 Commercial suppliers of diagnostic parasitology products (continued)

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<tbody>
<tr>
<td>TechLab, Inc.</td>
<td>1861 Pratt Dr., Suite 1030</td>
<td>(800) 832-4522, (540) 231-3942, E-mail: <a href="mailto:techlab@techlabinc.com">techlab@techlabinc.com</a>, <a href="http://www.techlabinc.com">http://www.techlabinc.com</a></td>
<td>EIA antigen detection (amebiasis, <em>Entamoeba histolytica</em>/<em>E. dispar</em> group, <em>Giardia</em>), Parasitology starter kit, stains, reagents, control slides</td>
</tr>
<tr>
<td>Volu-Sol, Inc. (a division of Biomune, Inc.)</td>
<td>5095 West 2100 South, Salt Lake City, UT 84120</td>
<td>(800) 821-2495, (801) 974-9474, Fax: (800) 860-4317, <a href="http://www.labspec.com">http://www.labspec.com</a></td>
<td>Parasitology starter kit, stains, reagents, control slides</td>
</tr>
<tr>
<td>Wampole Lab</td>
<td>P.O. Box 1001, Cranbury, NJ 08512</td>
<td>(800) 257-9525, Fax: (800) 532-0295, <a href="http://www.wampolelabs.com">http://www.wampolelabs.com</a></td>
<td>EIA antigen detection (amebiasis, <em>Entamoeba histolytica</em>/<em>E. dispers</em> group, <em>Giardia</em>)</td>
</tr>
</tbody>
</table>

* Much of the updated immunology testing information provided by Marianna Wilson (CDC). Abbreviations: DFA, direct fluorescent antibody; IB, immunoblot; IFA, indirect fluorescent antibody; IgG and IgM, immunoglobulins G and M; LA, latex agglutination; Rapid, rapid immunochromatographic. Company names may not reflect recent mergers and subsequent name changes.

* Not Food and Drug Administration cleared for in vitro diagnostic use.

* No updated information received since publication of the previous edition of this book.
Table 9.10.6–A6 Sources of parasitologic specimens (catalogs of available materials and price lists available from the companies and person listed)

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<th>Suppliers</th>
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<tr>
<td>Ann Arbor Biological Center</td>
<td>6780 Jackson Rd. Ann Arbor, MI 48103</td>
<td>(800) 334-5551</td>
<td>(919) 584-0381</td>
</tr>
<tr>
<td>Carolina Biological Supply Co.</td>
<td>2700 York Rd. Burlington, NC 27215</td>
<td>(800) 334-5551</td>
<td>(919) 584-3399</td>
</tr>
<tr>
<td>Dako</td>
<td>6392 Via Real Carpinteria, CA 93013</td>
<td>(800) 235-5634</td>
<td>(919) 584-0381</td>
</tr>
<tr>
<td>Meridian Bioscience, Inc.</td>
<td>3471 River Hills Dr. Cincinnati, OH 45244</td>
<td>(800) 543-1980, Ext. 335</td>
<td>(513) 272-5271</td>
</tr>
<tr>
<td>Scientific Device Laboratory, Inc.</td>
<td>411 E. Jarvis Ave. Des Plaines, IL 60018</td>
<td>(800) 448-4855</td>
<td>(708) 803-9495</td>
</tr>
<tr>
<td>Triarch, Inc.</td>
<td>N8028 Union St. Ripon, WI 54971</td>
<td>(414) 748-5125</td>
<td>(414) 748-3034</td>
</tr>
<tr>
<td>Tropical Biologicals</td>
<td>P.O. Box 139 Guaynabo, PR 00657</td>
<td>(800) 848-0810</td>
<td></td>
</tr>
<tr>
<td>TURTOX</td>
<td>P.O. Box 92912 Rochester, NY 14692</td>
<td>(800) 826-6164</td>
<td></td>
</tr>
<tr>
<td>Ward’s Natural Science Establishment, Inc.</td>
<td>P.O. Box 92912 Rochester, NY 14692</td>
<td>(800) 962-2660</td>
<td></td>
</tr>
<tr>
<td><strong>Table 9.10.6–A7</strong> Sources of Kodachrome slides (35 mm, 2 by 2)</td>
<td></td>
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<tr>
<td>-------------------------------------------------------------</td>
<td></td>
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</tr>
<tr>
<td>Armed Forces Institute of Pathology</td>
<td>W. H. Curtin and Co.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dept. ID/Parasitic Disease Pathology</td>
<td>P.O. Box 1546</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6825 16th St., NW</td>
<td>Houston, TX 77001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bldg. 54, Room 4015</td>
<td>TURTOX</td>
<td></td>
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</tr>
<tr>
<td>Washington, DC 20306-6000</td>
<td>P.O. Box 92912</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rochester, NY 14692</td>
<td></td>
<td></td>
</tr>
<tr>
<td>American Society of Clinical Pathologists</td>
<td>Dr. Herman Zaiman</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASCP Press</td>
<td>(A Pictorial Presentation of Parasites)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2100 West Harrison St.</td>
<td>P.O. Box 543</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chicago, IL 60612-3798</td>
<td>Valley City, ND 58072</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(312) 738-4890</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human Parasitology, teaching slide set</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Ash and Orihel, 1990)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supplement to Human Parasitology, teaching slide set</td>
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<td></td>
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<tr>
<td>(in preparation)</td>
<td></td>
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<tr>
<td>Parasites in Human Tissues, teaching slide set</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Orihel and Ash, 1996)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical Diagnostic Parasitology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Visual Teaching Aids (set of 100 2 by 2 slides)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diagnostic Protozoa, Helminths, and Blood Parasites</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>(Lynne S. Garcia)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>512 12th St.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Santa Monica, CA 90402</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Appendix 9.10.7–1—Current OSHA Regulations on the Use of Formaldehyde

