Procedures for the Recovery and Identification of Parasites From the Intestinal Tract; Approved Guideline—Second Edition

This guideline addresses the collection, processing, and examination of intestinal tract specimens for the identification of parasites.

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Abstract

The diagnosis of parasites from the intestinal tract depends on the recovery and identification of the etiologic agents. Therefore, the ability to collect, process, and examine fecal specimens is important in terms of clinical relevance and patient care. Parasitic infections are not normally treated without demonstration of the specific causative agent. Thus, the ability to recover and identify these organisms is an important part of the overall microbiological responsibilities of the diagnostic laboratory.1,2

Communication of instructions to the patient, specimen collection and handling techniques, diagnostic tests, and result reporting are key components in proper patient management. Major sections of this document cover these topics, as well as equipment, reagents, and specific techniques used in diagnosing intestinal parasitic infections.

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Foreword

Although it is common to think of parasitic diseases as occurring only in the tropical areas of the world, many of the infections seen in the intestinal tract are endemic within the more temperate regions of the world, including the United States. In addition to these more common organisms, laboratories are also required to identify some of the less common intestinal parasites seen in travelers and proficiency testing specimens. The diagnosis of parasites from the intestinal tract depends on the recovery and identification of the etiologic agents. The ability to collect, process, and examine specimens from this body site is important in terms of clinical relevance and patient care. Parasitic infections are not normally treated without demonstration of the specific causative agent. Thus, the ability to recover and identify these organisms is an important part of the overall microbiological responsibilities of the diagnostic laboratory.

The Subcommittee on Parasitology, as part of the Area Committee on Microbiology, identified the need for a guideline for the examination of fecal specimens. The subcommittee also identified this topic as one that would apply to most laboratories providing diagnostic procedures in microbiology, specifically parasitology. This aspect of diagnostic parasitology often represents the majority of specimens submitted by both in- and out-patients. Even in a relatively small laboratory, the submission of fecal specimens for examination for parasites may occasionally occur.

Communication of instructions to the patient, specimen collection and handling techniques, diagnostic tests, and result reporting are key components in proper patient management. Major sections of this document cover these topics, as well as equipment, reagents, and specific techniques used in diagnosing intestinal parasitic infections.1-8

The CLSI Working Group on Recovery/Identification of Parasites From the Intestinal Tract has revised the document to the second edition of the approved guideline. Last published in December of 1997, the document now includes the following enhancements:

- Newer technologies have been addressed. Diagnostic kits that detect specific organism antigens are discussed.
- Appropriate definitions are now included.
- Additional modified acid-fast staining techniques for the identification of intestinal coccidia have been added to the document.
- Additional modified trichrome staining methods for the identification of intestinal microsporidia are now included.
- The section on fecal immunoassays and gene probes has been greatly expanded to include the newer diagnostic options.
- Additional information on chemofluorescent agents has been added.
- Specific ordering options, including the routine O&P examination and fecal immunoassays, have been included to serve as guidelines for clinician ordering recommendations.

Comments submitted on the first edition of the approved M28 document are addressed in an appendix in this publication. The working group urges the reader to send constructive suggestions for improving this document to CLSI so that we can evaluate the practical usefulness of the document to members of the healthcare community. We look forward to receiving comments and to the reader’s active participation in the CLSI consensus process.
Key Words

Diagnostic procedures, etiologic agents, intestinal tract, parasites
Procedures for the Recovery and Identification of Parasites From the Intestinal Tract; Approved Guideline—Second Edition

1 Scope

This guideline is intended to provide the readers with standardized procedures used for the recovery and identification of parasites from the intestinal tract. The intended audience includes those on the healthcare team, including laboratorians, microbiologists, parasitologists, physicians, public health personnel, and those in academic settings who are involved in teaching diagnostic medical parasitology.

The document is not intended to provide didactic training related to human parasite life cycles, organism morphology, clinical disease, pathogenesis, treatment, or epidemiology and prevention. However, the procedures provided are very comprehensive and discuss in detail the actual method, procedure notes and limitations, and information related to quality control and reporting of results.

2 Equipment for Fecal Specimen Examination

2.1 Microscope

High-quality microscopes with good resolving power are mandatory for the examination of specimens for parasites. Identification of the majority of organisms depends on morphologic differences, most of which should be seen using dissecting or regular microscopes.

2.1.1 Dissecting

A dissecting microscope should be available for examination of larger specimens (arthropods, some helminths, and various artifacts). The total magnification usually ranges from 10x to 45x. Some of the microscopes have a zoom capacity from 10x to 45x and others have fixed objectives (0.66x, 1.3x, and 3x) that can be used with 5x or 10x oculars. It is helpful to be able to use a light source either from under the specimen or directed onto the top of the specimen.

2.1.2 Brightfield

A binocular, brightfield microscope, with a minimum of 10x, 40x, and 100x (oil immersion) objectives and 10x oculars, should be available for use. Some laboratories also use a 4x objective. In addition to the above objectives, some laboratorians find the 40x, 50x, or 60x oil immersion lenses helpful, particularly for screening stained smears. Although 10x oculars are most commonly used, 12.5x and 5x are also available, but the smaller magnification of the 5x oculars may make final organism identification more difficult. Preferably, the microscope should have a built-in lamp, an adjustable substage condenser with an iris diaphragm, and a blue daylight filter. In the event that an adjustable condenser is not available, a fixed condenser is acceptable. The numerical aperture of the condenser should match the highest numerical aperture of the objective lenses (usually the lens with the highest magnification).

2.1.3 Fluorescence

For completing direct fluorescent antibodies (DFAs) for *Giardia/Cryptosporidium*, fluorescence for microsporidia or autofluorescence for *Cyclospora*, a fluorescent microscope with FITC, Calcofluor, and a blue filter set are also necessary.
2.1.4 Care of the Microscope

Microscopes should be covered when not in use, and all lenses should be cared for with lens paper only. Remember to use several layers of lens paper when cleaning the objective; one layer is insufficient for total oil removal. It is particularly important to remove all oil when work is finished. Avoid the use of xylene for cleaning optical surfaces. Follow the manufacturer’s guidebook when making any adjustments or changes.

2.2 Calibration (Ocular Micrometer)

One of the most important factors in the identification of parasites is size. It is essential that any laboratory performing procedures for the recovery and identification of parasites have a calibrated ocular micrometer available. Measurements are performed using a micrometer disk (usually calibrated as a line divided into 50 units) that is placed in the ocular of the microscope. Depending on the objective magnification used, these unit divisions will represent different measurements. Therefore, the ocular disk should be compared with a known calibrated scale, usually a stage micrometer with a scale of 0.1- and 0.01-mm divisions. After each microscope objective has been calibrated, the ocular containing the disk and/or the objectives cannot be interchanged with oculars and/or objectives from another microscope. Each microscope should be calibrated as a total package; the original oculars and objectives used to calibrate the microscope should be used when measuring organisms. Some laboratories use a separate ocular (containing the micrometer disk), which has been used to calibrate a number of microscopes. Thus, the value per unit would be unique for each. The procedure is as follows:

1. Unscrew the eye lens of a 10x ocular (this may be the top or bottom, depending on the model) and place the micrometer disk (engraved side down) within the ocular. Use lens paper to handle the disk, and try to keep all surfaces free of lint or dust. Replace the ocular containing the micrometer disk in the microscope.

2. Place the calibrated micrometer on the stage and, with the low power (10x objective), focus on the calibrated scale. It will be possible to distinguish the difference between the 0.1- and 0.01-mm divisions.

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

4. After these two “0” lines are lined up (without moving the stage micrometer), look to the right of the “0” lines for another set of lines that is superimposed. Find a set as far to the right of the “0” lines as possible (the distance will vary with different objectives).

5. Count the number of ocular divisions between the “0” lines and the point where the second set of lines is superimposed. Count the number of 0.1-mm divisions between the “0” lines and the second set of superimposed lines on the stage micrometer.

6. Calculate the number of millimeters that is measured by a single, small ocular unit:

*Example:* \(27\) ocular units = 0.20 mm; 1 mm = 1000 µm

\[
\frac{0.20 \text{ mm}}{27 \text{ ocular units}} = 0.0074 \text{ mm/ocular unit}
\]

To convert mm to µm, multiply by 1000:
1000 µm · 0.0074 mm = 7.4 µm/ocular unit

NOTE: This measurement applies to this objective (low power, 10x) only.

(7) When the high-dry (40x) and oil immersion (100x) objectives are being calibrated, the “0” line of the stage micrometer will increase in size while the ocular “0” line remains the same size. The thin ocular line should be lined up 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 at the corresponding edge of the broad stage micrometer line.

(8) Once the microscope is calibrated, the factors for the three objectives (low, high-dry, and oil immersion) should be posted on the microscope. Make sure the posting is dated and initialed. Then, when measuring with the ocular micrometer, the distance can be multiplied by the factor (depending on the objective used).

(9) If the microscope receives heavy use, is moved frequently, is repaired or has been bumped or hit, the microscope should be recalibrated. If the microscope receives routine use, there is no need to recalibrate on a yearly basis.

2.3 Centrifuge

2.3.1 Requirements

A table or floor model centrifuge is acceptable and should be able to accommodate 15-mL and/or 50-mL centrifuge tubes (for the concentration procedures), as well as being capable of achieving and maintaining the gravitational “g” force required by the procedures selected. If a table model is selected, do not get one with an angle head. A swinging bucket-type head, rather than an angle head, is much more effective in concentration procedures, especially in flotation techniques.

2.3.2 Calibration

Since models and sizes of centrifuges vary, the use of “g” forces, instead of revolutions per minute (rpm), is suggested. A nomograph for calculating speeds is recommended. The rotating radius of the centrifuge head is the basis for the calculation, and it should be carefully determined. It is measured from the rotor axis to the tip of liquid inside the tube at the greatest horizontal distance from the rotor axis. Once the centrifuge is calibrated, it should be given periodic checks of speed (rpm) and maintenance (a minimum of every six months). Please refer to Appendix A for instructions on how to determine the relative centrifugal force.

2.4 Fume Hood

While the presence of a fume hood is not mandatory when processing stool specimens containing formalin, an OSHA-compliant monitoring program should be in force. Even with the substitution of dehydrating reagents other than xylene, fume hoods may be preferred in order to eliminate fecal and solvent odors. A small, table-top model is acceptable.

2.5 Biological Safety Cabinet

Although a biological safety cabinet is not required for processing fecal specimens in the parasitology laboratory, some laboratories use Class I (open-face) or Class II (laminar-flow) biological safety cabinets for processing all unpreserved specimens. However, many laboratories now receive their fecal specimens in preservative. Use of a biological safety cabinet is recommended if the laboratory is processing fresh
specimens and performing cultures for parasite isolation. This provides protection from viral and bacterial pathogens as well as parasites that might be infectious.

2.6 Miscellaneous Equipment

- Autoclave
- Magnetic stirrer and stir bar
- Analytical balance
- Hydrometer (with a range that includes 1.18 to 1.20)
- Staining rack
- 70 °C heating rack
- Alcohol lamp or Bunsen burner
- Refrigerator
- Freezer
- 35 or 37 °C incubator
- Timers
- Fluorescence microscope with FITC and Calcofluor filter set
- Blue filter

2.7 Basic Supplies

- Funnel
- Gauze
- Applicator sticks, plain and cotton-tipped
- Wire loop (bacteriology)
- Microscope slides (1" x 3" or larger)
- Coverslips (No. 1, 22-mm square or larger)
- Lens paper
- Absorbent wipes (facial tissues)
- Disposable glass or plastic pipets
- Pasteur pipets and bulbs
- Screw-capped test tubes (16 x 125 mm)
- Test tube racks
- Covered Coplin jars (12) or staining dishes (with slide rack)
- Forceps (for handling slides during staining)
- Centrifuge tubes (15 mL or 50 mL with caps)
- Sealable, leakproof urine/specimen containers
- Graduated cylinders–various sizes
- Beakers–various sizes
- 250 mL brown glass-stoppered bottle
- Two brown glass dropper bottles
- Xylene or xylene substitute\(^1\text{-}\text{5}\)
- Distilled water
- Ethanol (70, 95, and 100%)
- Hematoxylin, crystals or powder
- Trichrome stain or appropriate ingredients
- Ferrous ammonium sulfate \([\text{Fe(NH}_4\text{)}_2(\text{SO}_4)_2 \times 6\text{H}_2\text{O}]\)
- Ferric ammonium sulfate \([\text{FeNH}_4(\text{SO}_4)_2 \times 12\text{H}_2\text{O}]\)
- Formaldehyde (5 or 10% formalin)
- Ethyl acetate or other defatting agent\(^1\text{-}\text{5}\)
• PVA fixative
• Hydrochloric acid, concentrated (HCl)
• Sulfuric acid, concentrated (H₂SO₄)
• Potassium iodide (KI)
• Sodium chloride (NaCl)
• Iodine crystals, powdered (I₂)
• Distilled water
• Mounting medium
• Biohazard container with disinfectant for proper disposal of slides, tubes, and pipets
• Biohazard container for proper disposal of patient specimens

NOTE: Some protocols may require other specific supplies; read every protocol thoroughly before beginning work.

3 Standard Precautions

Because it is often impossible to know what might be infectious, all patient and laboratory specimens are treated as infectious and handled according to “standard precautions.” Standard precautions are guidelines that combine the major features of “universal precautions and body substance isolation” practices. Standard precautions cover the transmission of all infectious agents and thus are more comprehensive than universal precautions which are intended to apply only to transmission of blood-borne pathogens. Standard and universal precaution guidelines are available from the U.S. Centers for Disease Control and Prevention (Guideline for Isolation Precautions in Hospitals. Infection Control and Hospital Epidemiology. CDC. 1996;17(1):53-80 and MMWR 1988;37:377-388). For specific precautions for preventing the laboratory transmission of all infectious agents from laboratory instruments and materials and for recommendations for the management of exposure to all infectious disease, refer to the most current edition of CLSI document M29—Protection of Laboratory Workers from Occupationally Acquired Infections.

4 Definitions

cestode – tapeworm.

Charcot-Leyden crystals – slender crystals that are formed from the breakdown products of eosinophils; shaped like double, elongated pyramids with pointed ends; can be found in feces, sputum, and tissues; indicates an immune response that may or may not be related to a parasitic infection.

chromatin – deep-staining DNA-containing portion of the nucleus (protozoa).

chromatoidal bar/body – deep-staining, bar-shaped, round, or splinter-shaped inclusions found in the cytoplasm of certain amoebae (Entamoeba spp.)

CLB (cyanobacterium-like, or coccidian-like body) – organism thought to be a new pathogen, possibly an oocyst, a flagellate, an unsporulated coccidian, a large Cryptosporidium spp., or a blue-green alga and now thought to be coccidia in the genus Cyclospora (Cyclospora cayetanensis).

concentration techniques – procedures, usually in fecal examinations, allowing the examination of large amounts of feces (flotation or sedimentation procedures; some available for blood specimens and urine specimens).

cyst – the nonfeeding encysted stage of the protozoa.
direct smear (stool) – approximately 2-mg suspension of feces in water or saline for the purpose of examination for parasites; primary aim is to see motility.

duodenum – the proximal portion of the small intestine (Strongyloides stercoralis, Giardia lamblia\(^a\)).

eosinophilia – abnormal increase in the number of eosinophils found in the blood; often found in helminthic infections, especially with tissue invasion (visceral larval migrans, trichinosis, schistosomiasis, ascariasis, strongyloidiasis); also present with other infectious processes, allergic reactions (including drug induced) and with some malignant diseases.

filariform larvae – slender, infective larvae of Strongyloides stercoralis and hookworm.

glycogen – principal carbohydrate reserve; glucosan of high molecular weight; found within vacuoles of many of the protozoa.

helminth – may refer to a nematode (roundworm), cestode (tapeworm), or trematode (fluke).

karyosome – concentrated clumps of chromatin material within the nucleus; position and morphology often used to differentiate intestinal protozoa.

macrophage – motile, phagocytic, mononuclear cell that originates in the tissues and may be confused morphologically with protozoan trophozoites (particularly those of Entamoeba histolytica).

miracidium – free-living, ciliated larva released from a trematode egg and infective for the snail intermediate host.

nucleus – a cellular inclusion composed of chromatin; morphology often used to help identify intestinal protozoa (Entamoeba and Dientamoeba spp.).

occult blood – blood present in very small amounts; usually detectable by chemical means; specimen is most often stool; may or may not be related to parasitic infection.

oocyst – the encysted form of the fertilized macrogamete, or zygote, in coccidian parasites; may contain one or two immature sporonts, with the eventual development of sporocysts (Isospora belli).

parasite – an organism living on or in, and at the expense of, another organism.

permanent-stained smear – fecal material (fixed or unfixed) spread onto a glass slide, which is subsequently stained (trichrome, iron-hematoxylin, modified acid-fast, modified trichrome, etc.) and examined microscopically using an oil immersion objective (total magnification of 1000x).

proglottid – segments of the tapeworm containing male and female reproductive systems; may be immature, mature, or gravid (Taenia and Diphyllobothrium spp.).

rhabditoid (rhabditiform) larvae – noninfective, thick, rod-shaped larvae of hookworm and Strongyloides stercoralis; rhabditoid refers to the shape of the larval esophagus.

scolex – the head or attachment portion of a tapeworm; attachment may be by suckers or hooklets (Taenia, Diphyllobothrium spp.).

\(^a\) Although some individuals have changed the species designation for the genus Giardia to G. intestinalis or G. duodenalis, there is no general agreement. Therefore, for this listing, we will retain the name Giardia lamblia.
sigmoidoscopy – visual examination of the rectum and sigmoid flexure of the colon by using a lighted tube; often performed for suspect amebiasis cases (Entamoeba histolytica).

spore – one of the stages in the life cycle of the microsporidia; contains a polar tubule apparatus used for infecting cells; the number of polar tubule coils in the spore is used in organism classification (electron microscopy). **NOTE:** The size is usually 1 to 2 μm.

trophozoite – the feeding, motile stage of protozoa.

vacuole – cavity in the cytoplasm of a cell that may contain ingested bacteria, yeast cells, or debris; excess vacuolization can be found in poorly preserved organisms or multiple vacuoles can be found in poorly preserved organism; vacuole contents and/or morphology may be helpful in identification of some of the intestinal protozoa (Entamoeba spp., Iodamoeba bütschlii).

5 Collection, Preservation, and Shipment of Specimens

5.1 Fresh Specimen Collection

5.1.1 Types of Specimens

Specimens may range in consistency from watery to formed. If fresh specimens are submitted to the laboratory, it is helpful to determine and report the consistency of the stool (watery, loose, soft, formed). The stool consistency will also give some clues concerning the stages of the intestinal protozoa that might be present in the specimen. The trophozoite stage is more likely to be found in watery or loose specimens, while the cyst forms are more often found in formed specimens. Both stages may be present in soft specimens.

**NOTE:** The presence of gross blood on or in the specimen should also be reported. A few laboratories may perform occult blood testing on every specimen submitted; however, most laboratories would only perform the procedure if requested to do so.

5.1.2 How to Collect the Specimen

Specimens should be collected in clean, wide-mouthed containers (half-pint, waxed cardboard carton, or plastic containers). Regardless of the type of container, the lid should provide a tight fit. The specimens should not be contaminated with water or urine, because this could lyse any trophozoites present. Accidental contamination with soil or water could result in the presence of free-living organisms from the environment, which can be easily confused with parasitic protozoa or nematodes.

Although many diagnostic antigen detection tests (ELISA, FA, immunochromatographic strips) can be performed using stool preserved in 5% formalin, 10% formalin or SAF, some fecal immunoassays require fresh or frozen stool. It is important to verify specimen collection requirements for each diagnostic kit that may be used in the laboratory.

5.1.3 Numbers of Specimens

A minimum of three specimens (every other day or every day but not within the same day) should be collected for examination. If the patient does not have diarrhea (see Section 5.1.1 for details regarding stool consistency and stages of intestinal protozoa), then two specimens from normal movements can be collected with the third specimen being collected after using a cathartic.9-13 According to Nazer, et al (1993), examination of the first stool yielded 58.3% of the population tested; the second specimen added an additional 20.6% and the third specimen added another 21.2%.9 In a publication by Hiatt, et al (1995), the sensitivity of one examination was compared with that of three examinations; with the additional
examinations, the yield increased 22.7% for Entamoeba histolytica, 11.3% for Giardia lamblia, and 31.1% for Dientamoeba fragilis. Six specimens are recommended for the diagnosis of amebiasis; however, with the emphasis on cost-containment, few physicians will order this many specimens. In many laboratories, two fecal specimens are requested, rather than three; statistically, the difference between the examination of two versus three may not be critical. In publications by Cartwright (1999) and Hanson and Cartwright (2001), examinations of two specimens by either EIA or microscopy were necessary to achieve a diagnostic sensitivity of greater than 90%. Both options are certainly acceptable. Additional collection options can be seen in Appendix C. It is important to realize that not all laboratories will handle specimen collection the same way; however, in terms of clinical relevance, it is very important to understand the pros and cons of the approach selected.

5.1.4 Times of Collection

Stool specimens should be collected every other day or within a time frame of no more than ten days. If a series of six specimens is ordered, then they should be submitted within a 14-day period. Many organisms, particularly the intestinal protozoa, appear in the stool on a cyclic basis, and the series of two or three specimens is considered a minimum for an adequate examination. It is inappropriate for more than one specimen from the same patient to be collected on a single day.

5.1.5 Time Frame for Examination

Fresh specimens are necessary for the recovery of motile protozoa; however, many laboratories recommend that all specimens be collected in preservatives. Fresh specimens could be requested on an individual, case-by-case basis. The following recommendations for fresh specimens should be considered when the laboratorian is deciding whether to accept fresh specimens or whether a preservative collection system is more appropriate:

- Liquid: Examine within 30 minutes of passage.
- Soft: Examine within 30 minutes of passage.
- Semiformed: Examine within one hour of passage.
- Formed: Examine on the same day or the next day of passage.

(A refrigerated specimen is not acceptable for direct wet smear).

If these time frames cannot be met, then the specimens should be placed in preservative for examination at a later time.

5.1.6 Interfering Substances

There are certain substances and medications that interfere with the detection of intestinal protozoa: mineral oil, barium, bismuth, antibiotics, antimalarials, and nonabsorbable antidiarrheal preparations. After administration of any of these substances, parasites may not be recovered for one to several weeks. Two of the most commonly used substances, barium and antibiotics, such as tetracycline, modify the intestinal flora. In such cases, specimen collection should be performed seven days after completion of product use or antibiotic therapy, regardless of earlier collections.
5.1.7 Labeling the Specimen

Each specimen should be identified with the patient’s name, physician's name, identification number, and the date and time of collection. The requisition slip should accompany the specimen. It is also helpful to have any additional information such as the presumptive diagnosis and relevant travel history.

5.1.8 Examination Requested

The examination requested will depend on the infection suspected and the tests offered by the laboratory. It is important for all clients to know that the routine ova and parasite (O & P) examination does not include specific testing for the coccidia (Cryptosporidium and Cyclospora) or the microsporidia. Special stains and/or fecal immunoassays must be ordered as separate, billable tests.