OSHA requires all workers to be protected from dangerous levels of vapors and dust. Formaldehyde vapor is the air contaminant most likely to exceed the regulatory threshold in the clinical laboratory. Current OSHA regulations require vapor levels not to exceed 0.75 ppm (measured as a time-weighted average [TWA]) and 2.0 ppm (measured as a 15-min [short-term] exposure). OSHA requires monitoring for formaldehyde vapor wherever formaldehyde is used in the workplace. The laboratory must have evidence at the time of inspection that formaldehyde vapor levels have been measured, and both 8-h and 15-min exposures must have been documented (1, 3, 4).

If each measurement is below the permissible exposure limit and the 8-h measurement is below 0.5 ppm, no further monitoring is required providing the laboratory procedures remain constant. Changes in the laboratory that would require repeat monitoring include the following: large increase in the overall number of specimens processed each day (for example, from 20 to >100), change in location of reagents from inside a fume hood to outside the fume hood (on the open laboratory bench), or introduction of new procedures that require the use of formalin. If either the 0.5-ppm 8-h TWA or the 2.0-ppm 15-min level is exceeded, monitoring must be repeated every 6 months. If either the 0.75-ppm 8-h TWA or the 2.0-ppm 15-min level is exceeded (unlikely in a clinical laboratory setting), employees are required to wear respirators. Accidental skin contact with aqueous formaldehyde must be prevented by the use of proper clothing and equipment (gloves, laboratory coats).

The amendments of 1992 add medical removal protection provisions to supplement the existing medical surveillance requirements for employees suffering significant eye, nose, or throat irritation and for those experiencing dermal irritation or sensitization from occupational exposure to formaldehyde. In addition, these amendments establish specific hazard labeling requirements for all forms of formaldehyde, including mixtures and solutions composed of at least 0.1% formaldehyde in excess of 0.1 ppm. Additional hazard labeling, including a warning label that formaldehyde presents a potential cancer hazard, is required where formaldehyde levels, under reasonably foreseeable conditions of use, may exceed 0.5 ppm. The final amendments also provide for annual training of all employees exposed to formaldehyde at levels of 0.1 ppm or higher.

NOTE: Monitoring badges are not sensitive enough to correctly measure the 15-min exposure level. Contact the occupational health and safety office within your institution for monitoring options. Usually, the accepted method involves monitoring airflow in the specific area(s) within the laboratory where formaldehyde vapors are found.

Chemical Hygiene Plan
OSHA also requires each laboratory to develop a comprehensive, written chemical hygiene plan (CHP). Regardless of type of risk, volume, or concentration, every hazardous chemical in the laboratory must be included in the CHP. The plan should include storage requirements, handling procedures, location of OSHA-approved material, safety data sheets, and the medical procedures that are to be followed should exposure occur. The CHP must specify the clinical signs and symptoms of the environmental conditions (such as a spill) that would give the employer reason to believe exposure had occurred. When such conditions exist, the CHP should indicate the appropriate medical attention required (2).

REFERENCES

The following regulations appeared in the Federal Register on Friday, 4 December 1987 (vol. 52, no. 233: Occupational Exposure to Formaldehyde).
Current Procedural Terminology (CPT) comprises a list of descriptive terms and identifying codes for reporting medical services and procedures performed by physicians/laboratorians. The CPT list is published by the American Medical Association (AMA) (website, www.ama-assn.org/cpt). The most recent version is CPT 2003. The purposes of the CPT codes are to (i) provide a uniform language that will accurately describe medical, surgical, and diagnostic services and (ii) provide an effective means for reliable nationwide communication among physicians, patients, and third parties. The CPT list is the most widely accepted nomenclature for the reporting of physician/laboratory procedures and services under government and private health insurance programs.