5.2 Preservation of Specimens

Often there will be a lag time between the time the specimen is passed and the time it reaches the laboratory or the time it can be examined. If these time lags are longer than the times recommended in Section 5.1.5, then specimens should be preserved immediately after collection or after receipt by the laboratory. Many institutions use preservative collection kits for outpatients, and some have even decided to use them for inpatient collections, particularly if there are time lag problems related to specimen delivery. There are a number of fixatives available; regardless of which one is used, adequate mixing of the specimen and preservative is mandatory. One of the more common collection kits consists of a vial of 5% or 10% formalin (to be used for the concentration) and a vial of polyvinyl alcohol fixative (PVA) (to be used for the permanent stained smear). Any fixative should be mixed with the specimen using the ratio of three parts fixative and one part stool. Generally, fixation time should be no less than 30 minutes; preserved fecal specimens can be stored at room temperature. Local, state, and federal regulations must be followed for disposal of specimens, including specimens with fixatives (e.g., formalin, PVA). It is helpful to the laboratory if the form of the specimen (watery, loose, soft, formed, or hard) is communicated on the requisition as this cannot be determined from a well-mixed, preserved specimen.

5.2.1 Formalin

Formalin is an all-purpose fixative that is used for helminth eggs and larvae and protozoan cysts and oocysts. Formaldehyde is normally purchased as a 37% aqueous solution of formaldehyde gas. When making dilutions for parasitological studies, use the 37% solution of formaldehyde as if it were a 100% solution of formalin. Two concentrations are used (5% or 10%), and often the solutions are buffered. Although 5% is often recommended for all-purpose use, most commercial manufacturers provide 10%, which is more likely to kill all helminth eggs. Specimens collected in either concentration of formalin can be examined using the concentration techniques; specimens preserved in this fixative cannot be used for the permanent stained smear. The OSHA regulations on the use of formalin can be seen in Appendix B.

To prepare 10% buffered formalin:

(1) Combine 100 mL of formaldehyde solution (USP) with 900 mL of 0.85% saline or distilled water.

(2) Store dry in a tightly closed bottle.

(3) When ready to use, add 0.8 g buffer mixture (6.10 g Na₂HPO₄ or 0.15 g NaH₂PO₄) to 1 L of 10% formalin.

To prepare 5% buffered formalin:

(1) Combine 50 mL of formaldehyde solution (USP) with 950 mL of 0.85% saline or distilled water.
(2) Store dry in a tightly closed bottle.

(3) When ready to use, add 0.8 g buffer mixture (6.10 g Na₂HPO₄ or 0.15 g NaH₂PO₄) to 1 L of 5% formalin.

5.2.2 Sodium Acetate-Acetic Acid-Formalin (SAF)

This preservative can be used for both the concentration method and the permanent stained smear, and it does not contain mercuric chloride (as do Schaudinn’s and the classic PVA fixative). SAF is a liquid fixative (much like formalin), and the sediment is used to prepare the permanent stained smear. When using this method, consider that (1) the slides should be coated with albumin or albumin mixed with the stool sediment before the application of stool material onto the slide to assure adherence, and (2) SAF smears stained with iron-hematoxylin demonstrate better organism morphology than those stained with trichrome stain.

5.2.3 Merthiolate-Iodine-Formalin (MIF)

As a single-vial collection system, this fixative is designed for field surveys and provides some stain for the organisms. It is used for wet preparations (direct wet smear, concentration sediment) that are made either immediately after fixation or performed weeks later. Although some laboratories indicate that permanent stained smears can be prepared from this fixative, it is not recommended for use by inexperienced laboratory personnel. The clear, morphologic details necessary to identify many of the protozoa, especially trophozoites, are not seen in the permanent stained smear prepared from MIF.

5.2.4 Schaudinn's Fixative

This preservative can be used for either fresh stool specimens or other samples (sigmoidoscopy specimens) from the intestinal mucosal surface. When the specimen is received in the laboratory, slides can be prepared by spreading some of the stool onto the slides using applicator sticks and immediately immersing the slides into Schaudinn’s fixative. It is important that the smears not dry before fixation.

To prepare stock solution of Schaudinn’s fixative, prepare a saturated aqueous solution of mercuric chloride, which is then combined with ethyl alcohol:

(1) Using a beaker as a water bath, combine 110 g of mercuric chloride (HgCl₂) with 1000 mL distilled water, and boil (use a fume hood) until the mercuric chloride is dissolved.

(2) Let stand until room temperature has been reached and crystals have formed.

(3) Combine 600 mL of mercuric chloride (saturated aqueous solution) with 300 mL of ethyl alcohol (95%).

(4) Immediately before use, add 5 mL glacial acetic acid per 100 mL of stock solution. The mixture containing acetic acid is stable for two weeks.

5.2.5 Polyvinyl Alcohol Fixative

Polyvinyl alcohol (PVA) is a plastic resin that is combined with Schaudinn’s fixative. The PVA serves as an adhesive that helps “glue” the stool onto the glass slide. Fixation is still accomplished by the Schaudinn’s fixative. Although both a concentration and permanent stained smear can be prepared from PVA-fixed material, it is recommended that the concentration be performed on formalin-fixed material and the permanent stained smear be prepared from the PVA-fixed material. PVA fixative is very helpful when used with liquid specimens and should be used as other fixatives in the ratio of three parts fixative
to one part stool. Preserved specimens should be stored at room temperature. Commercially prepared PVA fixative is available from many suppliers; consequently, most laboratories do not prepare their own solutions.

**NOTE:** Due to hazardous waste disposal concerns, there has been a great deal of interest in developing fixatives that do not have a mercuric chloride base\(^{7,21}\); however, to date, those available do not give the same quality of fixation as Schaudinn’s PVA fixative, which contains the mercuric chloride base. The best substitute to use with trichrome staining appears to be zinc sulfate-based PVA or one of the single-vial collection systems.\(^8\)

### 5.3 Collection Kits

#### 5.3.1 In-House Kit Preparation

Depending on the fixative system selected, one would need vials containing the various fixatives and appropriate vial labels containing the poison warning, expiration date, and the name of the product, etc. Often the vials are purchased and then put into the kit package (containing stool collection carton, applicator sticks, and written instructions). These components may be packaged into a paper bag and given to the patient for stool collection on an outpatient basis. Depending on the geographic area and population serviced, instructions in multiple languages may be needed (these are usually provided with commercial kits).

#### 5.3.2 Commercial Kits

There are a number of companies that provide stool collection kits (premade), kit components, or bulk reagents. The majority of the kits available offer any combination desired for stool collection (clean vial, formalin, PVA, SAF, MIF, or one of the single-vial systems). The most widely used/recommended kit components include one vial of PVA fixative (for the preparation of the permanent stained smear) and one vial of 5\% or 10\% formalin (for the concentration method). These kits come with a set of written instructions. Some have a stool collection cup enclosed, but many do not. This does not seem to present any problem on the part of the user.

### 5.4 Shipment of Specimens\(^{22}\)

#### 5.4.1 Slides

Prepared slides do not require a double mailing container like that required for vials containing liquid. The slides can be packed in boxes or any other suitable container that will prevent breakage during shipping. Slides should be individually wrapped in tissue. If the slides are mounted with mounting medium, make sure they are completely dry before shipping. Containers made specifically for the safe transport of microscope slides are available commercially and are a safe and economical alternative to tissue and boxes. Flat cardboard containers are less protective for glass slides which often arrive broken. The plastic containers, which hold five slides and include a snap-top lid, provide better protection during transit.

#### 5.4.2 Collection Vials

Double mailing containers should be used in shipping any parasitological material other than prepared microscope slides. The inner tube is usually an aluminum screw-cap tube and the outer tube is cardboard. To absorb leaks that might occur during shipping, the vials or tubes in the inner container should be padded with cotton or other absorbent material. There are other mailing kits available using resealable bags with absorbent padding as the secondary container and then a plastic or cardboard box as the mailing container. All packages should conform to postal regulations. Contact the postal service with questions.
Make sure that the appropriate labels are used and that an information sheet is enclosed inside the container (with the patient’s name, specimen identification, person to whom it is being sent, and the return address). The sheet should be between the inner tube and outer tube (not in contact with the specimen container). b

6 Macroscopic Examination

A macroscopic examination should be performed on unpreserved specimens and should provide information on the age of the specimen and its physical characteristics.

6.1 Age

The age of fresh fecal specimens is an important factor in the diagnosis of parasitic infections. Because protozoans in the trophozoite stage degenerate soon after passage from the body, the date and time of passage of the stool specimen is mandatory. A “No Parasites Found” report on a 24-hour-old unpreserved watery stool may be meaningless. However, the same report on a formed stool may be significant. See Section 5.1.5.

Unfortunately, the majority of fecal specimens submitted to the laboratory do not have the time of collection noted. Frequently, the time indicated is the time the specimen was received in the laboratory. For this reason, as well as potential time lag issues, most laboratories require fecal specimens to be submitted in one or more of the available stool preservatives.

6.2 Consistency

The consistency of the stool specimen should be noted (as watery, loose, soft, formed, or hard). Trophic amoebae or flagellates are found most frequently in watery or loose specimens, and they tend to disintegrate rapidly at room temperature. Trophozoites and cyst stages may be found in soft specimens. Cyst stages are found most frequently in formed specimens and will not lose characteristic morphology at room temperature for approximately one day. 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, and infective Strongyloides stercoralis filariform larvae may develop from the noninfective rhabditiform larvae normally found in the stool of an infected patient.

6.3 Abnormalities

The presence of blood and/or excessive mucus can be noted by visual inspection. The observation of proglottides or scolecies and/or helminth adults can be enhanced by mixing a small amount of specimen with water and slowly straining the stool through a piece of wet gauze (of single thickness).

6.4 Clinical Relevance of Fresh Specimens and Organism Recovery

Although refrigeration of the fresh fecal specimen will delay the deterioration of the parasitic organisms, preservation of trophozoite morphology is best accomplished using fecal preservatives. Freezing the fecal specimen is not recommended, because the characteristic morphology of the parasitic organisms may be altered. Fecal specimens should never be incubated. Based on the stool consistency (watery, loose, soft, formed, or hard), the more liquid the stool, the more critical the need for stool preservatives to preserve

b In the U.S., there are several sites providing regulations regarding transportation/shipping of specimens that may be helpful for the reader: (International Air Transportation Association, IATA) www.iata.org, (Department of Transportation, Research and Innovative Technology Administration) www.myregs.com/dotspa/, www.rspa.dot.gov, and www.hazmat.DOT.gov. Also, hazardous materials questions can be submitted via telephone at: 1.800.HMR.4922 (1.800.467.4922).
organism morphology. The handling of the stool specimen and adherence to, or noncompliance with examination and/or fixation guidelines determines whether the etiologic agent is found and identified. Fresh specimens should be examined as:

- **Liquid:** Examine within 30 minutes of passage.
- **Soft:** Examine within 30 minutes of passage.
- **Semiformed:** Examine within one hour of passage.
- **Formed:** Examine on same day or next day of passage. (A refrigerated specimen is not acceptable for direct wet smear).

**NOTE:** See Section 5.1.5.

7 Microscopic Examination (Wet Preparation)

For formalin-preserved or other preserved specimens, it usually is not necessary to perform the direct wet mount examination. This is consistent with the College of American Pathologists (CAP) checklist. The O&P examination for fresh specimens includes examination of the direct wet mount (liquid, very soft stool), concentration wet mount, and permanent stained smear. The O&P examination for preserved specimens includes examination of the concentration wet mount and permanent stained smear.

7.1 Direct Smear of Fresh Specimen (Unfixed)

Direct smears require the least amount of equipment to prepare, time to read and, if positive, provide the physician with some preliminary information.

7.1.1 Saline or Unstained Mount

This preparation is used for the examination of most intestinal parasites in various stages (trophozoites, cysts, larvae, and eggs). Trophozoite motility and chromatoidal bodies within a cyst may be more easily seen in the direct wet mount than in other types of preparations. This type of preparation is suggested if fresh specimens are submitted for examination. If no organisms are found, there is little need for examining an iodine preparation. To allow for the optimal identification of the parasites, the density of the specimen (fluid to fecal solids ratio) is important. The specimen, when placed on a microscope slide and coverslipped, should be just thick enough to be able to read fine newsprint through it. If the specimen is too thin, organisms may be overlooked; if it is too thick, the search is too difficult. For fresh, unpreserved specimens, a 0.85% NaCl solution may be used as the diluent if motility of organisms is to be seen.

7.1.2 Iodine Mount

The iodine-stained preparation is used to render the nuclei and most other inclusions within protozoan cysts more visible. Trophozoites are usually distorted in this preparation. Helminth eggs and larvae are recognizable; however, the internal morphologic details are occasionally obscured by staining. Some laboratories use an iodine mount for the routine detection of Cryptosporidium oocysts, because these oocysts usually do not take up the iodine and remain highly refractile, while yeasts and other fecal debris take on the yellow color. However, the iodine stain is not as sensitive a method as the modified acid-fast stains or fecal immunoassay methods. Iodine stain has some variability in the intensity of color. A strong iodine solution tends to coagulate the fecal particles and reduce the refractile nature of the organism. However, if the solution is too weak, the internal morphology of the organisms will not stain properly. Follow preparation protocols carefully for either of the iodine solutions given below and heed the recommended stability periods.

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7.1.3 Direct Smear Procedure

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, and to check on organism motility. Following are instructions on how to perform the direct smear procedure and what supplies are needed.

7.1.3.1 Specimen

Any fresh stool specimen (liquid or very soft) that has not been refrigerated and is less than 30 minutes old may be used.

7.1.3.2 Reagent

The following reagent is needed: 0.85% NaCl.

To prepare 0.85% NaCl:

1. Dissolve NaCl (0.85 g) and distilled water (100 mL) in a flask or bottle, using a magnetic stirrer.
2. Distribute 10 mL into each of ten 16 x 125 mm, screw-capped tubes.
3. Label as 0.85% NaCl with an expiration date of one year.
4. Sterilize by autoclaving at 121 °C for 15 minutes.
5. When cool, store at 4 °C.
6. Prior to each use, saline should be clear and visibly free of any bacterial or fungal contamination.

- D’Antoni’s Iodine

To prepare D’Antoni’s iodine, the following ingredients are necessary: potassium iodide (KI) (1.0 g); powdered iodine crystals (1.5 g); and distilled water (100 mL).

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

- Lugol’s Iodine 1:5 Dilution

To prepare Lugol’s iodine, the following ingredients are necessary: potassium iodide (KI) (10 g); powdered iodine crystals (5 g); and distilled water (100 mL).

1. For solution preparation, follow the same directions listed above for D’Antoni’s iodine, including the expiration date of one 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 the color of strong tea, and it should be discarded when it lightens in color, usually within 10 to 14 days.

7.1.3.3 Quality Control (QC)

- The working iodine solution should be checked each time it is used or periodically (once a week). The iodine should be free of any signs of bacterial and/or fungal contamination. The color should be that of strong tea (discard if too light). Protozoan cysts should contain yellow-gold cytoplasm, brown glycogen material, and light to dark brown chromatin in the nuclei. The nucleus of *Iodamoeba butschlii* usually will not stain; instead it will be greenish and refractile. If organisms containing glycogen have been stored in formalin for several weeks or longer, the glycogen may not stain dark brown; it may be much lighter or unstained.

- Human white blood cells (WBCs) mixed with stool that is negative for parasites can be used as a QC specimen. The white cells will stain with the same color as that seen in the protozoa.

All QC results should be appropriately recorded.

7.1.3.4 Procedure

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

(2) 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 solutions (use separate sticks for each). If the specimen is watery or mucoid and difficult to pick up on a stick, try placing two sticks tightly together and use them to “scoop” the specimen. Again, mix well.

(3) Place a 22-mm coverslip on each suspension.

(4) Systematically scan the unstained preparation using the 10x objective. The entire coverslip area should be examined.

(5) If something suspicious is seen, the 40x objective can be used for more detailed study. At least one-third of the coverslip should be examined using the 40x objective, even if nothing suspicious has been seen.

(6) If any organisms are suspected, systematically examine the iodine-stained preparation in the same manner as the unstained preparation. If possible, identify those organisms present.

7.1.3.5 Results

Protozoan trophozoites, cysts, oocysts, and helminth eggs and larvae may be seen and should be identified.

7.1.3.6 Reporting Results

(1) Identify motile trophozoites. These may or may not be identified (depending on the clarity of the morphology). An example of a positive report is: *Giardia lamblia* trophozoites present.

(2) Identify protozoan cysts. These may or may not be identified (depending on the clarity of the morphology). An example of a positive report is: *Entamoeba coli* cysts present.
(3) Identify helminth eggs and/or larvae. An example is: *Ascaris lumbricoides* eggs present; *Strongyloides stercoralis* rhabditiform larvae present.

(4) Identify and report artifacts and/or other structures as follows: Moderate Charcot–Leyden crystals present; few red blood cells (RBCs) present.

7.1.3.7 Limitations of the Procedure

- Protozoa found in wet smears usually need to be confirmed by permanent stained smears. Some protozoa are small and difficult to identify using only the direct wet smears.23
- Confirmation is particularly important in the case of *Entamoeba histolytica/E. dispar* versus *Entamoeba coli*.

7.1.3.8 Procedure Notes

- Some laboratorians prefer to make the saline and iodine mounts on separate 1" x 3" slides. Often, there is less chance of getting fluids on the microscope stage if separate slides are used (less total fluid on the slide/under the coverslip). Others prefer to use 2" x 3" slides and place both preparations on the same slide.
- The microscope light should be reduced for low-power observations, because most organisms are overlooked when bright light is used. Reduce the light by closing the iris diaphragm rather than by racking the condenser down. 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.

7.1.4 Clinical Relevance of Direct Wet Mounts

Direct examination of unpreserved stool specimens is used primarily for the examination of motile protozoan trophozoites. However, with a few exceptions, protozoans should not be identified solely by this procedure.23-25 Furthermore, studies 26-30 show that rarely will a parasite be found by the direct wet mount examination only and not found by the concentration and permanent stained slide methods. If the fecal specimen has been received in preservative, do not perform the direct wet mount examination, but proceed to the concentration and permanent stained smear procedures.

7.2 Concentration Procedures

Fecal concentration has become a routine procedure as a part of the complete ova and parasite examination, and it 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 cysts, oocysts, and helminth eggs and larvae from fecal debris by centrifugation and/or differences in specific gravity. The principle of the sedimentation technique lies in the relative density of the organism to that of the solution in which it is contained. The more dense organisms will settle out of the less dense solution, either by gravity or by centrifugation. Most of the currently used techniques incorporate straining and defatting steps. The straining step usually requires a piece of wet, medium-mesh gauze of single thickness to remove larger pieces of fecal debris. Defatting agents, such as ether, ethyl acetate,4,5 or other xylene substitutes, are used. The defatting agents dissolve fecal fats and make the fecal debris float, thereby clarifying the specimen without distorting helminth eggs and larvae or the protozoan cysts. Protozoan trophozoites are usually distorted or destroyed by concentration procedures.

A rough estimate of the adult worm load in a patient can be made from an estimate of the total number of eggs in a 24-hour stool specimen.
NOTE: Concentrated stool sediment can be used for the detection of *Cyclospora cayetanensis* oocysts using UV epifluorescence. On the wet mount examination of concentrated stool sediment, oocysts measure approximately 8 to 10 µm, and will autofluoresce green (450 to 490 DM excitation filter) or blue (365 DM excitation filter) under UV epifluorescence.

7.2.1 Formalin-Ether, Formalin-Ethyl Acetate, or Other Defatting Agent

This is probably the most commonly used concentration procedure today. Ethyl acetate, and more recently xylene substitutes have been substituted for ether for safety reasons. These defatting reagents are used exactly as ether and perform acceptably on both formalinized and fresh specimens. Ethyl acetate and ether procedures can be used on PVA-preserved specimens.

7.2.1.1 Specimen

The specimen should be a fresh or preserved stool (5 or 10% buffered formalin, SAF, or one of the single-vial systems). PVA-preserved specimens can be used, but precipitates may form in the defatting step and some organisms, especially protozoa, may be removed or distorted. For very watery specimens, a single centrifugation step (ten minutes at 500 x g) will replace the typical concentration method; do not use the defatting agent.

7.2.1.2 Reagents

The following reagents are needed:

- Defatting agent (ethyl acetate or other defatting agent. See Section 7.2.)
- Formalin (5% or 10% buffered, SAF or one of the single-vial fixatives. See Section 5.3.2.)
- 0.85% NaCl (see Section 7.1.3.2.)
- D’Antoni’s iodine or 1:5 dilution of Lugol’s iodine (see Section 7.1.3.2.)

7.2.1.3 Quality Control

- The reagents should be checked each time they are used. The formalin and saline should appear clear and without any visible contamination.
- Known positive specimens should be concentrated and organism recovery verified at least quarterly, particularly after the centrifuge has been recalibrated. Record results on an appropriate sheet.
- Human white blood cells (WBCs) mixed with stool that is negative for parasites can be used as a QC specimen. The white cells will stain with the same color as that seen in the protozoa.

7.2.1.4 Procedure

1. Transfer one-half teaspoon 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/formalin thoroughly. Let the mixture stand a minimum of 30 minutes for fixation. If the specimen is already in 5% or 10% formalin, SAF, or one of the single-vial system fixatives, restir the stool/formalin mixture.

2. Depending on the density of the specimen, strain a sufficient quantity through wet gauze into a conical 15-mL or 50-mL centrifuge tube to give the desired amount of sediment (0.5 to 1 mL) in
Step 3 below. Usually, 8 mL of the stool/formalin mixture prepared (as outlined in Section 7.2.1.1) will be sufficient. If the specimen is received in vials of preservative (5% or 10% formalin) SAF or one of the single-vial fixatives), then approximately 3 mL will be sufficient unless the specimen has very little stool in the vial.

3) Add 0.85% NaCl almost to the top of the tube and centrifuge for 10 minutes at 500 x g. The amount of sediment obtained should be approximately 0.5 to 1.0 mL.

4) Decant the supernatant fluid and resuspend the sediment in saline. Add saline almost to the top of the tube, mix, and centrifuge again for 10 minutes at 500 x g. This second wash may be eliminated when there is little color distinction between the supernatant fluid and the sediment after the first wash. If the supernatant fluid is cloudy or dark, decant the fluid, resuspend the sediment in saline, and recentrifuge. However, remember that the more the specimen is rinsed/manipulated, the more likely it is that some organisms may be lost from the specimen in the wash solutions.

5) Decant the supernatant fluid and resuspend the sediment on the bottom of the tube in 10% formalin. Fill the tube two-thirds full only. Let the tube stand for at least ten minutes before adding the defatting agent in Step 6. If the amount of sediment left in the bottom of the tube is very small or the original specimen contained a lot of mucus, or the specimen is too old, do not add defatting agent. Instead, merely add the formalin, spin, decant, and examine the remaining sediment.

6) Add 4 to 5 mL of defatting agent. Stopper the tube and shake it vigorously for at least 30 seconds. Hold the tube so the stopper is directed away from your face or that of a coworker.

7) After a 15- to 30-second wait, carefully remove the stopper.

8) Centrifuge for ten minutes at 500 x 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 the remainder of the defatting agent at the top.

9) Free the plug of debris by ringing the plug with an applicator stick; decant all of the supernatant fluid. While continuing to hold the tube upside down, use a cotton-tipped applicator to remove all fluid from the wall of the tube. Then, turn the tube right-side up. This is particularly important when using plastic centrifuge tubes where fluid will adhere to the sides of the tube; the fluid tends to flow from the walls of the tube more completely if the tube is glass, rather than plastic. If the specimen contains a lot of mucus or the amount of sediment is minimal, do not turn the tube upside down, because the sediment may be accidentally poured off with the supernatant fluid. Keep the tube in a more horizontal position. The supernatant fluid can also be aspirated while the tube is upright.

10) While the sediment is still somewhat solid, add a drop or two of saline or formalin to the sediment; mix, and add a small amount of material to a slide; add a coverslip (22 x 22 mm is most commonly used; 22 x 40 mm may also be acceptable), and examine.

11) Systematically scan using the 10x objective. The entire coverslip area should be examined.

12) If something suspicious is seen, the 40x objective can be used for more detailed study. At least one-third of the coverslip should be examined using the 40x objective, even if nothing suspicious has been seen. As in the direct wet smear, iodine can be added to enhance morphological detail. Although some people seal these slides and examine the mounts using the 100x oil immersion objective, this is impractical and not routinely done, and definitive identification of most of the protozoa will be made from the permanent stained smear.
(13) Wet mounts can be held before examination or for teaching by placing the slide in a Petri dish containing a wet paper towel with the slide resting on broken applicator sticks; the atmosphere will be saturated but not wet.

7.2.1.5 Results

Protozoan cysts, oocysts, and helminth eggs and larvae in concentrated stool preparations may be seen in greater numbers than in a direct preparation.

7.2.1.6 Reporting Results

(1) Identify protozoan cysts. These may or may not be able to be identified depending on the clarity of the morphology. An example of a positive report is: *Giardia lamblia* cysts present.

(2) Identify helminth eggs and/or larvae. An example of a positive report is: *Trichuris trichiura* eggs present.

(3) Report artifacts and/or other structures. For example: Few Charcot–Leyden crystals present; or moderate WBCs present.

7.2.1.7 Procedure Notes

- Tap water may be substituted for 0.85% NaCl throughout this procedure; some laboratorians prefer to use 10% formalin for all the rinses throughout the procedure. However, if tap water rinses are used on fresh specimens (and occasionally formalinized specimens), *Blastocystis hominis* is frequently destroyed, and free-living contaminants might be introduced.

- Ethyl acetate is widely recommended as a substitute for ether. It can be used the same way in the procedure. Other biodegradable solvents are safer and have been used successfully for the most commonly found human parasites.

- Although definitive studies have not been reported, over the years one of the committee members has noted the following: As a formalin-preserved specimen ages, using a defatting agent might result in a decrease of parasites. Smaller parasites are affected in as little as one week. If it is suspected that the specimen is too old do not add the defatting agent.

  — After the plug of debris is rimmed and excess fluid decanted, while the tube is still upside down, the sides of the tube can be swabbed with a cotton-tipped applicator stick to remove excess defatting agent. This is particularly important to do if one is working with plastic centrifuge tubes. After swabbing the tube, add several drops of saline or formalin before preparing the wet smear for examination.

  — If excess defatting agent is in the smear of the sediment prepared for examination, there will be bubbles present under the coverslip, which will obscure the material and make it difficult to see. The suspension will tend to spread across the slide, and it will be difficult to make a good preparation.

- If specimens are received in SAF, then begin at Step 2, Section 7.2.1.4.

- If specimens are received in PVA, the first two steps of the procedure (see Steps 1 and 2, Section 7.2.1.4) should be modified as follows:
— Immediately after stirring the stool/PVA mixture with applicator sticks, pour approximately half of the material into a tube (container optional) and add 0.85% saline almost to the top of the tube.

— Filter approximately 3 mL of the stool/PVA/saline mixture through wet gauze into a 15-mL centrifuge tube. The standard procedure is followed from here to completion, beginning with Step 3 in Section 7.2.1.4.

- Regardless of the preservative used, too much or too little sediment will result in an ineffective concentration.

### 7.2.1.8 Limitations of the Procedure

- Protozoa seen in wet smears should usually be confirmed by permanent stained smears. Some protozoa are small and difficult to identify using just the wet smears. Even though a positive identification can be made from a wet preparation, detection of the same organism(s) in a permanent stain confirms the identification.

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

- Certain organisms, *Giardia lamblia*, hookworm eggs, and occasionally *Trichuris* eggs may not concentrate as well from PVA-preserved specimens as they do from those preserved in formalin. In moderate-to-heavy infections with those eggs mentioned above, the number of helminth eggs present ensures detection, regardless of the type of preservative used. Also, the morphology of *Strongyloides stercoralis* larvae and *Entamoeba coli* cysts are not as clear from PVA as from specimens fixed in formalin.

- *Isospora belli* oocysts are routinely missed in the concentrate sediment when concentrated from PVA-preserved specimens.

- Centrifugation at 500 x g for a minimum of ten minutes is sufficient to recover *Cryptosporidium* oocysts and microsporidial spores, as well as protozoa and helminth eggs and larvae.

### 7.2.2 Flotation: Zinc Sulfate

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, most helminth eggs (operculated eggs and/or very dense eggs such as unfertilized *Ascaris* eggs) do not concentrate well with the flotation method.\(^1\,^2\,^3\,^0\) Formalin-fixed specimens have much less distortion, and opercula are less likely to pop. To ensure detection of all possible organisms, both the surface film and the sediment should be examined. The specimen should be examined within 15 to 20 minutes of the final centrifugation; beyond this time frame, organisms begin to distort and disintegrate.

#### 7.2.2.1 Specimen

- The specimen should be fresh or preserved stool (5% or 10% buffered formalin, SAF, or one of the single-vial fixatives).

- For extremely watery specimens, a single centrifugation step (10 minutes at 500 x g) replaces the typical concentration method.
7.2.2.2 Reagents

The following reagents are used:

- Formalin (5% or 10% buffered), SAF, or one of the single-vial fixatives
- 0.85% NaCl
- Zinc sulfate (33% aqueous solution)

To prepare zinc sulfate (33% aqueous solution), the necessary ingredients are zinc sulfate (~330 g) and distilled water (670 mL).

1. Using a magnetic stirrer, dissolve the zinc sulfate in distilled water in an appropriate flask or beaker.

2. Using a hydrometer, adjust the specific gravity to 1.20 for use with formalinized specimens (or 1.18 for use with fresh specimens) by the addition of more zinc sulfate or distilled water.

3. Store in a tightly capped bottle with an expiration date of 36 months or when the specific gravity changes ±0.02.

7.2.2.3 Quality Control

- The reagents should be checked each time they are used. The formalin, saline, and zinc sulfate should appear clear, without any visible contamination. The specific gravity of the zinc sulfate should be checked monthly: it should be 1.18 when using fresh specimens and 1.20 when concentrating formalin-preserved specimens.

- Known positive specimens should be concentrated and organism recovery verified at least quarterly, particularly after the centrifuge has been recalibrated or a new lot of zinc sulfate is being used. Record results on the appropriate sheet.

7.2.2.4 Procedure

1. Transfer one-half teaspoon 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/formalin thoroughly. Let the mixture stand a minimum of 30 minutes for fixation. If the specimen is already in 5% or 10% formalin (or SAF), restir the stool/formalin mixture.

2. Depending on the size and density of the specimen, strain a sufficient quantity through wet gauze into a conical, 15-mL, round-bottom tube to give the desired amount of sediment (0.5 to 1 mL) in Step 3 below. Usually, 8 mL of the stool/formalin mixture prepared in Step 1 is sufficient. If the specimen is received in vials of preservative (5% or 10% formalin or SAF), then approximately 3 to 4 mL is sufficient, unless the specimen has little stool in the vial.

3. Add 0.85% NaCl (see Section 7.2.1.7, Procedure Notes) almost to the top of the tube and centrifuge for ten minutes at 500 x g. The amount of sediment obtained should be approximately 0.5 to 1 mL. Too much or too little sediment results in an ineffective concentration.

4. 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 within 2 to 3 mm of the rim with additional zinc sulfate.

5. Centrifuge for one minute at 500 x 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.

(6) Without removing the tube from the centrifuge, or carefully removing it to a rack and allowing it to stand for one minute, remove one or two drops of the surface film with a Pasteur pipet 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 that the loop is parallel with the surface of the fluid. Make sure the pipet tip or wire loop is not below the surface film.

(7) Add a coverslip (22 x 22 mm or 22 x 24 mm) to the preparation. Iodine may be added to the preparation, preferably after the scan for helminths is completed.

(8) Systematically scan using the 10x objective. The entire coverslip area should be examined.

(9) If something suspicious is seen, the 40x objective can be used for more detailed study. At least one-third of the coverslip should be examined using the 40x objective, even if nothing suspicious has been seen. As in the direct wet smear, iodine can be added to enhance morphological detail of cysts.

(10) Wet mounts can be held before examination or for teaching by placing the slide in a petri dish containing a wet paper towel with the slide resting on broken applicator sticks; the atmosphere will be saturated but not wet.

7.2.2.5 Results

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

7.2.2.6 Reporting Results

(1) Identify protozoan cysts. These may or may not be identified (depending on the clarity of the morphology). An example of a positive report is: *Giardia lamblia* cysts present.

(2) Identify helminth eggs and/or larvae. An example of a positive report is: *Trichuris trichiura* eggs present.

(3) Report artifacts and/or other structures. Examples might be: Few macrophages present; moderate WBCs present.

7.2.2.7 Procedure Notes

- Tap water may be substituted for 0.85% NaCl throughout this procedure. Some laboratorians prefer to use 5% or 10% formalin for all the rinses throughout the procedure.

- If fresh stool specimens are used (no preservatives), then the zinc sulfate should be prepared with a specific gravity of 1.18.

- Remember to use a round-bottom tube, rather than a centrifuge tube.

- If specimens are received in SAF, then begin at Step 2, Section 7.2.2.4.

- If fresh specimens are received, the standardized procedure requires the stool to be rinsed in distilled water before the addition of zinc sulfate in Section 7.2.2.4. However, the addition of fresh stool to distilled water frequently destroys any *Blastocystis hominis* present; it is not a recommended approach.
- Some prefer to carefully remove the tubes from the centrifuge before sampling the surface film. This is acceptable; however, there is more chance the surface film will be disturbed before sampling.

- Instead of collecting the surface film with a loop, some laboratorians prefer to gently add a small amount of zinc sulfate to the tube, so the fluid forms a slightly convex meniscus. A coverslip is then placed on top of the tube so that the under-surface touches the meniscus. The tube is left undisturbed for five minutes. The coverslip is then carefully pulled up from the tube and placed on a slide for examination.

- When using the hydrometer (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 of the 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 and subtracted if below 20 °C.

7.2.2.8 Limitations of the Procedure

- Protozoa found in wet preparations should usually be confirmed by permanent stained smears. Some protozoa are small and difficult to identify using just the direct wet smears.

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

- Protozoan cysts and thin-shelled helminth eggs are subject to collapse and distortion when left for more than a few minutes in contact with the high specific gravity zinc sulfate. The surface film should be removed for examination within five minutes of the time the centrifuge comes to a stop. The longer the organisms are in contact with the zinc sulfate, the more distortion will be evident on microscopic examination of the surface film.

- If zinc sulfate is the only concentration method used, both the surface film and the sediment should be examined to ensure detection of all possible organisms.

It is highly recommended that the sedimentation concentration method be used to obtain sedimented specimen for special stains (modified acid-fast, modified trichrome) for the coccidia and microsporidia. It is also recommended that concentration sediment be used as the specimen of choice for FA immunoassays for Giardia lamblia and/or Cryptosporidium spp. Concentration sediment from PVA-fixed specimens is not acceptable for immunoassay and special stains (e.g., modified acid-fast stain).

7.2.3 Commercial Concentrators

There are a number of commercially available systems designed after the formalin-ether concentration technique. If used as intended by the manufacturer, they compare favorably to the classical method. Generally, the systems contain all disposable supplies, conical tubes, caps, filtration devices, and reagents needed to concentrate a fecal specimen. Some are designed as a completely closed system that can concentrate the entire contents of its companion collection vial. The advantages of this type of system are that it minimizes exposure of the user to potentially infectious agents and hazardous chemicals, and it reduces the variation of results due to specimen sampling. A disadvantage is that there is no reserve specimen if a problem arises (the original vial containing the stool can be saved, but in most commercial systems there is no appropriate lid for the container). Use of commercial concentration systems will vary, depending on user preferences.
7.2.4 Clinical Relevance

Because concentration procedures increase the chances of detecting organisms by insuring a more thorough examination, they should be part of the laboratory’s workup of all fecal specimens. Note, however, that these procedures are not designed to recover protozoan trophozoites. The formalin-ether concentration technique and its modifications using other defatting agents is the best overall concentration technique available today. It is the easiest to perform and least subject to technical error.

8 Microscopic Examination (Permanent Stained Smears/Other Methods)

8.1 General Considerations

Permanent stained fecal smears are used to identify intestinal protozoan cysts, especially trophozoites, and to provide a permanent record of findings. They also provide a means of consulting with a specialist when necessary. It is generally recognized that stained fecal films are the single most productive means of examining a stool specimen for intestinal protozoa. Small protozoa that are missed by direct smear and concentration techniques are often seen on the stained smear. It also gives laboratories the ability 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. According to the CAP checklist, the O&P examination for fresh specimens includes examination of the direct wet mount (liquid, very soft stool), concentration wet mount, and permanent stained smear. The O&P examination for preserved specimens includes examination of the concentration wet mount and permanent stained smear.

8.1.1 Essential Ingredients of a Permanent Smear

Fixative: Histological fixatives are required to preserve the structure of the cells in as near a life-like condition as possible. The fixative should penetrate the material rapidly; kill the cell and prevent post-mortem changes; preserve the physical structure of the cell; render the cell insoluble to subsequent processing; increase the refractive index; and render the material receptive to stains. Mercuric chloride is the most commonly used and best histologic fixative used in the preparation of permanent smears in parasitology, although mercury substitutes are now available and are becoming more widely used.

Adhesive: This component of the process provides an adhesive that is used for adherence of the cells to the slide while remaining freely permeable to the staining reagents. PVA is used for this purpose. Other adhesives are used in other methods, not including the use of PVA.

Washings: This step removes the histological fixative. The mercuric chloride should be removed, because it forms a residue on the slide that interferes with the identification of the parasites. Iodine alcohol is used for this purpose in the trichrome and Heidenhain’s hematoxylin staining methods.

Mordant: Because many dyes are insufficiently charged to remain attached, a mordant is used to increase the charge. It appears that the mordant becomes attached to the cell membranes by a covalent bond, and the dye is rendered insoluble in neutral solutions used in dehydration. The trichrome procedure does not use a true mordant; however, in the Heidenhain’s hematoxylin procedure, ferric alum is used as the mordant.

Staining: A regressive-type staining procedure (i.e., excess stain is allowed to accumulate in the cells, and the material is then destained until the proper differentiation is obtained) is used in both the trichrome and Heidenhain’s hematoxylin procedures.

Destaining: The most critical step in the regressive-type staining procedure is the destaining step. Because the destaining of the material continues as long as the destaining reagent is in contact with the
material, this time interval should be considered in the protocol. The destaining reagent usually contains an acid. Acetic acid is used in the trichrome procedure and picric acid in the Heidenhain’s hematoxylin procedure.

**Dehydration:** This process uses a series of alcohols of increasing strength to remove water from the material; this is necessary because moisture will cause the material to decolorize and because most mounting media are not miscible with water.

**Clearing:** Clearing agents remove the dehydrating agent from the material and leave it in a condition for mounting. This reagent is needed because the mounting medium is no more miscible with alcohol than with water. The clearing agent causes the specimen to attain a high index of refraction, therefore becoming more transparent or clear. Carbolxylene, xylene, or xylene substitutes\(^1,3\) are used as the clearing agents in the trichrome and Heidenhain’s hematoxylin procedures, as well as other staining methods that require clearing agents.

**Mounting:** This consists of cementing the material to be examined between the slide and a coverslip for the purpose of preserving the smear for further microscopic study. A mounting material and a coverslip of No. 1 thickness are generally used in parasitology.

**Mounting Alternative:** Since many laboratories elect not to mount their permanent stained smears using a mounting medium, there is an alternative method that works quite well.

1. Remove the slide from the last xylene substitute dish and allow the slide to completely air dry.
2. About five to ten minutes prior to microscopic examination of the slide, add a drop of immersion oil directly onto the dry permanent stained smear. Allow the oil to sink into the dry fecal material on the slide.
3. Just prior to reading, add a No. 1 coverslip, add one additional drop of immersion oil onto the top of the coverslip, and proceed to examine the smear. **NOTE:** Make sure to add the coverslip, since dry fecal material is quite hard and might accidentally scratch the oil immersion objective lens if not protected.
4. If the smear is positive and the slide will be kept, the oil can be removed in xylene (or xylene substitute) and then prepared as a permanent mount. It can also be stored unmounted.

### 8.2 Trichrome Stain

The trichrome technique of Wheatley 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 the intestinal protozoa, human cells, yeast, and artifact material. The reagents are comparatively stable, and because chromatin and cytoplasm stain different colors, organisms are usually easier to detect in this stain as compared to iron hematoxylin, which provides a more monotone color. The Wheatley trichrome stain is available commercially.

#### 8.2.1 Specimen

The specimen usually consists of fresh stool smeared on a microscope slide in such a way to provide areas of both thin and thick density (apply stool to the slide by using applicator sticks and “rolling” the stool onto the slide). The slide is immediately fixed in Schaudinn’s fixative. Using applicator sticks, PVA-preserved stool can also be smeared on a slide (after the excess PVA is absorbed on a paper towel for three to five minutes) and allowed to air dry. As an option to expedite rapid drying and enhance the adhesion of fecal material to the slide during staining, PVA-prepared stool smears may be heat-fixed on a
slide warmer at 60 °C until dry (about four minutes) or placed in an incubator at 35 to 37 °C for an hour or dried overnight at room temperature.

Stool preserved in SAF or other fixatives can also be used.

### 8.2.2 Reagents

- **Trichrome stain**

  To prepare trichrome stain, the following reagents are necessary: chromotrope 2R (6 g) and light green SF (3 g); or (light green SF–1.5 g and fast green FCF–1.5 g) phosphotungstic acid (7 g); glacial acetic acid (10 mL); and distilled water (1000 mL).

  1. Prepare the stain by adding 10 mL of glacial acetic acid to the dry components in a dry volumetric flask.

  2. Allow the mixture to stand (ripen) for 15 to 30 minutes at room temperature. (Cover the flask with foil and assure all dry components are dampened with the acid.)

  3. Add 1000 mL of distilled water while mixing slowly. Mix thoroughly. Properly prepared stain is purple.

  4. Store in a glass or plastic bottle at room temperature. The shelf life is 24 months, although, as long as the control smears are within normal limits, the stain can continue to be used.

- **70% ethanol**

- **70% ethanol plus iodine**

  To prepare 70% ethanol plus iodine, use the following procedure:

  1. Prepare a stock solution by adding iodine crystals to 70% alcohol until a dark solution (1 to 2 g per 100 mL) is obtained.

  2. To use, dilute the stock solution with 70% alcohol until a dark, reddish brown (port wine) or strong tea color is obtained. As long as the color is acceptable, new working solution does not have to be replaced. Replacement time depends on the number of smears stained, the size of the container, and the amount of light exposure (one week to several weeks).

- **90% ethanol, acidified**

  To prepare 90% ethanol, acidified:

  1. Slowly add 90% ethanol (99.5 mL) to glacial acetic acid (0.5 mL).

  2. Mix thoroughly.

- **100% ethanol**

- **xylene, or xylene substitutes**
To prepare carbolxylene/xylene substitute:

1. Add one volume of liquefied phenol to three volumes of xylene or xylene substitute.

2. Liquefy the phenol crystals by heating the jar in a water bath. Liquid phenol solution contains water and, therefore, cannot be used.

8.2.3 Quality Control

- For QC of Schaudinn’s, SAF, PVA, or other fixatives, refer to the QC recommendations described for each specific procedure.

- Stool samples used for QC can be fixed stool specimens known to contain protozoa or PVA-preserved negative stools to which buffy coat cells have been added. Cultured protozoa can also be used. A QC smear prepared from a positive PVA or PVA containing buffy coat cells should be used when new stain is prepared or at least once each month.

- A QC slide can be included with each run of stained slides, although most laboratories do not use this approach, which is not required.

- Three changes of 100% alcohol may be necessary in geographic areas with high humidity.

- If the xylene becomes cloudy or has an accumulation of water in the bottom of the staining dish, use fresh 100% ethanol and xylene; either of these may contain water, which is only visible in the xylene.

- All staining dishes should be covered to prevent evaporation of reagents.

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

- When the smear is thoroughly fixed and the stain is performed correctly, the cytoplasm of protozoan trophozoites will have a blue-green color, sometimes with a tinge of purple. Cysts tend to be slightly more purple. Nuclei and inclusions (chromatoid bodies, RBCs, and bacteria), and Charcot–Leyden crystals have a red color and are sometimes tinged with purple. Glycogen is dissolved by the fixatives and appears as a clear area in the organism. The background material usually stains green, which provides a nice color contrast with the protozoa. This contrast is more distinct than that obtained with the hematoxylin stain.

- Known positive microscope slides, 2 x 2 projection slides, CDs, photographs, or digital images (reference books, atlases) should be available at the workstation.

- All QC results should be recorded.

8.2.4 Procedure for Fresh and PVA-Preserved Feces

1. Prepare the slide for staining. If the stool is fresh, place the smear immediately in Schaudinn’s fixative for a minimum of 30 minutes. If the stool is preserved in PVA, allow the smear to dry completely (air dry for several hours or at 35 to 37 °C for one hour). For other fixatives, the general approach is to use either preserved stool from the vial or sedimented stool (that has not been rinsed with water, saline, or formalin) to prepare the fecal smears. The smears are allowed to dry prior to staining.
NOTE: In some cases, adhesives such as albumin are recommended as coatings for the slides prior to adding the fecal material.

(2) Remove the slide from Schaudinn’s fixative and place it in 70% ethanol for five minutes. This step can be eliminated for PVA-air-dried smears; they can be placed immediately into 70% ethanol plus iodine (Step 3).

(3) Place the slide in 70% ethanol plus iodine for one minute for fresh specimens or for five to ten minutes for PVA-air-dried smears. This step removes the mercuric chloride from the smear.

If mercuric chloride fixatives are not being used, this step can be eliminated. However, proficiency testing (PT) specimens are routinely prepared from mercuric chloride-based PVA, and the iodine step will be required in the staining process for stool smears submitted for proficiency testing results, unless the participants are given other directions.

(4) Place the slide in 70% ethanol for five minutes.\(^c\)

(5) Place the slide in a second 70% ethanol solution for three minutes.\(^c\)

(6) Place it in trichrome stain for ten minutes.

(7) Place the slide in 90% ethanol plus acetic acid for one to three seconds. Immediately drain the rack (see Section 7.2.2.7, Procedure Notes) and proceed to the next step. Do not allow the slide to remain in contact with this solution longer than three seconds.

(8) Dip the slide several times in 100% ethanol. Use this step as a rinse.

(9) Place the slide in two changes of 100% ethanol for three minutes each.\(^c\)

(10) Place the slide in xylene or xylene substitute for five to ten minutes.\(^c\)

(11) Place the slide in second solution of xylene or xylene substitute for five to ten minutes.

(12) Mount the slide with a coverslip (No. 1 thickness) using mounting medium while the slide is still wet, or use an alternative approach using immersion oil.

(13) Allow permanently mounted smears to dry overnight at room temperature or for one hour at 37 °C. Direct oil examination can be done after the smear has dried 15 minutes before adding oil.

(14) Examine the smear microscopically using the 100x objective. Examine at least 200 to 300 oil immersion fields.

8.2.5 Results

- Protozoan trophozoites and cysts are readily seen. *Balantidium coli* trophozoites and cysts and *Cryptosporidium* spp. oocysts may or may not stain. Also, sometimes *Entamoeba coli* cysts appear pink and/or shrunken.

\(^c\) Slides may be kept up to 24 hours in these solutions without harming the quality of the smear or the stainability of organisms.
• Helminth eggs and larvae may not be easily identified; therefore, wet mount examinations of concentrates should be performed.