CPT comparisons are used for claims processing and the development of guidelines for medical care review. They also provide a useful basis for local, regional, and national utilization comparisons. The CPT code format is a five-digit code; one of the six sections is Pathology and Laboratory Medicine.

The following information should allow you to code the diagnostic parasitology procedures correctly for billing purposes. In addition, if a procedure requires the use of multiple codes, you should have this information in written form within your protocol manuals, preferably the appendix or another separate section so changes can be easily made without requiring changes in every individual protocol.

### CPT codes for diagnostic parasitology

<table>
<thead>
<tr>
<th>Procedure name</th>
<th>New CPT*</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar plate culture (<em>Strongyloides</em>)</td>
<td>87081 + 87210</td>
<td>Includes culture and wet mount examination</td>
</tr>
<tr>
<td>Baermann concentration (plus examination)</td>
<td>87015 + 87210</td>
<td>Includes concentration and wet mount examination</td>
</tr>
<tr>
<td>Cyclospora examination (modified AFB*)</td>
<td>87207</td>
<td>Modified acid-fast stain</td>
</tr>
<tr>
<td>Cryptosporidium examination (modified AFB)</td>
<td>87207</td>
<td>Modified acid-fast stain</td>
</tr>
<tr>
<td>Cryptosporidium examination (FA)</td>
<td>87272</td>
<td>Immunoassay</td>
</tr>
<tr>
<td>Cryptosporidium examination (EIA)</td>
<td>87328</td>
<td>Immunoassay</td>
</tr>
<tr>
<td>Cryptosporidium/Giardia examination (FA)</td>
<td>87272</td>
<td>Immunoassay</td>
</tr>
<tr>
<td>Cryptosporidium/Giardia examination (EIA)</td>
<td>87328 (negative)</td>
<td>Screen that detects both <em>Giardia</em> and <em>Cryptosporidium</em> but does not differentiate between the two. If positive (and individual antigens are subsequently tested), then a total of 3 87328 procedures is appropriate; if negative, then a single 87328.</td>
</tr>
<tr>
<td></td>
<td>87328 (positive) + 2 more (87328)</td>
<td></td>
</tr>
<tr>
<td>Cryptosporidium/Giardia examination (cartridge)</td>
<td>87328</td>
<td>Possibility of two answers, but only one test (limit billing to once); immunoassay (immunochromatographic format)</td>
</tr>
<tr>
<td>Cryptosporidium/Giardia/Entamoeba histolytica-<em>E. dispar</em> group examination (cartridge)</td>
<td>87328</td>
<td>Possibility of three answers, but only one test (limit billing to once); immunoassay (EIA immunoassay format)</td>
</tr>
<tr>
<td>Ectoparasite identification (macroscopic) (arthropod)</td>
<td>87168</td>
<td>Macroscopic exam</td>
</tr>
<tr>
<td>Ectoparasite identification (wet mount) (arthropod)</td>
<td>87210</td>
<td>Wet mount examination</td>
</tr>
<tr>
<td>Ectoparasite identification (skin, scabies)</td>
<td>87220</td>
<td></td>
</tr>
<tr>
<td>Fat, stool (qualitative)</td>
<td>87205</td>
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</tr>
<tr>
<td><em>Giardia</em> examination (FA)</td>
<td>87269</td>
<td>Immunoassay</td>
</tr>
<tr>
<td><em>Giardia</em> examination (EIA)</td>
<td>87329</td>
<td>Immunoassay</td>
</tr>
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</table>
## CPT codes for diagnostic parasitology (continued)