• Yeasts and human cells can be identified. Human cells include macrophages, polymorphonuclear leukocytes (PMNs), RBCs, and epithelial cells. Yeasts include single and budding cells and pseudohyphae. The development of both budding yeast and pseudohyphae can continue in a fresh stool specimen that is not examined immediately after passage or is not preserved within a short time after collection.

8.2.6 Reporting Results

(1) Report the organism and stage (do not use abbreviations), for example: *Giardia lamblia* trophozoites.

(2) Quantitate protozoa, if relevant (see Note below), as well as host cells, yeasts, and artifacts.

<table>
<thead>
<tr>
<th>Quantity</th>
<th>No./10 Oil Immersion Fields (1000x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Few</td>
<td>≤2</td>
</tr>
<tr>
<td>Moderate</td>
<td>3-9</td>
</tr>
<tr>
<td>Many</td>
<td>≥10</td>
</tr>
</tbody>
</table>

**NOTE 1:** As a result of ongoing debate on the clinical significance of *Blastocystis hominis*, laboratories may choose to report and quantitate the number of *Blastocystis hominis* organisms seen (rare, few, moderate, or many). Do not quantitate other protozoa.

**NOTE 2:** When examining proficiency testing (PT) specimens, some directions require you to quantitate the protozoa. This information is used as a quality control check to determine the overall quality of the manufacturing process used to prepare the PT specimens.

(3) Note and quantitate the presence of human cells; for example: moderate WBCs, many RBCs, few macrophages, rare Charcot–Leyden crystals.

(4) Report and quantitate yeast cells; for example: moderate budding yeast cells, and few pseudohyphae.

(5) Save positive slides for future reference. Label the slides before putting them in storage (name, patient number, and organisms present). Each institution should establish its own retention time and which slides should be kept. Well-stained slides can be stored for months or even years for technical purposes.

8.2.7 Procedure Notes

• The fixation of specimens is important. Improperly fixed specimens will result in protozoan forms that are nonstaining or predominantly red.

• Slides should always be drained between solutions. Touch the end of the slide to a paper towel for two seconds to remove excess fluid before proceeding to the next step.

• The incomplete removal of mercuric chloride (Schaudinn’s fixative and PVA fixative) may cause the smear to contain highly refractive residue, which may prevent finding or identifying any organisms present. Because the 70% ethanol iodine solution removes the mercury complex, it should be changed whenever necessary (at least weekly) to maintain the port wine or strong tea color.

• To restore a weakened trichrome stain, remove the stain container, cap or lid and allow the ethanol that was carried over from previous dish to evaporate. After a few hours, fresh stain may be added to
restore lost volume. Older, more concentrated stain produces more intense colors and may require slightly longer destaining times (an extra dip).

- Smears that are predominantly green may be due to the inadequate removal of iodine by the 70% ethanol (Steps 4 and 5). Lengthening the time of these steps or more frequent changing of the 70% ethanol will help. Greenish staining may also result from too much destaining due to acid carryover to the following alcohol. Stain variation is normal and may also reflect the stool contents.

- In the final stages of dehydration, the 100% ethanol and the xylene should be kept as free from water as possible. Coplin jars should have tight-fitting caps to prevent both the evaporation of reagents and the absorption of moisture from air. If the xylene becomes cloudy after the addition of slides from the 100% ethanol, return the slides to fresh 100% ethanol and replace the xylene. Three changes of 100% alcohol may be necessary in geographic areas with high humidity.

- 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. Generally, slides do not have to be cleaned with alcohol before use, but they should be free of grease.

- Upon examination, if the stain appears unsatisfactory and it is not possible to obtain another slide to stain, it may be restained. Place the slide in xylene or xylene substitute to remove the coverslip and reverse the dehydration steps, adding 50% ethanol as the last step. Destain the slide in 10% acetic acid for several hours, then wash it thoroughly, first in water and then in 50% and 70% ethanol, respectively. Place the slide in the trichrome stain for eight minutes and complete the staining procedure.

- Some laboratorians use a 20x objective and a 50x or 60x oil immersion objective to review the slides. This approach allows the switching of objectives back and forth without getting oil on the high-dry objective. If either a 50x or 60x oil immersion objective is used with a 100x oil immersion objective, this also allows switching back and forth between two oil immersion objectives at different magnifications. These various screening approaches can be helpful and are selected on the basis of personal preference and skill.

- If coverslipping the slides is not preferred, they can be allowed to air dry. About ten minutes before examination, immersion oil can be placed directly on the smear and allowed to stand. Immediately before examination, some prefer to add a No. 1 coverslip to the preparation, add another drop of oil, and then examine the smear. If the smear is positive and the slide will be kept, the oil can be removed in xylene (or xylene substitute) and then prepared as a permanent mount.

### 8.2.8 Limitations of the Procedure

- The permanent stained smear is not recommended for staining helminth eggs or larvae. Occasionally, however, they may be recognized and identified.

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

- If the viewer wants to screen the smear using low magnification (10x objective), one might see eggs or larvae; however, this is not recommended as a routine approach. Using the 10x low power objective, mucus strands may be seen. Careful examination at a higher magnification may reveal *Giardia* trophozoites lined up side-by-side or end-to-end in the mucus.
• If the viewer wants to screen the smear using a 50x or 60x oil immersion objective, this is acceptable; however, a portion of the smear should also be examined using the 100x oil immersion objective prior to sending the final report.

• Helminth eggs and larvae, *Balantidium coli* trophozoites and cysts, *Entamoeba coli* cysts, and *Isospora belli* oocysts are best seen in wet preparations.

• *Cryptosporidium* spp., *Cyclospora cayetanensis*, and *Isospora belli* are usually not seen on a trichrome-stained smear (acid-fast stains are recommended). A modified trichrome stain is recommended to identify microsporidia.

### 8.3 Iron-Hematoxylin Stain

*Modified Spencer-Monroe Method*: The iron-hematoxylin stain is one of a number of stains that allows one 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.

#### 8.3.1 Specimen

The specimen usually consists of fresh stool smeared on a microscope slide that is immediately fixed in Schaudinn’s fixative or PVA. The preserved stool is smeared on a slide and allowed to air dry (see Section 8.2.1). As an option to expedite rapid drying and enhance the adhesion of fecal material to the slide during staining, PVA-prepared stool smears may be heat-fixed on a slide warmer at 60 °C until dry (about four minutes) or placed in an incubator at 35 to 37 °C for an hour.

Stool preserved in SAF or one of the single-vial fixatives can also be used. An iron-hematoxylin stain is recommended for SAF-preserved stool specimens.

#### 8.3.2 Reagents

The following reagents are needed:

• Iron-hematoxylin stain

To prepare iron-hematoxylin stain, first prepare Solutions 1 and 2, and from these a working solution.

1. Prepare Solution 1 with hematoxylin (crystal or powder) (10 g) and ethanol (absolute) (1000 mL). Place this solution in a stoppered clear flask or bottle and allow it to ripen in a lighted room for at least one week at room temperature. The shelf life is at least five years if the bottle is stoppered tightly. The stock stain improves with age.

2. Prepare Solution 2 with ferrous ammonium sulfate [Fe(NH₄)₂ (SO₄)₂ · 6H₂O] (10 g), ferric ammonium sulfate [FeNH₄ (SO₄)₂ · 12H₂O] (10 g), hydrochloric acid [HCl] (concentrated) (10 mL), and distilled water (1000 mL). The shelf life is at least one year.

3. To prepare the working solution, mix equal volumes of Solutions 1 and 2. The working solution should be made fresh every week.

• D’Antoni’s iodine

To prepare D’Antoni’s iodine, see Section 7.1.3.2.
8.3.3 Quality Control

- For QC of Schaudinn’s SAF or PVA fixatives, refer to the QC recommendations described for each specific procedure.

- Stool samples used for QC can be 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 or PVA containing buffy coat cells should be used when new stain is prepared or at least monthly. Cultured protozoa can also be used.

- If staining is performed very sporadically, laboratories may include a QC slide with each run of stained slides.

- If the xylene becomes cloudy or has an accumulation of water in the bottom of the staining dish, use fresh 100% ethanol and xylene, because either of these may contain water, which is only visible in the xylene.

- All staining dishes should be covered to prevent evaporation of reagents.

- Depending on the volume of slides stained, staining solutions are changed on an as-needed basis.

- Background material stains a blue-dark gray color. Cells and organisms stain varying intensities of blue-gray. Inclusions, chromatoidal bodies, and nuclear structures stain darker than the surrounding cytoplasm.

- Known positive microscope slides, 2 x 2 photographic projection slides, CDs, photographs, or digital images (reference books, atlases) should be available at the workstation.

- Record all QC results.

8.3.4 Procedure

1. Prepare the slide to be stained as previously described in Section 8.2.1.

2. Place the slide in 70% ethanol for five minutes.

3. Place the slide in the 70% ethanol containing D’Antoni’s iodine solution for two to five minutes.

4. Place the slide in 70% ethanol for five minutes. Begin the procedure for SAF- or single vial-fixed slides at this point.

5. Wash the slide in running tap water for ten minutes.

6. Place the slide in iron-hematoxylin working solution for four to five minutes.

7. Wash the slide in running tap water for ten minutes.

8. Place the slide in 70% ethanol for five minutes.

9. Place the slide in 95% ethanol for five minutes.

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*d Slides may be held up to 24 hours in these solutions without harming the quality of the smear or stainability of organisms.
(10) Place the slide in two changes of 100% ethanol for five minutes each.

(11) Place the slide in two changes of xylene for five minutes each.

(12) Add mounting media to the stained area of the slide and cover it with a No. 1 coverslip, or use the alternative mounting method using immersion oil.

(13) Using the 100x objective, examine the smear microscopically. Examine at least 200 to 300 oil immersion fields.

8.3.5 Results

- If present, protozoan trophozoites and cysts will be stained blue to black.

- Helminth eggs and larvae may be distorted; therefore, wet mount examinations of concentrates should be examined.

- Yeast and human cells can be identified. Human cells include macrophages, PMNs, and RBCs. Yeasts include single and budding cells, and some have pseudohyphae. The development of both budding yeast and pseudohyphae can continue in a fresh stool specimen that is not examined immediately after passage or is not preserved within a short time after collection.

8.3.6 Reporting Results

(1) Report the complete scientific name (genus and species) of the organism and the stage seen, for example: *Giardia lamblia* rather than *G. lamblia* trophozoites.

(2) As a result of ongoing debate on the clinical significance of *Blastocystis hominis*, quantitate the number of *Blastocystis hominis* seen (rare, few, moderate, or many). Do not quantitate other protozoa. When examining proficiency testing (PT) specimens, some directions require you to quantitate the protozoa. This information is used as a quality control check to determine the overall quality of the manufacturing process used to prepare the PT specimens.

- Quantitation of protozoa, cells, yeast, and artifacts

  Quantity: No./10 Oil Immersion Fields (1000x)

  Few: \( \leq 2 \)

  Moderate: 3-9

  Many: \( \geq 10 \)

(3) Note and quantitate the presence of human cells, for example: moderate WBCs, many RBCs, few macrophages, rare Charcot–Leyden crystals.

(4) Report and quantitate yeast cells; for example: moderate budding yeast cells and few pseudohyphae.

(5) Save positive slides for future reference. Label them before placing them in storage (name, patient number, and organisms present). Each institution should establish its own retention time and criteria under which slides should be kept. Well-stained slides can be stored for months or even years for teaching purposes.
8.3.7 Procedure Notes

- Once the staining process has begun, the slides should not be allowed to dry until they have been coverslipped.

- Slides should always be drained between solutions. Touch the end of the slide to a paper towel for two seconds, to remove excess fluid, before proceeding to the next step.

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

- When staining large numbers of slides, the working hematoxylin solution may be diluted by carryover, and it may affect the quality of the stain. If dilution occurs, discard the working solution and prepare a fresh working solution.

- 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.

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

- 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. However, slides generally do not have to be cleaned with alcohol before use.

8.3.8 Limitations of the Procedure

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

- The smear should be examined using the oil immersion objective (100x) for the identification of protozoa, human cells, Charcot-Leyden crystals, yeasts, and artifact material.

- If the viewer wants to screen the smear using low magnification (10x), one might see eggs or larvae; however, this is not recommended as a routine approach. Using the 10x low power objective, mucus strands may be seen. Careful examination at a higher magnification may reveal *Giardia* trophozoites lined up side-by-side or end-to-end.

- Helminth eggs and larvae, *Balantidium coli* trophozoites and cysts, and *Isospora belli* oocysts are best seen in wet preparations.

- *Cryptosporidium* spp., *Cyclospora cayetanensis*, and *Isospora belli* will not be seen on an iron-hematoxylin-stained smear. Acid-fast stains are recommended.
8.4 Modified Iron-Hematoxylin Stain Incorporating the Carbol Fuchsine Step

*Modified Palmer Method*: The iron-hematoxylin stain is one of a number of stains that allows one 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. The following combination staining method using SAF- and other fixative-preserved fecal specimens was developed to allow the microscopist to screen for acid-fast organisms in addition to other intestinal parasites. For laboratories using iron-hematoxylin stains in combination with SAF-fixed material and modified acid-fast stains for *Cryptosporidium* spp., *Cyclospora cayetanensis*, and *Isospora belli*, this modification represents an improved approach to current staining methods. This combination stain can provide savings of both time and personnel.

8.4.1 Specimen

The specimen usually consists of fresh stool smeared on a microscope slide that is immediately fixed in Schaudinn’s fixative or PVA. The preserved stool is smeared on a slide and allowed to air dry (see Section 8.2.1). As an option to expedite rapid drying and enhance the adhesion of fecal material to the slide during staining, PVA-prepared stool smears may be heat-fixed on a slide warmer at 60 °C until dry (about four minutes) or placed in an incubator at 35 to 37 °C for an hour.

Stool preserved in SAF or one of the single vial fixatives can also be used. An iron-hematoxylin stain is recommended for SAF-preserved stool specimens.

8.4.2 Reagents

The following reagents are needed:

- Iron-hematoxylin stain

To prepare an iron-hematoxylin stain, first prepare Solutions 1 and 2, and from these a working solution.

1. Prepare Solution 1 with hematoxylin (crystal or powder) (10g) and ethanol (absolute) (1000 mL). Place this solution in a stoppered clear flask or bottle and allow it to ripen in a lighted room for at least one week at room temperature.

2. Prepare Solution 2 with ferrous ammonium sulfate \([\text{Fe} (\text{NH}_4)_2 (\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}]\) (10 g), ferric ammonium sulfate \([\text{FeNH}_4 (\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}]\) (10 g), hydrochloric acid \([\text{HCl}]\) (concentrated) (10 mL), and distilled water (1000 mL).

3. To prepare the working solution, mix equal volumes of Solutions 1 and 2. The working solution should be made fresh every week.

- D’Antoni’s iodine

To prepare D’Antoni’s iodine, see Section 7.1.3.2.

- 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 three months. Mayer’s albumin from commercial suppliers can normally be stored at 25 °C for one year.
• Picric acid

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

• Acid-alcohol decolorizer

Hydrochloric acid (HCl) (concentrated) 30 mL
70% alcohol to 1000 mL

• 70% alcohol and ammonia

70% ethanol 50 mL
ammonia 0.5 to 1.0 mL

Add enough ammonia to bring the pH to approximately 8.0.

• Kinyoun carbol fuchsin (available commercially)

To prepare Kinyoun carbol fuchsin, one should first prepare two solutions:

(1) To prepare Solution A, dissolve 4 g of basic fuchsin (color index No. 42510) in 20 mL of 95% ethanol.

(2) Add the dye to the alcohol slowly while stirring constantly.

(3) To prepare Solution B, dissolve 8 g of phenol crystals in 100 mL of distilled water.

(4) Mix solutions A and B together.

(5) Store the mixture at room temperature. It is stable for two years.

(6) Note the expiration date on the label.

8.4.3 Quality Control

• For QC of Schaudinn’s SAF or PVA fixatives, refer to the QC recommendations described for each specific procedure.

• Stool samples used for QC can be 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 or PVA containing buffy coat cells should be used when new stain is prepared or at least monthly. Cultured protozoa can also be used.

• If staining is performed very sporadically, laboratories may include a QC slide with each run of stained slides.

• If the xylene becomes cloudy or has an accumulation of water in the bottom of the staining dish, use fresh 100% ethanol and xylene, because either of these may contain water, which is only visible in the xylene.
• All staining dishes should be covered to prevent evaporation of reagents.

• Depending on the volume of slides stained, staining solutions are changed on an as-needed basis.

• Background material stains a blue-dark gray color. Cells and organisms stain varying intensities of blue-gray. Inclusions, chromatoidal bodies, and nuclear structures stain darker than the surrounding cytoplasm. With the incorporation of the modified acid-fast stain into this protocol, Cryptosporidium spp., Cyclospora cayetanensis, and Isospora belli should be stained and visible for identification. (Remember that Cyclospora cayetanensis may be acid-fast variable, and not all oocysts will take up the stain.)

• Known positive microscope slides, 2 x 2 photographic projection slides, CDs, or photographs, digital images (reference books, atlases) should be available at the workstation.

• Record all QC results.

8.4.4 Procedure

Prepare the slide to be stained as follows:

(1) One drop of concentration sediment (SAF or one of the single-vial systems) should be added to one drop of the Mayer’s albumin and mixed thoroughly. Spread the mixture across the slide in bands of varying thickness.

(2) Allow the slide to dry at room temperature; the smear will appear opaque when dry. This usually takes 10 to 15 minutes, depending on room humidity. Slides can be dried in an incubator (35 °C) for ten minutes when humidity is high.

(3) Place the slide in 70% ethanol for five minutes.

(4) Wash in container of tap water (not running water) for two minutes.

(5) Place the slide in Kinyoun stain for five minutes.

(6) Wash the slide in running tap water (constant stream of water into container) for one minute.

(7) Place slide in acid/alcohol decolorizer for four minutes.

(8) Wash the slide in running tap water (constant stream of water into container) for one minute.

(9) Place the slide in iron-hematoxylin working solution for ten minutes.

(10) Wash the slide in distilled water (in container) for one minute.

(11) Place the slide in picric acid solution for three to five minutes.

NOTE: Steps 7 to 11 can also be performed as follows:

(7) Place slide in acid/alcohol decolorizer for two minutes.

(8) Wash slide in running tap water (constant stream of water into container) for one minute.

(9) Place slide in acid/alcohol decolorizer for two minutes.
(10) Wash slide in running tap water (constant stream of water into container) for one minute.

(11) Continue staining sequence with Step 9 of the above initial procedure (iron-hematoxylin working solution).

(12) Wash slide in running tap water (constant stream of water into container) for five minutes.

(13) Place slide in 70% ethanol plus ammonia for three minutes.

(14) Place slide in 95% ethanol for two minutes.

(15) Place slide in 100% ethanol for two minutes.

(16) Repeat Step 15.

(17) Place the slide in two changes of xylene (or xylene substitute) for five minutes each.¢

(18) Add mounting media to the stained area of the slide and cover it with a No. 1 coverslip, or use the alternative mounting method using immersion oil.

(19) Using the 100x objective, examine the smear microscopically. Examine at least 200 to 300 oil immersion fields.

### 8.4.5 Results

- If present, protozoan trophozoites and cysts will be stained blue to black, while the coccidia will stain pink to purple.

- Helminth eggs and larvae may be distorted; therefore, wet mounts of concentrates should be examined.

- Yeast and human cells can be identified. Human cells include macrophages, PMNs, and RBCs. Yeasts include single and budding cells, and some have pseudohyphae. The development of both budding yeast and pseudohyphae can continue in a fresh stool specimen that is not examined immediately after passage or is not preserved within a short time after collection.

### 8.4.6 Reporting Results

(1) Report the complete scientific name (genus and species) of the organism and the stage seen, for example: *Entamoeba histolytica/E. dispar* trophozoites or *Cryptosporidium* spp. oocysts.

(2) As a result of ongoing debate on the clinical significance of *Blastocystis hominis*, quantitate the number of *Blastocystis hominis* seen (rare, few, moderate, or many). Do not quantitate other protozoa. When examining proficiency testing (PT) specimens, some directions require quantitation of the protozoa. This information is used as a quality control check to determine the overall quality of the manufacturing process used to prepare the PT specimens.

- Quantitation of protozoa, cells, yeast, and artifacts

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¢ Slides may be kept up to 24 hours in these solutions without harming the quality of the smear or the stainability of organisms.
(3) Note and quantitate the presence of human cells, for example: moderate WBCs, many RBCs, few macrophages, rare Charcot-Leyden crystals.

(4) Report and quantitate yeast cells, for example: moderate budding yeast cells and few pseudohyphae.

(5) Save positive slides for future reference. Label them before placing them in storage (name, patient number, and organisms present). Each institution should establish its own retention time and criteria under which slides should be kept. Well-stained slides can be stored for months or even years for teaching purposes.

8.4.7 Procedure Notes

- Once the staining process has begun, the slides should not be allowed to dry until they have been coverslipped.

- Slides should always be drained between solutions. Touch the end of the slide to a paper towel for two seconds, to remove excess fluid, before proceeding to the next step.

- The first 70% ethanol step acts with the Mayer’s albumin to adhere 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.

- When staining large numbers of slides, the working hematoxylin solution may be diluted by carryover, and it may affect the quality of the stain. If dilution occurs, discard the working solution and prepare a fresh working solution.

- 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.

- 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.

- 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 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.

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

- 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. However, slides generally do not have to be cleaned with alcohol before use.
8.4.8 Limitations of the Procedure

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

- The smear should be examined using the oil immersion objective (100x) for the identification of protozoa, human cells, Charcot-Leyden crystals, yeasts, and artifact material.

- If the viewer wants to screen the smear using low magnification (10x), one might see eggs or larvae; however, this is not recommended as a routine approach. Using the 10x low power objective, mucus strands may be seen. Careful examination at a higher magnification may reveal *Giardia* trophozoites lined up side-by-side or end-to-end.

- Helminth eggs and larvae, *Balantidium coli* trophozoites and cysts, and *Isospora belli* oocysts are best seen in wet preparations. Microsporidia are best seen with a modified Trichrome stain.

- With the incorporation of the carbol fuchsin step, *Cryptosporidium* spp., *Cyclospora cayetanensis*, and *Isospora belli* will be seen as acid-fast structures; however, many *C. cayetanensis* will not stain with carbol fuchsin. The hot safranin stain provides more uniform staining.

8.5 Modified Kinyoun’s Acid-Fast Stain (Cold) for *Cryptosporidium* spp., *Cyclospora cayetanensis*, and *Isospora belli* Oocysts

*Cryptosporidium*, *Cyclospora*, and *Isospora* organisms cause 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. Although auramine/rhodamine fluorescent stains can be used as a screening method, this approach is usually not as sensitive as some of the other methods. To demonstrate these organisms, modified acid-fast stains are recommended. Unlike the Ziehl-Neelsen modified acid-fast stain, this stain does not require the heating of reagents for staining.

8.5.1 Specimen

Concentrated sediment of fresh or formalin-preserved stool should be used (concentration: 500 x g for ten minutes). Other types of clinical specimens, such as duodenal fluid, bile, and those from pulmonary sources (induced sputum, bronchial wash, and biopsies) may also be stained.

8.5.2 Reagents

To perform modified Kinyoun’s acid-fast stain, the following reagents are needed:

- distilled or tap water
- absolute methanol
- 50% ethanol

To prepare 50% ethanol:

1. Add 50 mL of absolute ethanol to 50 mL of distilled water.

2. Store the solution at room temperature. It is stable for two years.
(3) Note the expiration date on the label.

- Kinyoun Carbol Fuchsin (available commercially)

To prepare Kinyoun carbol fuchsin, one should first prepare two solutions.