<table>
<thead>
<tr>
<th>Procedure name</th>
<th>New CPT&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harada-Mori concentration (plus examination)</td>
<td>87015 + 87210</td>
<td>Includes concentration and wet mount examination</td>
</tr>
<tr>
<td>Helminth egg hatching (concentration, wet mounts)</td>
<td>87177</td>
<td>Includes concentration and wet mount examination</td>
</tr>
<tr>
<td><em>Isospora</em> examination (modified AFB only)</td>
<td>87207</td>
<td>Without concentration. 87015 + 87207 would be an option if this is an orderable test, which includes concentration and modified AFB stain.</td>
</tr>
<tr>
<td>Microsporidia, special stain (modified trichrome)</td>
<td>87015 + 87207</td>
<td>Modified trichrome on concentration sediment; includes concentration and modified trichrome stain</td>
</tr>
<tr>
<td>Microsporidia, fluorescent stain (calcofluor) (on concentration sediment)</td>
<td>87015 + 87206</td>
<td>Includes concentration and fluorescent stain (optical brightening agent) on sediment</td>
</tr>
<tr>
<td>Occult blood, stool</td>
<td>82270</td>
<td>Includes direct wet mount, concentration, and concentrate wet mount examinations. This <em>does not</em> include the permanent stain.</td>
</tr>
<tr>
<td>Occult blood, gastric</td>
<td>82271</td>
<td></td>
</tr>
<tr>
<td>Ova and parasite examination</td>
<td>87177</td>
<td>Permanent stain only</td>
</tr>
<tr>
<td>Ova and parasite examination (permanent stain) (example: trichrome)</td>
<td>87209</td>
<td>Includes direct wet mount, concentration, concentrate wet mount, and permanent stained smear examinations. (Note: There is an error in the code book under 87177 where 88312 is specified for trichrome—it should read 88313); CPT 2003 now states that 88313 should be used.</td>
</tr>
<tr>
<td>Ova and parasite examination (routine ova and parasite exam) (performed on <em>fresh stool</em>, includes direct wet mount)</td>
<td>87177 + 87209</td>
<td></td>
</tr>
<tr>
<td>Ova and parasite examination (routine ova and parasite exam) (performed on <em>preserved stool</em>, no direct wet mount)</td>
<td>87015, 87210, 87209</td>
<td>Includes concentration, concentrate wet mount, and permanent stained smear examinations</td>
</tr>
<tr>
<td>Parasite concentration, iodine prep (wet prep only)</td>
<td>87015 + 87210</td>
<td>Includes concentration and wet mount examination (iodine mount)</td>
</tr>
<tr>
<td>Parasite concentration, saline prep 1 (wet prep only)</td>
<td>87015 + 87210</td>
<td>Includes concentration and wet mount examination (saline mount)</td>
</tr>
<tr>
<td>Saline prep 2 (wet prep only)</td>
<td>87210</td>
<td>Multiple wet mounts may have to be examined in certain circumstances: duodenal aspirates, string test mucus, urine concentrations, etc.</td>
</tr>
<tr>
<td>Saline prep 3 (wet prep only)</td>
<td>87210</td>
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<tr>
<td>Saline prep 4 (wet prep only)</td>
<td>87210</td>
<td></td>
</tr>
<tr>
<td>Saline prep 5 (wet prep only)</td>
<td>87210</td>
<td></td>
</tr>
<tr>
<td>Parasite examination, wet prep only</td>
<td>87210</td>
<td></td>
</tr>
<tr>
<td>Parasite examination, duodenum (concentration, wet mounts)</td>
<td>87177</td>
<td>Includes direct wet mount, concentration, and concentrate wet mount examinations. This <em>does not</em> include any type of permanent stain.</td>
</tr>
<tr>
<td>Parasite examination, duodenum (permanent stain)</td>
<td>87209</td>
<td>Trichrome stain</td>
</tr>
<tr>
<td>Parasite examination, Entero-Test (wet mounts only)</td>
<td>87210</td>
<td>Entero-Test (string test, “fishing” test, capsule test): sampling of duodenal contents</td>
</tr>
<tr>
<td>Parasite examination, Entero-Test (permanent stain only)</td>
<td>87209</td>
<td>Trichrome</td>
</tr>
<tr>
<td>Parasite examination, urine (random) (concentration, wet mounts)</td>
<td>87015 + 87210</td>
<td>Includes concentration and wet mount examination; may include multiple wet mounts prepared/examined—use additional 87210 with modifier</td>
</tr>
<tr>
<td>Parasite examination, 24-h urine (concentration, wet mounts)</td>
<td>87015 + 87210</td>
<td>May be multiple wet mounts prepared/examined—use additional 87210 with modifier</td>
</tr>
<tr>
<td>Parasite concentration, blood (concentration, wet mounts)</td>
<td>87015 + 87210</td>
<td>May be multiple wet mounts prepared/examined—use additional 87210 with modifier (will include Knott and membrane concentrations using Nuclepore filters)</td>
</tr>
<tr>
<td>Parasite, calcofluor stain (direct material, no concentration)</td>
<td>87206</td>
<td>Optical brightening agent; fluorescence</td>
</tr>
<tr>
<td>Parasite concentration (miscellaneous)</td>
<td>87015</td>
<td>Concentration only</td>
</tr>
<tr>
<td>Giemsa stain, thin blood film</td>
<td>87207</td>
<td>Giemsa stain</td>
</tr>
<tr>
<td>Giemsa stain, thick blood film</td>
<td>87015 + 87207</td>
<td>Includes concentration and Giemsa stain</td>
</tr>
</tbody>
</table>
CPT codes for diagnostic parasitology (continued)

<table>
<thead>
<tr>
<th>Procedure name</th>
<th>New CPT(a)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parasite culture (intestinal and blood protozoa)</td>
<td>87081 + 87015 + 87210 + 87207 (Giemsa) or 88313 (trichrome)</td>
<td>Includes culture, concentration, wet mount, permanent stain of culture sediment (intestinal and blood protozoa)</td>
</tr>
<tr>
<td>Parasite culture, presumptive, screening only (Trichomonas)</td>
<td>87081</td>
<td>(Pouch) (screen) (microscope exam through plastic pouch)</td>
</tr>
<tr>
<td>Parasite culture, presumptive, screening only (Acanthamoeba)</td>
<td>87081 + 87210 + 87205 (Giemsa)</td>
<td>Includes culture (agar plates/bacterial overlay), wet mount, permanent stain (for the trichrome stain, use 88313, not 87205)</td>
</tr>
<tr>
<td>Parasite detection (E. histolytica/E. dispar group EIA)</td>
<td>87336</td>
<td>Immunoassay for the E. histolytica/E. dispar group; does not differentiate the true pathogen, E. histolytica; immunoassay</td>
</tr>
<tr>
<td>Parasite detection (E. histolytica EIA)</td>
<td>87337</td>
<td>Should read E. histolytica; specific for the true pathogen, E. histolytica; immunoassay</td>
</tr>
<tr>
<td>Parasite detection (Cryptosporidium/Giardia EIA)</td>
<td>87328 (negative) + 2 more (87328)</td>
<td>Immunoassay that detects both Giardia and Cryptosporidium, but does not differentiate between the two. If positive, then a total of 387328 procedures is appropriate; if negative, then a single 87328.</td>
</tr>
<tr>
<td>Parasite detection, EIA (Cryptosporidium only)</td>
<td>87328</td>
<td>Immunoassay</td>
</tr>
<tr>
<td>Parasite detection, EIA (Giardia only)</td>
<td>87329</td>
<td>Immunoassay</td>
</tr>
<tr>
<td>Parasite examination, blood (thin film)</td>
<td>87207</td>
<td>Giemsa stain</td>
</tr>
<tr>
<td>Parasite examination, blood (thick film)</td>
<td>87015 + 87207</td>
<td>Includes concentration and Giemsa stain</td>
</tr>
<tr>
<td>Parasite referral in (stool for ova and parasite exam)</td>
<td>87177 + 87209</td>
<td>Includes direct wet mount, concentration, concentrate sediment, and permanent stained smear examinations</td>
</tr>
<tr>
<td>Petri dish culture/concentration (plus examination)</td>
<td>87015 + 87210</td>
<td>Includes concentration and wet mount examination</td>
</tr>
<tr>
<td>pH, stool</td>
<td>83986</td>
<td>pH, body fluid, except blood (probably the best option), previously approved by consultants</td>
</tr>
<tr>
<td>Pinworm examination</td>
<td>87172</td>
<td>Single cellophane tape/paddle/other device examination</td>
</tr>
<tr>
<td>Reducing substances, stool (pH)</td>
<td>83986</td>
<td>Since this may not have been specifically ordered by physician, may not be billable unless part of written routine algorithm approved by medical staff and pathology for handling tissues</td>
</tr>
<tr>
<td>Tissue homogenization for culture</td>
<td>87176</td>
<td></td>
</tr>
<tr>
<td>Trypsin activity, stool</td>
<td>84488</td>
<td>Example: Ascaris adult worm</td>
</tr>
<tr>
<td>Worm identification (macroscopic)</td>
<td>87169</td>
<td>Example: Enterobius adult worm; small structures that resemble worms</td>
</tr>
<tr>
<td>Worm identification (wet mount)</td>
<td>87210</td>
<td></td>
</tr>
</tbody>
</table>

\(a\) Each time a procedure requires multiple codes for billing, this information should be included in your protocol manuals, possibly in a separate section within the appendix (specifying that the procedure includes multiple coded procedures, thus the need for multiple codes; define procedure and each code required [could be in table format]). This information is similar to that required for the use of laboratory algorithm testing, in which multiple steps/procedures are required to complete a more comprehensive test or series of tests.

\(b\) AFB, acid-fast bacilli.

REFERENCE