(1) To prepare Solution A, dissolve 4 g of basic fuchsin (color index No. 42510) in 20 mL of 95% ethanol.

(2) Add the dye to the alcohol slowly while stirring constantly.

(3) To prepare Solution B, dissolve 8 g of phenol crystals in 100 mL of distilled water.

(4) Mix solutions A and B together.

(5) Store the mixture at room temperature. It is stable for two years.

(6) Note the expiration date on the label.

- 1% sulfuric acid

To prepare 1% sulfuric acid:

(1) Add 1 mL of concentrated sulfuric acid to 99 mL of distilled water.

(2) Store the solution at room temperature. It is stable for two years.

(3) Note the expiration date on the label.

**NOTE:** Some prefer 3% to 5% sulfuric acid; however, *Cyclospora cayetanensis*, which is modified acid-fast variable, may lose too much color with this higher percentage decolorizer.

- Malachite green

To prepare malachite green:

(1) Dissolve 3 g of malachite green (color index No. 42000) in 100 mL distilled water.

(2) Store the solution at room temperature. It is stable for one year.

(3) Note the expiration date on the label.

- Methylene blue

To prepare methylene blue:

(1) Dissolve 0.3 g of methylene blue in 100 mL of 95% ethanol.

(2) Store the solution at room temperature. It is stable for two years.

(3) Note the expiration date on the label.
8.5.3 Quality Control

- A control slide of Cryptosporidium oocysts from a 10% formalin-preserved specimen should be included with each staining run. If the Cryptosporidium oocysts stain well, any Isospora or some Cyclospora oocysts present will take up the stain.

- Cryptosporidium oocysts stain a pinkish-red, are 4 to 6 µm, and contain four sporozoites, which may or may not be visible. Cyclospora oocysts stain from clear (do not take up the stain and appear like “wrinkled cellophane”) to pink or dark pink. The background should stain a uniform green. Usually the Cryptosporidium oocysts are used for quality control purposes.

- The specimen should be checked for adherence to the slide (macroscopically).

- All QC results should be recorded.

8.5.4 Procedure

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

2. Fix with absolute methanol for one minute.

3. Flood the slide with Kinyoun’s carbol fuchsin and stain for five minutes.

4. Rinse the slide briefly (three to five seconds) with 50% ethanol.

5. Rinse the slide thoroughly with water.

6. Decolorize using 1% sulfuric acid for two minutes or until no more color runs from the slide.

7. Rinse the slide with water, then drain it.

8. Counterstain with malachite green or methylene blue for two minutes.

9. Rinse the slide with water and air dry.

10. Mount the slide with mounting medium and coverslip (No. 1 thickness).

11. Examine the slide using the high-dry objective. To see internal morphology, use the oil immersion objective (100x).

8.5.5 Results

- With this cold Kinyoun acid-fast method, the oocysts of Cryptosporidium spp. and Isospora belli stain pink, to red, to deep purple. Some of the four sporozoites may be visible in the Cryptosporidium oocysts. Some of the immature Isospora oocysts (entire oocyst) stain, while those that are mature usually appear with the two sporocysts within the oocyst wall, which is stained a pink-to-purple color with a clear area between the stained sporocysts and the oocyst wall. The background stains blue or green, depending on the counterstain used.

- There is usually a range of color intensity among the organisms present; not every oocyst appears deep pink-to-purple, and not every oocyst stains. Remember that Cyclospora oocysts may not all stain
uniformly pink to purple (modified acid-fast variable). Some oocysts may lose the stain and even appear clear with an appearance of wrinkled cellophane (i.e., clear to pale pink).

8.5.6 Reporting Results

(1) Report the organism and the stage (oocyst). Do not use abbreviations. An example of a laboratory report is: Cryptosporidium spp. oocysts; Cyclospora cayetanensis oocysts; Isospora belli oocysts.

(2) Notify the physician when these organisms are identified.

8.5.7 Procedure Notes

- Routine stool examination stains are not recommended for the identification of Cryptosporidium organisms; however, the sedimentation concentration is acceptable (500 x g for ten minutes) for the recovery and subsequent identification of Cryptosporidium spp. Oocysts can be difficult to identify on wet smears, so the concentration sediment can be used to prepare smears for modified acid-fast staining. The routine concentration (formalin-ethyl acetate) can be used to recover Isospora belli oocysts, but routine permanent stains are not reliable for this purpose, because the oocysts are difficult to identify on the permanent stained smear.

- PVA-preserved specimens are not acceptable for staining with the modified acid-fast stain.

- Other organisms stain positive such as acid-fast bacteria, acid-fast yeast, and Nocardia spp. Detection of sporozoites within the oocysts is the differential characteristic. Measurement of the coccidian oocysts is highly recommended for confirmation (Cryptosporidium 4-6 µm, Cyclospora 8-10 µm, and Isospora, up to ~35 µm).

- It is important to ensure that smears are not too thick, because thicker smears may not adequately destain.

- A concentrated specimen is essential for demonstration of organisms (500 x g for ten minutes). The number of organisms seen in the specimen may vary from numerous to very few.

- Some specimens require treatment with 10% KOH (potassium hydroxide) because of their mucoid consistency. Add 10 drops of 10% KOH to the sediment and vortex until it becomes homogeneous. Rinse with 10% formalin and centrifuge (500 x g for 10 minutes). Without decanting the supernatant, take one drop of the sediment and smear it thinly on a slide.

- Concentrations of sulfuric acid (1% to 3%) are normally used. Stronger concentrations may remove too much stain, particularly for Cyclospora oocysts.

- There is some debate as to whether organisms lose their ability to take up the acid-fast stain after long-term storage in 10% formalin.

- Unlike Isospora and Cyclospora, Cryptosporidium oocysts are immediately infective when passed in the stool. Unpreserved specimens should be centrifuged in capped tubes, and gloves should be worn during all phases of specimen processing.

- Some laboratorians use Coplin jars for this staining procedure and report finding less debris in the final stain.
8.5.8 Limitations of the Procedure

- Light infections of *Cryptosporidium* (low number of oocysts) may be missed. The fecal immunoassay methods are more sensitive.

- Multiple specimens should be examined, because the numbers of oocysts present in the stool vary from day to day. A series of three specimens submitted on alternate days is recommended.

8.6 Modified Ziehl-Neelsen Acid-Fast Stain (Hot) for *Cryptosporidium* spp., *Cyclospora cayetanensis*, and *Isospora belli* Oocysts

*Cryptosporidium*, *Cyclospora*, and *Isospora* organisms cause 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. Although auramine/rhodamine fluorescent stains can be used as a screening method, this approach is usually not as sensitive as some of the other methods. Modified acid-fast stains are recommended to demonstrate these organisms. Application of heat to the carbol fuchsin appears to help in the staining process and it, possibly, provides better stain penetration.38

8.6.1 Specimen

Concentrated sediment of fresh or formalin-preserved stool should be used (concentration: 500 x g for ten minutes). Other types of clinical specimens, such as duodenal fluid, bile, and those from pulmonary sources (induced sputum, bronchial wash, and biopsies) may also be stained.

8.6.2 Reagents

To perform this stain, the following reagents are needed:

- Distilled or tap water

- Carbol fuchsin

To prepare carbol fuchsin, use basic fuchsin and phenol.

To prepare basic fuchsin (Solution A):

1. Dissolve 0.3 g of basic fuchsin in 10 mL of 95% ethanol.
2. Grind the dye and alcohol in a mortar with a pestle.

To prepare the phenol (Solution B):

1. Dissolve 5 g of phenol crystals in 100 mL of distilled water.
2. Use hot running water if gentle heat is needed. Do not use a Bunsen burner.
3. Mix Solution A with Solution B.
4. Store the mixture at room temperature. It is stable for two years.
5. Note the expiration date on the label.

- 3% sulfuric acid
To prepare 3% sulfuric acid:

1. Add 3 mL of concentrated sulfuric acid to 97 mL of distilled water (some laboratories like to use 1% sulfuric acid; the choice is optional).

2. Store this solution at room temperature. It is stable for two years.

3. Note the expiration date on the label.

- Methylene blue

To prepare methylene blue:

1. Dissolve 0.3 g of methylene blue chloride in 100 mL of distilled water.

2. Store this solution at room temperature. It is stable for two years.

3. Note the expiration date on the label.

8.6.3 Quality Control

- A control slide of Cryptosporidium oocysts from a 10% formalin-preserved specimen is included with each staining batch run. If the Cryptosporidium oocysts stain well, any Isospora organisms present will also take up the stain. Remember that Cyclospora cayetanensis will tend to stain acid-fast variable and may range from red-purple to clear.

- Cryptosporidium oocysts stain pink-red, are 4 to 6 µm, and contain four sporozoites, which may or may not be visible. The background should stain a uniform blue.

- The specimen should be checked for adherence to the slide (macroscopically).

- All QC results should be recorded.

8.6.4 Procedure

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

2. Dry on a heating block (70 °C) for five minutes.

3. Place the slide on the staining rack and flood it with carbol fuchsin.

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

5. Allow the slide to stain for five minutes. If the slide begins to dry, add more stain (without additional heating).

6. Rinse the slide thoroughly with water and drain it.

7. Decolorize the slide with 1 to 3% sulfuric acid for 30 seconds. (Thicker slides may require a longer destaining process.)
(8) Rinse the slide with water and drain it.

(9) Flood the slide with methylene blue for one minute.

(10) Rinse the slide with water; drain it, and allow it to air dry.

(11) Examine the slide using the high-dry objectives. To see the internal morphology, use the oil immersion objective (100x).

**8.6.5 Results**

- With this modified acid-fast method, the oocysts of *Cryptosporidium* spp. and *Isospora belli* stain pink, to red, to deep purple. Some of the four sporozoites may be visible in the *Cryptosporidium* oocysts. Some of the immature *Isospora* oocysts (entire oocyst) stain, while those that are mature usually appear with the two sporocysts within the oocyst wall, which is stained a pink-to-purple color with a clear area between the stained sporocysts and the oocyst wall. *Cyclospora* oocysts will stain from red-purple to pale pink; some oocysts will not stain at all and will appear clear. The oocysts of *Cyclospora* will often appear wrinkled with no internal structure. The background stains blue, and not every oocyst stains.

- There is usually a range of color intensity among the organisms present; not every oocyst appears deep pink-to-purple.

**8.6.6 Reporting Results**

(1) Report the organism and stage (oocyst). Do not use abbreviations. An example of a laboratory report is: *Cryptosporidium* spp. oocysts; *Cyclospora cayetanensis* oocysts; *Isospora belli* oocysts.

(2) Notify the physician when these organisms are identified.

**8.6.7 Procedure Notes**

- Routine stool examination stains are not recommended for the recovery and identification of *Cryptosporidium* spp. or *Cyclospora cayetanensis*; however, the sedimentation concentration is acceptable (500 x g for ten minutes). The routine concentration (formalin-ethyl acetate) can be used to recover *Isospora belli* oocysts, but routine permanent stains are not reliable for this purpose.

- PVA-preserved specimens are not acceptable for staining with the modified acid-fast stain.

- Other organisms stain positive, such as acid-fast bacteria and *Nocardia* spp. Detection of sporozoites within oocysts is the differential characteristic. Measurement of the coccidian oocysts is highly recommended for confirmation (*Cryptosporidium* 4 to 6 µm, *Cyclospora* 8 to 10 µm, and *Isospora*, up to ~35 µm).

- It is important to ensure that smears are not too thick, because thicker smears may not adequately destain.

- A concentrated specimen is essential for demonstration of organisms (500 x g for ten minutes). The number of organisms seen in the specimen may vary from numerous to very few.

- Some specimens require treatment with 10% KOH because of their mucoid consistency. Add ten drops of 10% KOH to the sediment and vortex until it becomes homogeneous. Rinse with 10%
formalin and centrifuge (500 x g for ten minutes). Without decanting the supernatant, take one drop of the sediment and smear it thinly on a slide.

- Do not boil the stain. Gently heat it until steam rises from the slide. Do not allow the stain to dry on the slide.

- Various concentrations of sulfuric acid (0.25% to 10%) may be used; however, destaining time varies according to the concentration used. Generally, 1 or 3% solutions are used.

- There is some debate as to whether organisms lose their ability to take up the acid-fast stain after long-term storage in 10% formalin.

- Unlike *Isospora* and *Cyclospora*, *Cryptosporidium* oocysts are immediately infective when passed in the stool; specimens should be centrifuged in capped tubes, and gloves should be worn during all phases of specimen processing. Oocysts of *Cyclospora* and *Isospora* are not immediately infectious.

### 8.6.8 Limitations of the Procedure

- Light infections (low number of oocysts) may be missed. The fecal immunoassay methods are more sensitive.

- Because the number of oocysts present in the stool varies from day to day, multiple specimens should be examined. A series of three specimens submitted on alternate days is recommended.

### 8.7 Modified Hot Safranin Technique for *Cryptosporidium* spp., *Cyclospora cayetanensis*, and *Isospora belli* Oocysts

*Cryptosporidium*, *Cyclospora*, and *Isospora* organisms cause 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. Although auramine/rhodamine fluorescent stains can be used as a screening method, this approach is usually not as sensitive as some of the other methods. To demonstrate these organisms, the hot safranin method is recommended.

#### 8.7.1 Specimen

Concentrated sediment of fresh or formalin-preserved stool should be used (concentration: 500 x g for ten minutes). Other types of clinical specimens, such as duodenal fluid, bile, and those from pulmonary sources (induced sputum, bronchial wash, and biopsies) may also be stained.

#### 8.7.2 Reagents

To perform the modified hot safranin stain, the following reagents are needed: distilled or tap water; acidic alcohol 3% HCl; 1% safranin in acidified water (pH 6.5); 1% methylene blue or 1% malachite green.

- Acidic alcohol 3% HCl

To prepare acidic alcohol 3% HCl in methanol:

1. Add 3 mL of HCl to 97 mL of methanol.

2. Store the solution at room temperature. It is stable for two years.
(3) Note the expiration date on the label.

- 1% safranin in acidified water (pH 6.5)

To prepare 1% safranin:

(1) Dissolve 1 g of safranin in 99 mL of acidified water (pH 6.5).

(2) Add the dye to the acidified water slowly while stirring constantly.

(3) Store the mixture at room temperature. It is stable for two years.

(4) Note the expiration date on the label.

- Methylene blue or 1% malachite green

To prepare 1% methylene blue or 1% malachite green (color index No. 42000):

(1) Add 1 g of dye to 99 mL of distilled water.

(2) Store the solution at room temperature. It is stable for one year.

(3) Note the expiration date on the label.

### 8.7.3 Quality Control

- A control slide of *Cryptosporidium* oocysts from a 10% formalin-preserved specimen should be included with each staining run. If the *Cryptosporidium* oocysts stain well, any *Isospora* or most *Cyclospora* oocysts present will take up the stain.

- *Cyclospora* oocysts stain a reddish orange, are 8 to 10 µm, and will have a crinkled or wrinkled oocyst wall. *Cryptosporidium* oocysts stain a reddish orange, are 4 to 6 µm, and may contain four visible sporozoites. The background should stain a uniform blue or green, depending on the counterstain. Usually the *Cryptosporidium* oocysts are used for quality control purposes.

- The specimen should be checked for adherence to the slide (macroscopically).

- All QC results should be recorded.

### 8.7.4 Procedure

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

(2) Dry the slide on a slide warmer at ~60 °C.

(3) Allow to cool to room temperature before staining.

(4) Place the slide in a Coplin jar containing 3% acidic alcohol and let stand for five minutes.

(5) Rinse the slide briefly (three to five seconds) with cold tap water.
(6) Place the slide in a Coplin jar containing 1% safranin and microwave at full power for one minute. It is recommended that the Coplin jar be placed in a glass or plastic tray so that the overflowing stain (due to boiling) will be caught in the tray and not on the microwave oven floor.

(7) Rinse the slide with tap water.

(8) Place the slide in a Coplin jar and counterstain with an aqueous solution of malachite green or methylene blue for one minute.

(9) Gently rinse the slide with tap water and allow to air dry.

(10) Mount the slide with mounting medium and coverslip (No. 1 thickness).

(11) Examine the slide using the high-dry objective. To see internal morphology, use the oil immersion objective (100x).

8.7.5 Results

- With this modified hot safranin method, the oocysts of Cryptosporidium spp. and Isospora belli will stain reddish orange. Some of the four sporozoites may be visible in the Cryptosporidium oocysts. Some of the immature Isospora oocysts (entire oocyst) stain, while those that are mature usually appear with the two sporocysts within the oocyst wall, which is stained a reddish orange color with a clear area between the stained sporocysts and the oocyst wall. The background stains blue or green, depending on the counterstain used.

- There is usually a range of color intensity among the organisms present; not every oocyst appears reddish orange, and not every oocyst stains. Remember that not all Cyclospora oocysts will stain uniformly. Some oocysts may lose the stain and even appear clear with an appearance of wrinkled cellophane (i.e., clear to pale red-orange). However, this stain method will provide more uniform staining results than may be seen using the modified acid-fast methods for Cyclospora.

8.7.6 Reporting Results

(1) Report the organism and the stage (oocyst). Do not use abbreviations. An example of a laboratory report is: Cryptosporidium spp. oocysts; Cyclospora cayetanensis oocysts; Isospora belli oocysts.

(2) Notify the physician when these organisms are identified.

8.7.7 Procedure Notes

- Routine stool examination stains (trichrome, iron hematoxylin, iodine) are not recommended for the identification of the coccidia; however, smears from the concentration sediment (500 x g for ten minutes) are acceptable for the recovery and subsequent identification of coccidia after special staining with modified acid-fast stains. The routine concentration (formalin ethyl-acetate) can be used to recover Isospora spp. oocysts, but routine permanent stains are not reliable for this purpose, because the oocysts are difficult to identify on the permanent stained smear.

- PVA-preserved specimens are not acceptable for staining with the modified hot safranin stain.

- Measurement of the coccidian oocysts is highly recommended for confirmation (Cryptosporidium 4 to 6 µm, Cyclospora 8 to 10 µm, and Isospora, up to ~35 µm).
• It is important to ensure that smears are not too thick, because thicker smears may not adequately stain.

• A concentrated specimen is highly recommended for demonstration of organisms (500 x g for ten minutes). The number of organisms seen in the specimen may vary from numerous to very few.

• Some specimens require treatment with 10% KOH (potassium hydroxide) because of their mucoid consistency. Add ten drops of 10% KOH to the sediment and vortex until it becomes homogeneous. Rinse with 10% formalin and centrifuge (500 x g for ten minutes). Without decanting the supernatant, take one drop of the sediment and smear it thinly on a slide.

• Unlike *Isospora* and *Cyclospora*, *Cryptosporidium* oocysts are immediately infective when passed in the stool. Unpreserved specimens should be centrifuged in capped tubes, and gloves should be worn during all phases of specimen processing.

8.7.8 Limitations of the Procedure

• Light infections of any of the coccidia (low number of oocysts) may be missed. The fecal immunoassay methods are more sensitive for *Cryptosporidium*.

• Multiple specimens should be examined, because the numbers of oocysts present in the stool vary from day to day. A series of three specimens submitted on alternate days is recommended.

8.8 Modified Trichrome Stain (Chromotrope 2R) for Microsporidia (Weber-Green and Ryan-Blue)\(^{37,42}\)

Microsporidia are obligate, intracellular, spore-forming protozoa. Microsporidia in the intestine were recently implicated as one of the causes of chronic diarrhea in patients with human immunodeficiency virus. A staining method was developed using various components of the trichrome staining method to differentiate these extremely small organisms from the background fecal material\(^{37-39,42}\).

8.8.1 Specimen

Prepare a thin smear (volume: 10 µL) of 10% formalin-fixed stool on a slide. Heat-fix the slide on a slide warmer at 60 °C until dry (several minutes). Although unconcentrated specimen can be used, material can be taken from the concentrated sediment (providing the centrifugation time is ten minutes at 500 x g).

8.8.2 Reagents for Weber-Green

To perform this stain, the following reagents are needed for chromotrope stain.

To prepare chromotrope stain:

1. Mix 6.0 g of chromotrope 2R, 0.15 g fast green FCF, 0.7 g of phosphotungstic acid, and 3 mL of glacial acetic acid.

2. Allow the mixture to stand for 30 minutes.

3. Add 100 mL of distilled water.

4. Prepare a fresh mixture for use every month.
NOTE: Since Wheatley’s trichrome, and the two formulas for chromotrope stain for the microsporidia use different amounts of various ingredients, the shelf life for each stain is stated according to the recommendation of the developer of that particular stain formula. Thus, the Weber-Green modified trichrome stain shelf life is stated as one month, while the Ryan-Blue modified trichrome stain shelf life is 24 months. (There is no reason why a fresh mixture of chromotrope 2R stain would need to be prepared monthly, unless it becomes contaminated or was giving unacceptable staining characteristics. Generally, this stain has a long shelf life that should be consistent with the 24 months indicated in Section 8.2.2.)

- Absolute alcohol
- Acid alcohol
  
  Acid alcohol is composed of 90% ethanol (995.5 mL) and glacial acetic acid (4.5 mL).
- 95% ethanol
- 100% ethanol
- Xylene (or xylene substitute)

### 8.8.3 Reagents for Ryan-Blue

To perform this stain, the following reagent is needed: Chromotrope stain.

To prepare chromotrope stain:

1. Mix 6.0 g of chromotrope 2R, 0.5 g aniline blue, 0.25 g of phosphotungstic acid, and 3 mL of glacial acetic acid.
2. Allow the mixture to stand for 30 minutes.
3. Add 100 mL of distilled water and adjust the pH to 2.5 with 1.0 N HCl. Properly prepared stain will be dark purple. The staining solution should be protected from light.
4. The shelf life is at least 24 months when stored at room temperature.

NOTE: Since Wheatley’s trichrome, and the two formulas for chromotrope stain for the microsporidia use different amounts of various ingredients, the shelf life for each stain is stated according to the recommendation of the developer of that particular stain formula. Thus, the Weber-Green modified trichrome stain shelf life is stated as one month, while the Ryan-Blue modified trichrome stain shelf life is 24 months. There is no reason why a fresh mixture of chromotrope 2R stain would need to be prepared monthly, unless it becomes contaminated or was giving unacceptable staining characteristics. Generally, this stain has a long shelf life that should be consistent with the 24 months indicated in Section 8.2.2.

- Absolute alcohol
- Acid alcohol

  Acid alcohol is composed of 90% ethanol (995.5 mL) and glacial acetic acid (4.5 mL).
- 95% ethanol
8.8.4 Quality Control for Weber-Green and Ryan-Blue Modified Trichrome Stain

- Include a microsporidia-positive control from a 10% formalin-fixed stool specimen in each staining batch run. Spore walls of microsporidia stain a pinkish red and measure about 1 µm. The background will stain green (Weber-Green) or blue (Ryan-Blue).

- The specimen should be checked for adherence to the slide (macroscopically).

- To obtain proper rinsing and dehydration, change all solutions subsequent to the modified trichrome staining after every ten slides.

- To see these tiny organisms, use a microscope with good optics and bright light. Use the 100x objective (oil immersion) magnification to detect organisms; lower magnifications will not reveal the spores.

- Review the positive control slide before the patient specimen, and review it intermittently after three negative specimens.

- Due to the difficulty in detecting these small spores, confirm positive results with at least one other examiner.

- All QC results should be recorded.

8.8.5 Procedure (Weber-Green or Ryan-Blue Modified Trichrome)

1. Prepare the slide for staining.

2. Place the slide in absolute methanol for five or ten minutes.

3. Allow the smear to air dry.

4. Place it in modified trichrome (either Weber-Green or Ryan-Blue) stain for 90 minutes.

5. Dip the slide in acid alcohol for one to three seconds only.

6. Rinse the slide in 95% alcohol by dipping.

7. Place the slide in 95% alcohol for five minutes.

8. Place the slide in two changes of 100% ethanol for three to ten minutes each.

9. Place the slide in two changes of xylene or xylene substitute for ten minutes each.

10. Drain the slide and mount it with the coverslip (No.1 thickness) using mounting media, or use the alternative mounting method using immersion oil.

11. Examine the smear after drying using the 100x objective (oil immersion). Lower magnifications will not reveal the organisms. Examine at least 300 fields.
8.8.6 Results

- Spores are oval and measure about 0.8 to 1.1 x 1.1 to 1.7 µm (and up to 2+ µm) with wall staining pinkish red. The content of the spore appears as either a transparent vacuole or with a pinkish red, belt-like stripe. The background will stain green (Weber-Green) or blue (Ryan-Blue).

- Confusing background material in the stool includes bacteria and small yeasts that may sometimes stain red. Usually, however, they are darker than microsporidia and stain evenly with no transparent vacuole in the center. Bacteria are usually solid and round or rod-shaped rather than oval. Yeasts and fungi are larger, more variable in size, and sometimes show budding.

8.8.7 Reporting Results

(1) Report the specimen as having tested positive or negative for microsporidia. Do not attempt to identify other organisms; instead, use the specific stains intended for the other protozoa. Although there are size differences between *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* spores seen in fecal specimens, these differences can be extremely difficult to determine. Therefore, it is not appropriate to report these organisms to the genus/species levels.

(2) Include an approximate quantification for the physician as to few, moderate, or many organisms present.

8.8.8 Procedure Notes

- Slides should be drained between solutions by touching them to a paper towel to remove excess fluids.

- To obtain proper rinsing and dehydration, change all solutions subsequent to the stain after every ten slides. Keep the lids on the staining jars sealed.

- If only one or two slides are to be stained, it saves stain to use a plastic slide shipping container which requires only 3 to 4 mL of stain.

For best results, prepare fresh stain for use every month. Prepare fresh stain more frequently if large volumes of slides are stained or if spores on the control slide become too pale to distinguish them from background material. Some laboratories are reporting good staining results with stain that should be three to six months old.

8.8.9 Limitations of the Procedure

- Because of the small size of the spore and the large amount of artifact material present in a stool, it can be extremely difficult to detect these organisms when they are few in number.

- It is helpful to have another examiner in the laboratory to confirm positive and negative results.

- If there is not a good microscope with bright light and at least an 100x (oil immersion) objective available, the spores may not be detected at all.

8.9 Chemofluorescence Agents

Another diagnostic approach for the confirmation of microsporidial spores requires the use of chemofluorescent agents (optical brightening agents) such as Calcofluor white (CFW). These reagents are
sensitive but nonspecific; objects other than microsporidial spores also fluoresce. This can be a problem when examining fecal specimens, since false-positive results have been seen with artifact material. However, when these reagents are used with other specimens, such as urine sediment, the interpretation of results is much easier and more reliable. In spite of the potential interpretation issues, this is a simple, sensitive screening method for the detection of microsporidial spores in clinical specimens. Slides prepared from fresh or formalin-fixed stool, and other specimens can be stained 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 active ingredient is the disodium salt of 4,4’-bis-(anilino-bis-diethylamino-5-triazin-2-ylamino)-2,2’-stilbene disulfonic acid, which is a nonspecific fluorescent dye binding to the polysaccharide polymers of amebic cysts and microsporidial spores.1,40,43

This staining approach can also be used to demonstrate Acanthamoeba cysts from clinical specimens.

The user will need an epifluorescence microscope equipped with an exciter filter that transmits the 250- to 400-nm group of intense mercury spectral emission lines. View through a barrier filter, which removes UV while transmitting visible blue light and longer wavelengths.

**8.9.1 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.

- **Stool**
  1. The specimen can be fresh stool or stool preserved in 5% or 10% formalin, SAF, or some of the newer single-vial system fixatives. The specimen sediment should be used after concentration at 500 x g for ten minutes.
  2. Any specimen other than tissue thought to contain microsporidia could be stained by this method.

- **CSF**
  1. Centrifuge at 250 x g for ten minutes.
  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.

- **Tissue**
  1. Triturate a small portion in sterile water or saline (brain, corneal scrapings or biopsy specimen, lung, or skin lesion.

- **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 the tube, and return it to the original holder.
4. The sterile water can be examined directly or concentrated by centrifugation.

- Contact lens and/or solutions
  1. Solutions must be from opened containers already used by the patient. If the volume is greater than 2 mL, centrifuge prior to examination (250 x g for ten minutes).
  2. Contact lenses should be submitted in 2 mL of sterile water or saline. Examine a small portion of lens and the fluid containing the lens.

- Slide submitted to the laboratory
  1. Submit at least two slides. Place the specimen in the center of the slide, covering about a dime-sized area.
  2. Circle the material with a wax pencil or marker to denote location of the specimen.
  3. Air dry slides thoroughly.
  4. Place the slide in a slide holder for subsequent staining in the laboratory.

8.9.2 Reagents

Commercially available solution of CFW with an Evans’s blue counterstain or solution prepared as follows:

- 0.1% CFW
  CFW, M2R, purified 0.1 g
  Distilled water 99.9 mL

  Mix, filter, and store in a dark container. The mixture is stable at room temperature for one year.

- 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 one year.

- Page’s amoeba saline (1 X)
  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 minutes. Store refrigerated in a glass bottle. The mixture is stable for one year.

- Absolute methanol
- Sterile distilled water
- Positive fecal specimen (microsporidial spores)
8.9.3 Quality Control

- A control slide should be included with each batch of specimens stained with CFW.

- It is important to use actual microsporidial spores or *Acanthamoeba* cysts in the QC process. Control slides containing spores can be prepared by using concentrated positive stool and spreading the material over an area 45 by 25 mm. Allow the smear to air dry. Control slides of amebae can easily be prepared from QC stock cultures. Control slides for the microsporidia can be obtained commercially. See the “Helpful Websites” section of this document.

- Control slides for the amoebae can be prepared as follows: Make a suspension of amoebae from stock culture with sterile water or Page’s amoeba saline. Make a suspension of *Escherichia coli* with sterile water or Page’s amoeba saline. Add one drop of each control to a “ring” slide (containing two rings). Allow the smear to air dry, fix in absolute methanol for three to five minutes, and store at room temperature. Smears are stable for one year.

- The specimen should be checked for adherence to the slide (macroscopically).

- *Acanthamoeba* cysts are double walled (10 to 25 µm), and the outer wall is wrinkled. The cysts will fluoresce.

- Microsporidial spores will be ovoid and refractile, and the spore wall will fluoresce. Occasionally, the polar tubule 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.

- All QC results should be recorded.

8.9.4 Procedure

1. Using a sterile swab, stick, or pipet (nonsterile is acceptable for the stool specimens), thinly spread the specimen evenly over the area circumscribed by the ring on the slide. Do not use too much specimen on the slide, because the smear may be too thick to visualize any organisms present, especially microsporidial spores.

2. Allow the smear to air dry.

3. Fix the smear in absolute methanol for three to five minutes.

4. Allow the smear to air dry.

5. Add three or four drops of CFW and three or four drops of Evan’s blue into a tube (12 by 75 mm) and mix well.

6. Add several drops of this mixture to the specimen, and allow it to stand for five minutes.

7. Turn the slide on its side and allow the excess stain to run off.

8. Add a coverslip, blot the excess stain from slide, and examine immediately.
8.9.5 Results

- Microsporidial spores and *Acanthamoeba* cysts will fluoresce. 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.

- Yeast cells, pseudophyphae, hyphae, and other fungal elements will stain with CFW, as will *Pneumocystis jiroveci* (now classified with the fungi).

- Bacteria will not fluoresce, while epithelial cells and blood cells will stain by Evan’s blue counterstain.

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

8.9.6 Reporting Results

1. Report as microsporidial spores or *Acanthamoeba* cysts seen.
2. Notify the physician when these organisms are identified.

8.9.7 Procedure Notes

- Collagen, elastic, and keratin will also fluoresce.

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

- 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.

- 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 or viscous specimens).

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

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

- If *Naegleria* cysts are present in the specimen, they may be seen using CFW.

8.9.8 Limitations of the Procedure

- Light infections (low number of microsporidial spores or *Acanthamoeba* cysts) may be missed. It is important that the procedure be performed using centrifuged stool sediment.

- Multiple specimens should be examined, because the numbers of microsporidial spores present in the stool vary from day to day.
9  Fecal Immunoassays and Gene Probes

Fecal immunoassays are now available for the detection and identification of *Cryptosporidium* spp., *Giardia lamblia*, the *Entamoeba histolytica/E. dispar* group, *Entamoeba histolytica*, and *Trichomonas vaginalis* (not recovered from the intestinal tract, so will not be discussed in this document). Additional immunoassays are under development for *Dientamoeba fragilis* and the microsporidia.41,44-47

Using purified or recombinant antigens, the detection of parasite-specific antigen indicates the presence of current disease. These fecal immunoassays are simple to perform and allow batch testing of numerous specimens at any one time. One disadvantage is that these methods detect only one or two pathogens at one time. In order to detect other parasitic pathogens, the O&P examination also must be performed if the fecal immunoassay is negative and the patient remains symptomatic (see Appendix D).

Commercially available immunoassays have sensitivity and specificity percentages of more than 90% when used according to the manufacturer’s instructions for specimen handling and preservation (some test kits require fresh or frozen fecal specimens only). Some combination reagents are available for the detection and differentiation of *Giardia lamblia*, *Cryptosporidium* spp., and the *E. histolytica/E. dispar* group. Many of the reported sensitivities and specificities are above 95%.

The selection of any particular fecal immunoassay test format is based on a number of factors, including: training and expertise of laboratory personnel; number of specimens submitted for testing; clients serviced; availability of relevant equipment; laboratory menu options; laboratory marketing capability; educational initiatives for clients; cost; and clinical relevance related to geographic area and types of patients seen. Since the sensitivity and specificity of all of the fecal immunoassay kits are comparable, selection of a specific test kit often depends not only on the test format, but the cost.

If one compares the pros and cons of the O&P examination to that of the fecal immunoassays, both types of tests play an important role in diagnosing patient infections with parasites. The approach that one laboratory may take regarding the test format selection of fecal immunoassays may vary from another laboratory. However, these differences should not be considered “good or bad” but merely choices that fit into the laboratory workflow and fulfill other required criteria for that particular laboratory.

When using any of the fecal immunoassays, it is important to remember the recommendations for specimen processing prior to testing. In the fluorescent antibody procedure, the actual organisms (*G. lamblia* cysts, *Cryptosporidium* spp. oocysts) will fluoresce. The sensitivity of the procedure will be enhanced by performing the test on centrifuged stool sediment (ten minutes at 500 x g). More organisms will be found in the centrifuged stool than in the uncentrifuged specimen. When using the enzyme immunoassay, the test system allows the detection of antigen in solution, so centrifugation is not required. The cartridge formats have parasite-specific antibody incorporated into the strip. As the specimen containing antigen in solution wicks up the membrane, antigen/antibody complexes are formed, and this linkage is indicated by a colored line appearing on the membrane. The cartridges have one or more internal control lines that are found toward the top of the membrane strip; coloration of this control line indicates the test reagents are performing correctly and that the specimen/buffer mix has wicked all the way to the end of the strip. It is important to include external controls (positive, negative) with each run of patient specimens. Therefore, many laboratories batch test their clinical specimens.

It is important to thoroughly read the package insert prior to testing patient samples, since color changes (negative to positive) can be somewhat subtle. Correct interpretation requires careful examination of the wells or strips, depending on the format used. It is also important to remember that, in general, the control lines will tend to be darker than those of the patient test specimen. Generally, any color change detected indicates a positive result, providing the results are read at the correct time. If the results are read past the time indicated in the package insert, there may be false-positive results.
Although there have been several product recalls over the past few years, it is not recommended that fecal immunoassay results be confirmed with other tests, such as the O&P examination. Although state and/or federal public health reference laboratories may wish to confirm results using additional tests, it becomes problematic for laboratories in terms of coding and billing, and the process of double testing becomes expensive. It is important to remember that the laboratory cannot code and bill for tests other than those specifically ordered by the physician.

The development of fecal immunoassays, in addition to the O&P examination and special stains for the coccidia and microsporidia, provides very specific options for patient testing (see Appendix D). The selection of any of these testing options depends on patient history and physical findings. Each assay should be available as a separate orderable and billable test, or they can be arranged in logical batteries addressing specific clinical situations. However, the development, approval process, and use of algorithm testing using test batteries can become complicated in terms of the approval process, coding, and billing. It is also appropriate to designate these tests on the order forms/computer screens as: Fecal Immunoassay for (list the organism). Although some test formats will provide results for more than one organism, each organism can be billed as a separate coded, billed test (effective 2004, CPT list). **However, it is mandatory that the laboratory code and bill only for those tests that have been ordered by the physician.** For example, if the immunoassay format tests for both *Giardia lamblia* and *Cryptosporidium* spp., then the physician should be checking off both tests as separate orders (i.e., *Giardia lamblia* fecal immunoassay, *Cryptosporidium* spp. fecal immunoassay. Both entries should appear on the order form or computer screen). Different CPT codes will be used for each organism, and the results for both can be coded and billed as separate test charges.

Although nucleic acid-based diagnostic tests for specific parasites tend to be available in specialized research or reference laboratories, PCR and probe tests have been reported for many human parasitic pathogens. The only nucleic acid-based probe test commercially available is for the detection of *Trichomonas vaginalis*. Since separate probes are required for each parasite, cost becomes an issue. However, as the cost of these tests decreases and test formats are automated, there will be increasing demands for such tests on a routine basis.

### 9.1 *Giardia lamblia*

Currently, commercially available immunoassays for the detection and identification of *G. lamblia* include the following formats: direct fluorescent antibody (FA), enzyme immunoassay (EIA), and the immunochromatographic assays using the cartridge format containing the membrane strips. These test kits can be used with fresh, frozen, or preserved specimens (5%, 10% formalin, SAF, some of the single-vial systems – ask the manufacturer for confirmation of fixative/immunoassay compatibility). **NOTE:** Since the freeze/thaw cycle may distort or rupture *G. lamblia* cysts, it is not appropriate to perform the FA procedure using frozen stool. If using human fecal samples for testing, report “*Giardia lamblia*” positive if the test result is positive. It is important to remember that some of the available tests will be positive with other species of *Giardia* when testing veterinary specimens or water samples. Combination tests with *Cryptosporidium* spp. are also available, as is a cartridge format testing for *G. lamblia*, *Cryptosporidium* spp. and the *E. histolytica/E. dispers* group.

### 9.2 *Cryptosporidium* spp.

Commercially available immunoassays for the detection and identification of *Cryptosporidium* spp. include the following formats: FA, EIA, and the cartridge. These test kits can be used with fresh, frozen, or preserved specimens (5%, 10% formalin, SAF, some of the single-vial systems – ask the manufacturer for confirmation of fixative/immunoassay compatibility). **NOTE:** Since the freeze/thaw cycle may distort or rupture *Cryptosporidium* spp. oocysts, it is not appropriate to perform the FA procedure using frozen stool. If using human fecal samples for testing, report “*Cryptosporidium* spp.” positive if the test result is positive. It is important to remember that some of the available tests will be positive with other
species of Cryptosporidium when testing veterinary specimens or water samples. Combination tests with Giardia lamblia are also available, as is a cartridge format tests for G. lamblia, Cryptosporidium spp., and the E. histolytica/E. dispar group.

9.3 Entamoeba histolytica/E. dispar Group

Differentiation of E. histolytica (pathogenic) and E. dispar (nonpathogenic) is not possible on morphological grounds unless ingestion of RBCs by trophozoites is recognized on the permanent stained smear (e.g., by the trichrome or iron-hematoxylin methods). Reagents are now available for the E. histolytica/E. dispar group, as well as for E. histolytica alone (EIA, cartridge). However, while formalinized specimens can be used with the available immunoassay kits for Giardia and Cryptosporidium, the reagents for E. histolytica/E. dispar or E. histolytica are limited to testing fresh and/or frozen fecal specimens. Since the laboratory is unable to determine whether the organism is E. histolytica or E. dispar on the basis of morphology (i.e., the absence of trophozoites containing ingested RBCs), the report should state the presence of Entamoeba histolytica/E. dispar. An additional comment can be added to the report indicating that if further identification of the true pathogen, E. histolytica, is required, a fresh fecal specimen can be forwarded to a reference laboratory (unless there is an appropriate testing kit in-house). This report (Entamoeba histolytica/E. dispar) indicates that this group of organisms is differentiated from Entamoeba coli and/or Entamoeba hartmanni, both of which are nonpathogens. Although rare, if trophozoites containing ingested RBCs are seen, the organism can be reported as Entamoeba histolytica.

9.4 Entamoeba histolytica

Differentiation of E. histolytica (pathogenic) and E. dispar (nonpathogenic) is not possible on morphological grounds unless ingestion of RBCs by trophozoites is recognized on the permanent stained smear (e.g., by the trichrome or iron-hematoxylin methods). Reagents are now available for the E. histolytica/E. dispar group, as well as for E. histolytica alone (EIA, cartridge). Although formalinized specimens can be used with the available immunoassay kits for Giardia and Cryptosporidium, the reagents for E. histolytica/E. dispar or E. histolytica are limited to testing fresh and/or frozen fecal specimens. Although rare, if trophozoites containing ingested RBCs are seen, the organism can be reported as Entamoeba histolytica. If the fecal immunoassay specific for E. histolytica is positive, then the report can state that Entamoeba histolytica is positive, implying the presence of the true pathogen. This report indicates that the organism is differentiated from Entamoeba coli and/or Entamoeba hartmanni, as well as E. dispar, all of which are nonpathogens.

10 Special Techniques for Organisms Found in Fecal Specimens

10.1 Larval Nematodes: Concentration and Culture Techniques

*Strongyloides stercoralis* rhabditiform larvae are usually the only larvae found in stool specimens. Depending on the bowel transit time and the patient's condition, rhabditiform and rarely filariform larvae may be present. If there is a delay (two or more days) in examination of the stool, then eggs, embryonated eggs, and rhabditiform larvae of hookworms may be present. Culture of feces for larvae is useful to (a) reveal their presence when they are too scanty to be detected by concentration methods; (b) distinguish whether the infection is due to *Strongyloides*, hookworms, or Trichostrongylus on the basis of rhabditiform larval morphology by allowing hookworm egg hatching to occur, thus releasing first stage larvae, and (c) allow development into the filariform larvae for further differentiation. To achieve these goals, one method for concentration and four alternative techniques for culture are described below.46-49
10.1.1 Baermann Concentration Technique

Using a special apparatus, the Baermann technique relies on the principle that active larvae will migrate out of the fecal specimen into water, where they settle out and can be collected and examined. Besides being useful for diagnostic purposes from stool specimens directly, or after enhancement by culture, this technique can be used for examination of soil specimens for the presence of larvae for epidemiologic purposes. Remember to always use universal precautions when handling stool specimens; infective larvae may be present in cases of strongyloidiasis. The Baermann technique procedure is as follows:

1. Use a fresh fecal specimen that has been obtained after administration of a mild saline cathartic, not a stool softener.
2. Set up a ring stand supporting a six-inch glass funnel. Attach a rubber tubing (approximately three-inch) and a pinch clamp to the bottom of the rubber tubing. Place a collection beaker underneath.
3. Place a wire gauze or nylon sieve (recessed 1.0 cm below the top of funnel) over the top of the funnel followed by two layers of gauze.
4. Close the clamp and fill the funnel with tap water until it just soaks the gauze padding.
5. Spread a large amount of fecal material on the gauze padding so that it is covered with water. If the fecal material is very firm, emulsify in water.
6. Allow the apparatus to stand for two or more hours, then draw off 10 mL of fluid by releasing the clamp, centrifuge for two minutes at 500 x g, and examine the sediment under the microscope for the presence of motile larvae.

10.1.2 Agar Plate Culture for Strongyloides stercoralis

Agar plate cultures are also recommended for the recovery of S. stercoralis larvae and tend to be more sensitive than some of the other diagnostic methods. One to two grams of stool is placed onto the center of an agar plate and the plate sealed to prevent accidental infections and held for two 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.

10.1.2.1 Agar

The formula for the agar is the following:

- 1.5% agar
- 0.5% meat extract
- 1.0% peptone
- 0.5% NaCl

**NOTE:** Positive tracking on agar plates has been seen on a number of different types of agar. However, this is the most appropriate agar formula for Strongyloides stercoralis.
10.1.2.2 Quality Control for Agar Plate Culture

(1) Follow routine procedures for optimal collection and handling of fresh fecal specimens for parasitologic examination.

(2) 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 surface of the plates.

(3) Review larval diagrams and descriptions for confirmation of larval identification.

NOTE: 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 posted on the body of the microscope.)

(4) Record all quality control (QC) results (condition of agar plates).

10.1.2.3 Procedure for Agar Plate Culture

(1) Place approximately 2 g of fresh stool in the center of the agar plate (area of approximately one inch in diameter).

(2) Replace the lid and seal the plate with cellulose tape.

(3) Maintain the agar plate (agar side up) at room temperature for two days.

(4) After two 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.

(5) With the end of hot forceps, make a hole in the top of the plastic petri dish.

(6) 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 minutes.

(7) 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 could be spilled onto the counter.

(8) Centrifuge formalin rinse fluid five minutes at 500 x g.

(9) Prepare wet smear preparation from sediment and examine using 10x objective (low power) for presence of larvae. If larvae are found, confirm identification using 40x objective (high dry power).

10.1.2.4 Results and Patient Reports from Agar Plate Culture for Strongyloides stercoralis

Larval nematodes of hookworm, S. stercoralis, or Trichostrongylus spp. may be recovered. If Strongyloides organisms are present, free-living stages and larvae may be found after several days on the agar plates.

(1) Report “No larvae detected” if no larvae could be detected at the end of incubation and rinse procedure.
(2) Report larvae detected by agar plate culture. Example: Strongyloides stercoralis larvae detected by agar plate culture.

10.1.2.5 Procedure Notes for Agar Plate Culture for Strongyloides stercoralis

- Infective larvae may be found any time 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.

- It is important to maintain the plates (agar side up) at room temperature. Do not incubate or refrigerate at any time; this also applies to the fresh stool specimen.

- 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.

- Bacteriology technologists should be alerted if they see random tracking on the agar (movement of larvae dragging bacteria across the plate) as it may be seen in clinical specimens for culture such as sputum and feces.

10.1.2.6 Procedure Limitations for Agar Plate Culture for Strongyloides stercoralis

- 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.

- Specimens that have been refrigerated or preserved are not suitable for culture. Larvae of certain species are susceptible to cold environments.

- In some instances, tracking can be observed on routine bacterial culture media. If this is seen, use extreme caution when handling these plates. These larvae can be infective.

10.1.3 Harada-Mori Culture

This filter paper test-tube culture is performed as follows:

(1) Use fecal material that has not been refrigerated. Some species are susceptible to cold and will not undergo subsequent development.

(2) Cut a narrow (3/8 x 5 inches) strip of filter paper, and make it slightly tapered at one end. Smear 0.5 to 1.0 g of feces in the center of the strip.

(3) Add 3 to 4 mL of distilled water to a 15 mL conical centrifuge tube.

(4) 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 (½ inch) below the fecal spot. It is not necessary to cap the tube. A cork stopper or a cotton plug may be used; however, air must be able to reach the “culture.”

(5) 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 two days, then the culture becomes stabilized).

(6) Keep the tube for ten days, and check it daily by withdrawing a small amount of fluid from the bottom of the tube and examining it for the presence of active larvae.
CAUTION: Infective larvae may be found anytime after the third day. Since infective larvae may migrate upward as well as downward on the filter paper strip, caution must be exercised in handling the fluid and the paper strip itself to prevent infection.

(7) Examine the larvae for typical morphological features to reveal whether hookworm, *Strongyloides*, or *Trichostrongylus* larvae are present. If *Strongyloides* organisms are present, larvae and free-living stages may be found after several days in culture.

(8) It is often difficult to observe details in rapidly moving larvae. If desired, a drop of iodine or formalin, or slight heating can be used to kill the larvae.

10.1.4 Petri Dish Slant Culture

This alternative technique utilizes a filter paper on a microscope slide placed slanted in a glass or plastic petri dish. This technique has the advantage of allowing direct examination of cultured fecal specimen through a stereoscope.

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

(2) Place the strip on a glass slide (1 x 3 inches). Place the slide inclined (about 10°) at one end in the petri dish by using a piece of glass rod.

(3) 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. Add water to maintain the original level.

(4) Keep the dish for ten days. Examine daily either by looking through a stereoscope or by withdrawing a small amount of fluid and placing on a microscope slide and a coverslip.

CAUTION: Infective larvae may be found anytime after the third day. Infective larvae may migrate up the side of the petri dish and be present in the moisture that accumulates under the lid. Great caution should be exercised in handling the fluid in the petri dish or underneath the lid.

(5) Examine the larvae for typical morphological features.

10.2 Helminth Eggs

10.2.1 Schistosomal Hatching Test

The diagnosis of schistosomal infections can be aided by this method. When eggs of *Schistosoma mansoni* or *S. japonicum* are scarce, their presence may be detected by allowing them to hatch and looking for the free-swimming miracidia. This is an especially sensitive test for indirectly demonstrating small numbers of viable eggs present in fecal specimens, because it allows a large sample of specimen to be examined. In addition, this technique allows examination to determine the viability of the eggs. The presence of living miracidia within the eggs indicates an active infection. This is not a concentration technique; therefore there is seldom any point in using it to look for schistosome eggs in urine in which they are readily concentrated by centrifugation. Miracidia are phototropic, and advantage is taken of this characteristic in the following procedure:

(1) Homogenize a stool specimen (40 to 50 g) in 0.85% saline and strain through two layers of gauze.

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1 Visual identification of the cilia on the flames cells (primitive excretory system) in the miracidium in schistosome eggs found in unpreserved stool or urine can also be used to confirm viable eggs.
(2) Allow the material to sediment for one hour, pour off the supernatant fluid and repeat this process at least twice. Although sedimentation is probably better, some laboratories perform low speed centrifugation and test the sediment. Schistosome eggs can be recovered in routine sedimentation procedures and used for the hatching procedure; however, this method is performed using no preservatives, so the egg shells may be a bit more fragile.

(3) Decant the saline solution, resuspend the sediment in a small quantity of chlorine-free water, and pour the suspension into a 500-mL side-arm flask or an Erlenmeyer flask.

(4) Add water to the flask so that the fluid level rises to 2 to 3 cm in the side arm.

(5) 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.

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

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

(8) Examine the lighted area with a magnifying lens to look for minute white organisms swimming rapidly in a straight course. Placing a dark cardboard behind the flask will help detect white miracidia against a black background.

10.2.2 Estimation of Worm Burden

The presence and severity of parasitic disease usually depends not only on the presence of parasitic infection, but also on its magnitude. Parasitic infection may be classified as light, moderate, or heavy. A rough estimate of the adult worm load in a patient can be made from an estimate of the total number of eggs in a 24-hour stool specimen. The human parasitic worms for which it is reasonably possible to correlate egg production with adult worm burdens are the schistosomes, Ascaris lumbricoides, Trichuris trichiura, Ancylostoma duodenale, and Necator americanus. There are various circumstances under which this determination may be advantageous: (1) to determine if the worm burden is sufficient to explain signs, symptoms or laboratory abnormalities, or if another cause should be sought; (2) to determine the intensity of infection when deciding on possible chemotherapy; (3) to evaluate the efficacy of specific anthelmintic therapy in a quantitative manner by periodic egg counts; (4) to estimate worm burdens in a population group to determine the efficacy of a control or an eradication program; and (5) to guide the decision, especially in an endemic area, whether or not to treat a particular patient for worm infection which may be of little or no clinical significance. For example, it is estimated that 25 N. americanus adults are necessary to produce clinical symptoms in an adequately nourished individual who is otherwise normal. A number of methods have been devised to calculate the total egg count in a specimen. Both dilution-based and direct smear-based methods are described.

10.2.2.1 Stoll Egg Counting Technique

This is a dilution method for determining the number of eggs per gram of feces. This technique is designed to obtain the specimen volumetrically by displacing an easily measurable quantity of liquid with feces. Stool displacement flasks for use in this procedure (Stoll flasks) have a long neck with marks at 56 to 60 mL to facilitate easy and proper filling. Stoll flasks and pipets are available commercially. Sodium hydroxide (0.1 N) is used as a diluent, because it saponifies fats, helps to free eggs embedded in debris, and makes the suspension clearer than water does. The procedure is as follows:
(1) Collect an entire 24-hour stool specimen; the specimen can be refrigerated.

(2) Fill the special calibrated Stoll flask with 0.1 N (4 g/L water) sodium hydroxide to the bottom mark etched on the neck of the flask (56 mL).

(3) Add fecal material to the fluid so that the level rises to the 60-mL mark. This amount is roughly equivalent to 4 g of feces.

(4) Add eight to ten glass beads and firmly insert a No. 4 rubber stopper at the neck of the flask. If the specimen is very hard, place the mixture in a refrigerator for a few hours or overnight before the next step.

(5) Shake vigorously with a straight up and down motion to make a uniform suspension. Avoid circular motion, which may concentrate the eggs in a small area. It is acceptable to set the suspension aside for two to three hours to aid the liberation of eggs from fecal debris.

(6) Reshake the contents of the flask. With a calibrated pipet, remove 0.15 mL from the center of the suspension and expel the entire contents onto a 3 inch x 2 inch slide. Do not use a cover glass.

(7) Place the slide on a mechanical stage and examine with the low power objective of the microscope. Count the eggs systematically making sure not to count the same egg twice.

(8) In the 0.15 mL examined, 0.01 mL of feces is present (4 mL feces in 60 mL suspension); therefore, the number of eggs counted should be multiplied by 100 to obtain the number of eggs per mL of feces, and by weight of specimen to get the total number of eggs per 24-hour specimen. **NOTE:** 1 mL of soft feces has been found to weigh 1.03 to 1.04 grams, whereas the same amount of formed stool weighs 1.05 to 1.07 grams. Counts ideally should be recorded as “eggs per mL” rather than “eggs per gram.” However, these counts are sufficiently accurate for routine purposes.

(9) The estimated number of eggs per gram of feces will vary, depending on the consistency of the stool. The most accurate counts will be obtained using formed or semiformed stools. Correction factors should be used to convert the estimate to a “formed stool” basis. For stools with the following consistencies: soft-formed, soft, loose, and watery, multiply the estimate with 1.5, 2, 3, and 4, respectively. Egg counts on liquid specimens are generally least reliable.

10.2.2.2 Simplified Stoll Egg-Counting Technique

Since stool flasks may not be available routinely in every laboratory, this modified technique may be easier to follow.

(1) Fill a 15 mL graduated centrifuge tube to the 14-mL mark with 0.1 N sodium hydroxide.

(2) Add fecal material to the fluid so that the fluid rises to the 15-mL mark.

(3) Mix well with a wooden applicator. If the stool is hard, leave for several hours at room temperature or overnight in the refrigerator.

(4) Shake vigorously to mix and quickly withdraw 0.15 mL from the center of the suspension.

(5) Transfer the material to a slide, then proceed as described in Steps 6 to 9 in **Section 10.2.2.1** above.

**Interpretation of Results:** Heavy infection *Trichuris trichiura* 30 000 eggs/mL; hookworm 2500 to 5000 eggs/mL; *Ascaris* 2000 to 3000 eggs/mL.
**NOTE:** The presence of even one adult *Ascaris lumbricoides* is potentially dangerous because of the migrating habits of this parasite, which can result in serious clinical manifestations.

### 10.2.2.3 Direct Smear Technique

In the original description of this direct smear for egg counting, Beaver (1949) used a light meter apparatus in preparing a fecal smear of a known standard density. For routine purposes, this technique is impractical. A modified procedure that utilizes direct wet mounts of a standard sample of feces was developed also by Beaver (1950). Eggs in the entire film are counted.

1. Place a drop of 0.85% saline in the center of a glass slide.
2. Pick approximately 1.5 to 2 mg of feces (this amount roughly corresponds to a low cone at the tip of an applicator stick).
3. Emulsify the feces thoroughly in the saline and place a coverslip on top.
4. Allow the preparation to settle for a few minutes. The smear may be sealed with petroleum jelly-paraffin (1:1) to prevent drying.
5. Examine the whole smear systematically, making sure not to count the same egg twice.
6. Obtain the estimated number of eggs/mg feces by multiplying the number of eggs counted by 750. This is based on the calculation in the original nephelometric method for which a factor of 500 or 1000 was used depending on whether 2-mg or 1-mg preparations were made.

**Interpretation of Results:** Same as with the Stoll dilution technique as in Section 10.2.2.2.

### 10.2.2.4 Kato Thick Smear

This cellophane-covered thick smear was described for use in field surveys for helminthic infections. It was later slightly modified for use in quantitative determinations. This method has the advantage of allowing examination of a large amount of feces (50 to 60 mg), which is about 25 times more than what is used in other methods. Stool stabilized in liquid preservatives is not suitable to prepare a Kato thick smear because of the dilution effect and the effect on clearing. However, powdered sodium azide adequately preserves stool specimens and can be used as a preservative if delay is anticipated in preparing the smears.

1. Obtain wettable, medium thickness cellophane coverslips (22 x 30 mm).
2. Soak the coverslips in a mixture of 100 mL pure glycerin and 100 mL water containing 1 mL malachite green (3% aqueous solution).
3. Press a portion of stool through a piece of stainless steel mesh (mesh No. 100) to separate out large fibers and particles. Omit this step if stool does not contain a lot of fibers or large particles.
4. Transfer 50 mg of feces from a well-mixed 24-hour stool to a clean slide and cover with a presoaked cellophane coverslip.
5. Invert the slide and press against an absorbent surface until the fecal mass covers an area of 20 to 25 mm in diameter.
(6) Leave the preparation for one hour at room temperature or for 30 minutes at 40 °C to allow clearing of the fecal material.

(7) Examine the entire preparation under the low power objective and count the number of eggs on the whole slide. To avoid overclearing, examine the slides within one hour of preparation.

(8) Report the number of eggs per smear and make appropriate calculations to determine the number of eggs per gram of feces or per 24-hour specimen.

*Interpretation of Results:* Same as with the Stoll dilution technique as in Section 10.2.2.2.

**NOTE:** Counts provide an estimate of the existing worm burden.

### 10.2.3 Recovery of *Enterobius* and *Taenia* Eggs

Since the eggs of *Enterobius vermicularis* are usually deposited outside the body when the adult female migrates to the perianal area rather than within the intestinal tract, diagnostic tests other than stool examination are essential to confirm the diagnosis of enterobiasis.1,43,67-69 *Taenia solium* eggs, while usually found free in feces, may also be recovered by cellophane tape techniques, because gravid proglottids often creep to the perianal area. The specimens should be taken upon rising, before bathing and/or a bowel movement. Multiple specimens are recommended before indicating the patient does not have the infection.1,68

#### 10.2.3.1 Graham Cellophane Tape Technique

(1) Anchor a piece of clear cellulose tape (0.75 inches wide and 4 to 5 inches long) on the underside of a 3 x 1 inch slide. Affix a strip of paper to the free end of the tape for labeling and identification. Hold slide against a tongue depressor starting ½ inch from one end. Running around the same end, loop tape over to expose sticky surface. Commercial collection paddles are also available and can be used instead of the clear tape.

(2) **CAUTION:** *Enterobius vermicularis* and *Taenia solium* eggs can be infectious when recovered. Put on gloves before obtaining and while working with specimen.

(3) To obtain the specimen from the perianal area, hold the tape and the slide against the tongue depressor and press against the right and left perianal folds.

(4) Replace the tape on the slide (with the adhesive side now on the glass), and press firmly into position. Clean the upper side of tape with a dry piece of cotton or gauze. Place name and date on the label.

(5) Lift the tape from the side and place a drop of toluene or xylene (xylene substitute is also acceptable) on the slide. Press tape back into place.

**NOTE:** Caution should be taken when using toluene or xylene because of the fumes.

(6) Examine the slide with the 10x objective.

**NOTE:** Toluene or xylene should not be added until the time of examination, because prolonged contact with these clearing agents may cause the eggs to degenerate. Tape preparations may be stored in the refrigerator for several days or weeks prior to application of toluene/xylene/xylene substitute without degeneration of the eggs.
10.2.3.2 Swube Tube Technique

Originally designed to collect pollen and plant spores, the “swube tube” is a useful tool for recovering eggs of Enterobius and Taenia. The tube consists of a paddle with one side labeled “sticky side” that fits inside of a larger holding tube.

CAUTION: Enterobius and Taenia solium eggs can be infectious when recovered. Wear gloves before obtaining and while working with the specimen.

(1) To obtain the specimen from the perianal area, remove the paddle from the tube and press “sticky side” against the right and left perianal folds.

(2) Replace the paddle in the tube. Label with name and date.

(3) Refrigerate until examination.

(4) To examine, remove the paddle from the tube and holder; place it on the microscope stage and move it manually while examining it with the 10x objective (low power). The paddle can also be placed on a glass slide to prevent contamination of the microscope stage. Also, the sticky side with the sample can be applied to a glass slide and the paddle cut.

10.3 Adult Helminths

10.3.1 Cestode Scolices and Proglottids

10.3.1.1 Search for Cestode Scolices and Proglottids

Occasionally, stool specimens have to be searched for the presence of scolices and gravid proglottids for positive species identification. Recovery of scolices after therapy is an indication of successful treatment. If scolices have not been passed, then they may be still attached to the mucosa, and the tapeworm is capable of producing more segments and the infection continues. Anthelmintics such as niclosamide and praziquantel tend to dissolve the tapeworm; therefore, the scolex may be difficult to recover unless the patient receives a saline purge soon after taking the medication. CAUTION: Taenia solium eggs are infectious; remember to wear gloves when handling these specimens.

(1) Examine grossly a 24-hour stool specimen for the presence of proglottids. If any are found, gently use cotton-tipped applicators to follow the narrowing proglottids in search of the scolex (the proglottids are not always attached to the scolex; it may be very small and difficult to find). If neither proglottids nor scolex is found, proceed to the next step.

(2) Mix a 24-hour stool specimen with water and thoroughly break up the specimen to make a watery suspension.

(3) 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). A double or triple layer of gauze taped over a deep pan is also acceptable (easy disposal into plastic bag for decontamination).

(4) Wash off the sediment remaining from each portion by running a slow current of water over it.

(5) Examine the cleansed debris with a hand lens to look for scolices and proglottids (a Taenia scolex is 0.5 to 1 cm long and 1 to 2 mm wide).

(6) Repeat Steps 3 and 4 for each portion of the suspension strained.
(7) Collect the strained sediment in a glass dish and place over a black surface to increase the contrast of organisms against the background.

(8) Observe with a magnifying hand lens and pick pieces of worms using an applicator stick or a camel hair brush.

**NOTE:** Wood’s lamp may be used to search for scolices if the patient has been given quinacrine dyes. The worms, having absorbed the dye, will fluoresce at a wavelength of 360 nm.

(9) Rinse gravid proglottids and/or scolices with tap water and place between two microscope slides separated at the edges by thin pieces of cardboard.

(10) Fasten the preparation by means of rubber bands at each end of the slides so that the segments become somewhat flattened.

(11) Observe under the low power of a dissecting microscope for the number of uterine branches in the segments and the presence or absence of a rostellum of hooks on the scolex.

10.3.1.2 Tapeworm Species Identification

Species identification on the basis of the number of uterine branches in gravid segments may be greatly facilitated by injecting gravid segments with India ink. This may be done as follows:

(1) Place the proglottids in water for several hours to relax spread evenly.

(2) Blot the segments with a piece of tissue and place on a microscope slide.

(3) Using a tuberculin syringe and a hypodermic needle, slowly inject India ink into the distal end of the gravid proglottid and into the central uterine stem. Ink can also be injected directly into the uterine pore.

(4) Wash the segment quickly in water, blot thoroughly, and place between two microscope slides held together by means of rubber bands at each end. The uterine branches will be black and can be counted.

(5) If desired, dehydrate the segments by placing through several changes of 50%, 70%, 90%, and absolute alcohol. Clear the segment in two changes of xylene and mount in mounting medium.

(6) Examine under a stereoscope for distinctive characteristics.

Tapeworm segments that are not relaxed in water before they are put in formalin may contract so much that injecting India ink is impractical. The uterine branches may then be highlighted by staining with Semichon’s acid carmine. This is done as follows:

(1) Prepare the stock stain by mixing:

- Carmine powder 5 g
- Distilled water 50 mL
- Acetic acid, glacial 50 mL

(The shelf-life is a minimum of five years, provided the bottle is tightly stoppered.)

(2) Place segment in 70% ethanol for 30 minutes to prepare it for staining.
(3) Prepare the working stain by adding drops of the stock stain to 70% ethanol until it is a medium pink.

(4) Place the segment in the working stain at least overnight. The stain must penetrate all the way through to stain the internal structures. It may take seven days to adequately stain extremely shrunken segments.

(5) Rinse the segment in 70% ethanol.

(6) Destain in weak acid alcohol (three drops concentrated HCl in 100 mL 70% ethanol) until the uterine branches are barely visible through the tissues. (They will be easier to see after clearing.) Destaining may take a few minutes to hours depending on the size of the segment and its condition.

(7) Rinse in 70% ethanol.

(8) Neutralize in Na₂CO₃-ethanol (one drop saturated Na₂CO₃ in 10 mL 70% ethanol) for 30 to 60 minutes.

(9) Rinse in 70% ethanol.

(10) Clear the segment in lactophenol or glycerin. If the uterine branches are not stained well enough to count the segment, they may be rinsed in 70% ethanol and returned to the stain solution.

10.3.1.3 Fixation

Often, and mostly for educational reasons, it is essential to preserve recovered worms. This may be done by placing the worms in 10% formalin that has been heated to 60 to 63 °C. The worms are stirred gently to allow fixation without excess contraction of the segments. This mixture can be stored indefinitely.

10.3.2 Trematodes

10.3.2.1 Recovery

Essentially the same procedure described for searching for tapeworm scolices and proglottids may be followed. For larger adult trematodes, a simple search of the specimen with the naked eye is satisfactory.

10.3.2.2 Fixation

(1) Prepare a fixative solution with the following components:

- Formaldehyde  10 mL
- 95% alcohol  50 mL
- Glacial acetic acid  5 mL
- Distilled water  45 mL

(2) Heat the solution to 60 to 63 °C. (Use a hot plate and because of the vapor, place solution in a fume hood while heating.)

(3) Fix the adult trematode by placing in the above solution for 24 hours. The solution does not have to be kept hot during the 24-hour period.

(4) Place the worm between two slides held together with a rubber band at each end.
(5) Examine under a stereoscope for distinctive characteristics.

10.3.3 Nematodes

10.3.3.1 Recovery

For small adult nematodes, follow the same procedure described for searching for tapeworm scolices and proglottids. For larger adult nematodes, a simple search of the specimen with the naked eye is satisfactory.

10.3.3.2 Fixation and Clearing of Small Nematodes

(1) Heat 70% alcohol to 75 °C (use a hot plate).

(2) Fix the adult worms by placing in the heated alcohol for three hours or longer. The alcohol is heated only initially. Do not continue to heat.

(3) Transfer the specimens to a shallow dish and add a larger volume (three to four times) of 10% glycerin in 70% alcohol (one part glycerin to nine parts 70% alcohol).

(4) Leave uncovered in a dust-free location for several days. This allows evaporation of alcohol, leaving the worms in increasingly stronger concentrations of glycerin.

(5) Mount on a slide in glycerin, place a coverslip and examine microscopically.

(6) Mount the specimen using glycerin jelly if a permanent mount is desired. Glycerin jelly is prepared as follows:

- Gelatin 8 g
- Distilled water 52 mL
- Glycerin 50 mL
- Egg albumin (fresh) 5 mL
- Phenol crystals 0.1 g

a. Soak the gelatin in water and heat gently to dissolve.
b. Add glycerin and egg albumin, stir until thoroughly mixed.
c. Use a hot plate to heat to 75 °C for 30 minutes.
d. Filter through several layers of fine flannel using a prewarmed funnel.
e. Add phenol crystals to the filtrate.

NOTE: This method is also good for preparing permanent mounts of helminth eggs (see Section 10.2). However, the preparation must be sealed with fingernail polish or other mounting medium.

10.3.3.3 Fixation and Clearing of Large Nematodes

(1) Prepare a dilute solution (1% to 2%) of formalin.

(2) Fix the adult worms (Ascaris lumbricoides) by placing them in the dilute formalin for two to three hours (worms die slowly over a period of a few hours).

(3) Once the worms are dead, transfer them to a larger container of 10% formalin. Do not use this higher percentage of formalin for killing purposes. Use of higher concentrations for killing may cause the worms to rupture.
NOTE: The fixative listed above (Section 10.3.3.2) can also be used for larger nematodes.

11 Other Specimens From the Intestinal Tract

11.1 Sigmoidoscopy Specimen: Direct Wet Smear

The direct smear is primarily used to detect motile parasites that are found in the colon (organism in question is usually Entamoeba histolytica; however, other protozoans, if present, can be recovered). Specific ulcerated areas should be sampled. If no lesions are seen, the mucosa should be randomly sampled from at least six different areas of the mucosa. On low power (100x) examination of the smear, motility of trophozoites and/or human cells might be detected. At high-dry power (400x), organisms might be tentatively identified based on size, nuclear/cytoplasm ratio, appearance of the cytoplasm, and the motility (saline only). The direct smear can be prepared with either 0.85% NaCl or with the addition of iodine (Lugol’s or D’Antoni’s; see Section 7). Presumptive findings using this procedure should always be confirmed using a permanent stained smear.

11.1.1 Specimen

Mucosal lining, mucus, stool, and/or a combination of the three may be used for the specimen, which is usually taken by the physician and either prepared at bedside for immediate examination as a wet preparation or submitted to the laboratory for subsequent examination as a wet preparation and permanent stained smear. Cotton swabs should not be used to collect the specimen; the material will be absorbed into the cotton. Material may also be submitted to pathology for routine histological preparation and examination. Prepare direct wet mounts on clean, scratch-free 1 inch x 3 inch glass slides. Depending on the specimen type, the following specimen to diluent ratio should be used:

- For mucoid specimens, two drops specimen to one drop diluent.
- For stool, one drop specimen to two to three drops diluent.
- For watery material, two drops specimen to zero to one drops diluent.

NOTE: Watery material may require centrifugation prior to smear preparation.

11.1.2 Specimen Transport

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 must be transported to the laboratory within 30 minutes from the collection time and must be examined immediately. If this is not possible, then the material should be preserved and processed as a permanent stained smear.

11.1.3 Reagents (Refer to Section 7.1)

- 0.85% NaCl
- Modified D’Antoni’s stock iodine
- Lugol’s iodine solution
11.1.4 Quality Control

The direct mount reagents should be checked each time they are used. The saline should appear clear, without any visible contamination. The stock iodine (D’Antoni’s or Lugol’s) should be a “strong tea” color, and there should be crystals in the bottom of the bottle. The working solution should also be “strong tea” color. If not, then discard the old working solution and dispense some stock solution into the dropper bottle (new working solution).

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.

11.1.5 Procedure

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

2. Examine the smear using the low power objective (10x) with low light. View each field for a few seconds, looking for any organism motility. **NOTE:** Do not have the light too bright or any organisms present could be missed. Any suspicious objects should be examined using the high dry objective (40x) with low light.

3. Prepare a wet mount using D’Antoni’s or Lugol’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 preparation. The iodine will diffuse into the saline suspension under the coverslip. If the specimen is thick or contains mucus, capillary action pulling the iodine under the coverslip and into the saline may not occur, and it will be necessary to make a separate iodine mount. The addition of the iodine will give the material some color (organisms may lose some refractility, but the internal contents may be easier to see); however, motility will be lost.

11.1.6 Results

- Protozoan trophozoites may be confused with human cells (macrophages), so any identification should be reported as “presumptive” until the permanent stained smears have been examined.

- Also, the presumptive identification and quantitation of the human cells (macrophages, polymorphonuclear leukocytes, eosinophils, red blood cells) can be determined from the wet preparations. However, this information should also be considered “presumptive” until the permanent stained smears have been examined.

11.1.7 Reporting Results

1. 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. An example of a laboratory report would be: *Cryptosporidium* spp. oocysts.

2. Note and quantitate the presence of human cells. An example would be: moderate PMNs, many RBCs, few macrophages.

3. Notify the physician if organisms are identified. If the results are negative, this should be reported as a “presumptive report” (based on wet examination only) prior to the examination of the permanent stained smear.
Table 1. Quantitation of Parasites, Host Cells, Yeasts, Artifacts

<table>
<thead>
<tr>
<th></th>
<th>Protozoa, Host Cells, Yeasts, Artifacts</th>
<th>Helminths</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Permanent Stained Smear</td>
<td>Wet Prep</td>
</tr>
<tr>
<td>Quantity</td>
<td>No. per 10 Oil Immersion Fields (1000x)</td>
<td>No. per 10 40x Fields (400x)</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>

This is a general chart for the quantitation of parasites, host cells, yeasts, and artifacts found in specimens from the intestinal tract. In general, protozoa are not quantitated on the laboratory slip (exception: Blastocystis hominis); however, human cells, yeasts, and artifacts like Charcot-Leyden crystals are normally reported and quantitated.

11.1.8 Procedure Notes

- Remember that the iodine working solution should be a “strong tea” color; if not, discard and prepare a new working solution.

- 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 1000x oil immersion to see morphologic details.

- In the saline mount human cells and/or protozoan trophozoites may exhibit some motility.

- In the iodine mount human cells and/or protozoan trophozoites may be seen, but will not exhibit motility.

- Presumptive findings (either positive or negative of other than helminths) must be confirmed using a permanent stained smear.

11.1.9 Limitations of the Procedure

- Multiple areas of the mucosa should be examined (six smears are recommended), and this technique should not take the place of the routine parasite examination.

- Wet preparations are normally not examined using oil immersion power (1000x); however, this may vary among laboratories. In any case, permanent stained smears should be used to confirm morphology and organism (or human cell) identification. Remember, some shrinkage of organisms can occur in permanent stained smears and is most visible with protozoan cysts.
If the specimen amount is limited, the specimen that is available should be processed as a permanent stained smear to maximize the amount and clinical relevance of the information obtained.

11.2 Sigmoidoscopy Specimen: Permanent Stained Smear

The permanent stained smear from sigmoidoscopy is used to detect parasites in the colon (the organism in question is usually *Entamoeba histolytica*). Specific ulcerated areas are sampled. If no lesions are seen, the mucosa should be randomly sampled from six different areas. On oil immersion power (1000x) examination of the smear, protozoan trophozoites and/or cysts might be detected. Coccidian oocysts, helminth eggs or larvae, and/or human cells may also be detected using this procedure. The permanent smear can be stained with trichrome or iron-hematoxylin stains. Permanent stained smears usually confirm those structures that might have been seen on the wet specimen examinations.

11.2.1 Specimen

The specimen may consist of mucosal lining, mucus, stool, and/or a combination of the three.

1. Prepare smears on clean, scratch-free 1 inch x 3 inch glass slides.
2. For mounts of mucus or similar material, place approximately one to two drops onto the slide.
3. For mounts of stool, place approximately one drop onto the slide.
4. If the material is very watery, you can add one to two drops onto the slide.

11.2.2 Reagents

- Schaudinn’s fixative
- Polyvinyl alcohol fixative (PVA)

See Section 5.2 for preparation instructions and for information regarding commercial availability of PVA.

11.2.3 Quality Control

- The fixatives should be checked weekly or when a new lot number is used. Fresh stool containing protozoa or negative stool seeded with human buffy coat cells can be used to evaluate the efficacy of the fixatives. Cultured protozoa can also be used. If the specimen is received in fixative from a referral laboratory, it is assumed that the QC on the fixative solution has been completed by the referral laboratory.

  — The Schaudinn’s fixative should appear clear, without floating debris or crystals. It is acceptable to have some crystal sediment on the bottom of the Coplin jar or dish.

  — The PVA fixative should be clear (slight milky or smoky color or slight precipitate on the bottom 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.

- All QC results should be recorded.
11.2.4 Procedure

(1) Gently smear a drop or two of patient material onto the slide and immediately immerse the slide into the Schaudinn’s fixative. The fixation and staining times are identical with those for routine fecal smears. Please refer to Section 8 for specific directions.

(2) If the material is bloody, contains a lot of mucus, or is a watery specimen, gently mix one drop of patient material with three drops of PVA fixative directly on the slide. The smear should be allowed to dry for at least two hours prior to staining either at room temperature or at 35 to 37 °C. The fixation and staining times are identical to those for routine fecal smears. Please refer to Section 8 for specific directions.

(3) Examine the stained smear using the oil immersion lens (100x) with appropriate light. At least 300 oil immersion fields of the smear should be examined.

11.2.5 Results

• With either the trichrome or iron-hematoxylin stains, the protozoan trophozoites and cysts can be seen. Oocysts will not be clearly delineated; if suspect organisms are seen, then additional procedures will need to be used for confirmation (see Section 8.4 or Section 8.5).

• Helminth eggs or larvae may not be easily identified on the permanent stained smear, and wet mount examinations should be performed.

• Human cells are readily identified (macrophages, polymorphonuclear leukocytes [PMNs], red blood cells [RBCs], etc.).

• Yeast cells (single cells, budding, presence of pseudohyphae) are also easily identified.

11.2.6 Reporting Results

(1) Report the organism and stage; do not use abbreviations. An example would be: *Entamoeba histolytica* trophozoites (containing red blood cells) or *Entamoeba histolytica/E. dispar* trophozoites (no ingested red blood cells).

(2) Note and quantitate the presence of human cells. Examples would be: moderate PMNs, many RBCs, few macrophages, etc.

(3) Report and quantitate yeast cells which are abundant (≥50/oil immersion field) or are budding or have pseudohyphae. The presence of pseudohyphae in a fresh preparation or one that was immediately preserved after collection is suggestive of invasive disease. Examples would be: moderate budding yeast cells and few pseudohyphae.

(4) Refer to Table 1.

11.2.7 Procedure Notes

• Sigmoidoscopy specimens are submitted to help differentiate between inflammatory bowel disease and amebiasis. It is critical that the specimens be preserved immediately after being obtained. Any delay will result in the disintegration of amebic trophozoites or distortion of human cells.
• It is critical that permanent stained smears of this material be carefully examined using the oil immersion lens (100x).

11.2.8 Limitations of the Procedure

• The more areas of the mucosal surface sampled, the more likely the organisms might be found (Entamoeba histolytica). The specimens should not be taken with a cotton swab, because the material will be absorbed into the cotton. If only one or two smears are submitted for examination, the physician must be informed (the recommendation is six smears from representative areas of the mucosal surface).

• The examination of sigmoidoscopy smears 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 ten days).

11.3 Duodenal Aspirate

Some organisms may be more difficult to recover in the stool, particularly those normally found in the duodenum. An alternative approach would be to sample the duodenal contents. Samples are obtained through the use of nasogastric intubation or the “string test”¹,⁷¹ (see Section 11.4). Fluid from the duodenum is examined for the presence of Strongyloides stercoralis, Giardia lamblia trophozoites, Cryptosporidium spp., and Isospora belli oocysts. The specimen can be examined as a wet preparation or as a permanent stained smear. In rare instances, Clonorchis sinensis, Opisthorchis viverrini, Fasciola hepatica, Trichostrongylus, and hookworm eggs may be recovered.

11.3.1 Specimen

Duodenal fluid must be transported STAT in a securely covered container placed in a plastic bag. A screw-capped urine container or plastic centrifuge tube with no preservatives is practical for this purpose. If examination or transport will be delayed, duodenal fluid may be added to fixatives at the collection site.

11.3.2 Reagents

The following reagents are used (see Section 5.2):

• Schaudinn’s fixative

• PVA

• 10 % formalin

• SAF

11.3.3 Quality Control

• The fixatives should be checked weekly or when a new lot number is used. Fresh stool containing protozoa or negative stool seeded with human buffy coat cells can be used to evaluate the efficacy of the fixatives. Cultured protozoa can also be used.

— The Schaudinn’s fixative should appear clear, without floating debris or crystals. It is acceptable to have some crystal sediment on the bottom of the Coplin jar or dish.
— The PVA fixative should be clear (slightly milky or smoky color or slight precipitate on the bottom 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.

— The 10% formalin should appear clear, without any visible contamination.

- All QC results should be recorded.

### 11.3.4 Procedure

**NOTE:** Gloves must be worn when handling this specimen; infectious *Strongyloides* larvae can penetrate the intact skin.

1. Examine the specimen within one hour after it is taken. Note the amount of yellow color present, which indicates bile staining and confirms that it is actually from the duodenum.

2. If necessary, centrifuge to concentrate the mucus and any organisms present (500 x g for ten minutes). Centrifugation should be routinely performed if the volume of fluid is ≥2 mL.

3. Place one drop of fluid on a clean slide and cover with a 22 x 22 mm coverslip. If the specimen is very viscous, a drop of saline may be added.

4. Examine the entire coverslip under low power (100x) for larvae or motile trophozoites, looking especially carefully around the mucus where *Giardia lamblia* may be entangled.

5. Examine the mucus present under high dry (400x), since *Giardia lamblia* may only be detectable by the flutter of the flagella rather than the motility of the organism.

6. Immediately after reading, place the slide in a Coplin jar containing Schaudinn’s solution, so permanent stained smears can be prepared. Do not dry the slide, or the coverslip will not float off and sink to the bottom. If you have enough specimen for a second sample, gently smear a drop or two of patient material on the second slide and immediately immerse the slide into the Schaudinn’s fixative. The fixation and staining times are identical with those for routine fecal smears. Please refer to Section 8 for specific directions.

7. If the material contains a lot of mucus, or is a watery specimen, gently mix one drop of patient material with three drops of PVA fixative directly on the slide. The smear should be allowed to dry for at least two hours prior to staining either at room temperature or at 35 to 37 °C. The fixation and staining times are identical to those for routine fecal smears. Please refer to Section 8 for specific directions.

8. Place a drop of the duodenal fluid on one or more slides to be stained for *Cryptosporidium* spp., *Cyclospora cayetanensis*, and *Isospora belli*. Make additional slides if microsporidia are suspected.

9. Repeat the wet mount procedure (Steps 4 to 7) until all the remaining mucus (after centrifugation) or sediment is gone.

10. Stain the *Cryptosporidium/Isospora* slide(s) with modified acid-fast stain and examine as usual. Please refer to Sections 8.4 or 8.5 for specific directions. Positive smears using Auramine-O should be confirmed by modified acid-fast staining.

11. Stain slide for microsporidia with modified trichrome stain. Refer to Section 8.8 for specific directions.
(12) Examine the permanent stained smear (using the oil immersion lens 100x) with appropriate light. At least 300 oil immersion fields on each smear should be examined.

11.3.5 Results

- With either the trichrome or iron-hematoxylin stains, the protozoan trophozoites and cysts can easily be seen. Oocysts will not be clearly delineated; if suspect oocysts are seen, then additional procedures will need to be used for confirmation. See Section 8.4 or Section 8.5.

- Oocysts of Cryptosporidium spp. and Isospora belli will be visible on permanent stained smears (modified acid-fast procedures). Please refer to Sections 8.4 or Section 8.5 for specific directions.

- Helminth eggs or larvae may not be easily identified on the permanent stained smear, but will be visible in the wet preparations.

11.3.6 Reporting Results

(1) 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 of a laboratory report would be: Giardia lamblia trophozoites or Strongyloides stercoralis larvae.

(2) Call the physician if organisms are identified.

(3) Quantitate any helminth eggs if they are recovered.

(4) If the results are negative on the wet smear examination, report as a “presumptive report (based on wet examination only)” prior to the examination of the permanent stained smear.

(5) Refer to Table 1.

11.3.7 Procedure Notes

- If more than 2 mL of specimen is received, centrifuge the specimen (500 x g for ten minutes), and examine the mucus or material in the bottom of the tube.

- Modified acid-fast methods (Cryptosporidium spp.) or monoclonal antibody direct detection methods (Cryptosporidium spp., Giardia lamblia) can be used. Isospora belli can be identified on the wet examination or on smears stained with the modified acid-fast methods.

11.3.8 Limitations of the Procedure

- Many of the parasites will be caught up in the mucus; therefore, it is very important to centrifuge the specimen to concentrate this material for examination. Centrifugation is strongly recommended if the specimen is visibly almost transparent and is more than 2 mL.

- Although duodenal aspirate specimens are normally examined as wet preparations, it is important to remember that some of the organisms may be missed without additional permanent stains (Cryptosporidium, Isospora).
11.4 String Test (Gelatin-Capsule Method)

This test is used to obtain specimens from the duodenum which are then examined for the presence of parasites. This test consists of a gelatin capsule lined with silicone rubber that contains a spool of nylon string and a weight.\textsuperscript{1,2,3} 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. After swallowing the capsule, the patient is allowed to relax for four hours. The patient is not allowed to eat during this time frame, but can drink small quantities 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 \textit{Strongyloides} larvae, \textit{Giardia} trophozoites, or \textit{Cryptosporidium} or \textit{Isospora} oocysts that are present will also adhere to the string and will be pulled up with the string when it is removed.\textsuperscript{1} The specimen can be examined as a wet preparation or as a permanent stained smear. In rare instances, \textit{Clonorchis sinensis}, \textit{Opisthorchis viverrini}, \textit{Fasciola hepatica}, \textit{Trichostrongylus} spp., or hookworm eggs may be recovered.

11.4.1 Specimen

- The physician must notify the laboratory when a capsule is swallowed so that a parasitologist is available to read the test four hours later when the string is removed.

- The string is placed in a securely covered container, which is transported in a plastic bag. Unless the specimen container is hand-delivered to the laboratory, 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.

- The specimen must be transported STAT and read within one hour.

11.4.2 Reagents

The following reagents are used (see Section 5.2):

- Schaudinn’s fixative

- Polyvinyl alcohol fixative (PVA)

- 10 % formalin

- Sodium acetate-acetic acid-formalin (SAF)

11.4.3 Quality Control

- The fixatives should be checked weekly or when a new lot number is used. Fresh stool containing protozoa or negative stool seeded with human buffy coat cells can be used to evaluate the efficacy of the fixatives. Cultured protozoa can also be used.

  - The Schaudinn’s fixative should appear clear, without floating debris or crystals. It is acceptable to have some crystal sediment on the bottom of the Coplin jar or dish.

  - The PVA fixative should be clear (slightly milky or smoky color or a slight precipitate on the bottom 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.

  - The 10% formalin should appear clear, without any visible contamination.
• All QC results should be recorded.

11.4.4 Procedure

NOTE: Gloves must be worn when handling this specimen; infectious *Strongyloides* stercoralis larvae can penetrate the intact skin. After putting on gloves, wash gloved hands with soap and water to remove any powder.

(1) Record the color of the string. Yellow bile stain indicates that the string did reach the duodenum.

(2) Place the specimen inside 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.

(3) Place one drop of mucus on a clean slide and cover with a 22 x 22 mm coverslip. If the mucus is very viscous, a drop of saline may be added before adding the coverslip.

(4) Store the remaining mucus in a transfer pipet placed in a labeled 16 x 125 mm test tube, so it will not dehydrate.

(5) Examine the entire coverslip under low power (100x) for eggs, larvae, or motile trophozoites, looking especially carefully at the mucus where *Giardia lamblia* may be entangled.

(6) Examine the mucus present under high dry (400x), since *Giardia lamblia* may only be detectable by the flutter of the flagella rather than the motility of the organism.

(7) Immediately after reading, place the slide in a Coplin jar containing Schaudinn’s solution, so permanent stained smears can be prepared. Do not dry the slide, or the coverslip will not float off and sink to the bottom. If there is enough specimen, gently smear a drop or two of patient material on the labeled slide, and immediately immerse the slide into the Schaudinn’s fixative. The fixation and staining times are identical with those for routine fecal smears. Please refer to Section 5 for specific directions.

(8) If the material contains a lot of mucus, or is a watery specimen, gently mix one drop of patient material with three drops of PVA fixative directly on the slide. The smear should be allowed to dry for at least two hours prior to staining either at room temperature or at 35 to 37 °C. The fixation and staining times are identical to those for routine fecal smears. Please refer to Section 8 for specific directions.

(9) Place a drop of the mucus on one or more slides to be stained for *Cryptosporidium* spp. and *Isospora belli*.

(10) Repeat the wet mount procedure (Steps 4 to 7) until all the remaining mucus is used.

(11) Stain the *Cryptosporidium/Isospora* slide(s) with modified acid-fast stain and examine as usual. Please refer to Section 8.4 or Section 8.5 for specific directions.

(12) Examine the permanent stained smear, using the oil immersion lens (100x) with appropriate light. At least 300 oil immersion fields on each smear should be examined.

(13) If *Strongyloides* eggs and/or larvae or *Cryptosporidium* or *Isospora* oocysts are found, the rest of the specimen can be preserved in 5% or 10% formalin for teaching purposes.
11.4.5 Results

- With either the trichrome or iron-hematoxylin stains, the protozoan trophozoites and cysts can easily be seen. Oocysts will not be clearly delineated; if suspect organisms are seen, then additional procedures will need to be used for confirmation. See Section 8.4 or Section 8.5.

- Oocysts of Cryptosporidium and Isospora will be visible on permanent stained smears (modified acid-fast procedures). Please refer to Section 8.4 or Section 8.5 for specific directions.

- Helminth eggs or larvae may not be easily identified on the permanent stained smear, but will be visible in the wet preparations.

11.4.6 Reporting Results

(1) 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 of a laboratory report would be: Giardia lamblia trophozoites or Strongyloides stercoralis larvae.

(2) Call the physician if organisms are identified.

(3) Quantitate Clonorchis sinensis eggs if they are recovered.

(4) If the results are negative on the wet smear examination, report as a “presumptive report” (based on wet examination only) prior to the examination of the permanent stained smear.

(5) Refer to Table 1.

11.4.7 Procedure Notes

- Modified acid-fast methods (or monoclonal antibody direct detection methods) can be used for the identification of Cryptosporidium spp.

- Isospora belli can be identified on the wet examination or from smears stained with the modified acid-fast method.

11.4.8 Limitations of the Procedure

- 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 (400x) with low light intensity in order to see the “flutter” of the Giardia flagella.

- If the duodenal specimens from the gelatin capsule are normally examined as wet preparations, it is important to remember that some organisms and other species may be missed without additional permanent stains (Cryptosporidium, Isospora).

12 Culture Technique for Amoebae

12.1 Entamoeba histolytica

Cultivation of feces for amoebae can be helpful to demonstrate the presence of organisms that were not found by concentration or direct microscopy. Cultivation, however, is not considered an alternative to other methods of detection, since specimens that are positive by microscopic examination can often be
Although Entamoeba histolytica may be cultured from clinical specimens for diagnostic purposes, culture has been used primarily for the growth of large numbers of trophozoites for research investigations. Cultured organisms can be used for antigen tests to differentiate E. histolytica from E. dispar. This organism has been successfully cultured from clinical specimens by using axenic or nonaxenic culture methods. A wide variety of media have been described for the cultivation of intestinal parasites. Among the more popular media are the modified Boeck and Drobohlav’s Locke-egg diphasic medium, Balamuth’s egg yolk infusion medium, Diamond’s trypticase yeast extract, iron-serum medium (TYI-S-33), and trypticase yeast extract serum gastric mucin medium (TYS GM-9).\textsuperscript{72-81}

12.2 Specimen

A fresh stool specimen should be collected in a clean, wide-mouthed container. If the specimen cannot be cultured immediately, it should be held at room temperature until it can be cultured. Specimens greater than 24 hours old should not be cultured. Biopsy tissues and liver aspirates have also been successfully cultured.

12.3 Tys Gm-9 Medium

12.3.1 Reagents

12.3.1.1 Nutrient Broth

(1) Dissolve 2.8 g monobasic potassium phosphate, 0.4 g dibasic potassium phosphate, 7.5 g sodium chloride, 2.0 g trypticase (BBL), and 1.0 g yeast extract in 600 mL deionized water.

(2) Add deionized water to bring the volume to 970 mL.

12.3.1.2 Rice Supplement

(1) Place 500 mg rice starch (purified rice starch is preferred, but unpolished rice flour is also satisfactory) into each of several 16 x 125 mm screw-cap glass tubes, and sterilize with dry heat at 150 °C for 2.5 hours. To assure sterilization, place the tubes horizontally and loosen the cap to allow free movement of the hot air.

(2) Place 9.5 mL sterile 0.85% saline or phosphate buffered saline (pH 7.2) into each tube.

(3) Bring the rice starch into suspension by vigorous shaking with a vortex-type mixer. Store at 4 °C.

12.3.1.3 Preparation of Complete Medium

(1) Place 200 mg gastric mucin into each of ten sterile, screw-cap 125 mL Erlenmeyer flasks.

(2) Add 96 mL nutrient broth to each flask and autoclave at 15 lbs. pressure and 121 °C for 15 minutes. This will solubilize the mucin and sterilize the medium.

(3) Allow the medium to cool to room temperature and aseptically add 3 mL bovine serum (previously inactivated for 30 minutes at 56 °C) and 0.5 mL polysorbate 80 to each of the flasks.

(4) Swirl vigorously to keep mucin particles suspended. Dispense 8-mL portions into 16 x 125 mm screw-cap tubes.

(5) Incubate the tubes of complete medium at 37 °C for 24 hours to test for sterility. Store at 4 °C.
12.3.2 Cultivation Procedure

12.3.2.1 Inoculation

(1) Bring four tubes of complete medium to room temperature.

(2) Add 0.5 mL of rice supplement to each tube of complete medium (approximate concentration of 3 mg rice supplement per milliliter of complete medium).

(3) Add 150 to 200 IU of potassium penicillin G and 150 to 200 mg streptomycin sulfate per milliliter of medium to two of the tubes. Do not add antibiotics to the other tubes, because the patient’s flora may be compatible with the amoebae to be cultured.

(4) Emulsify 50 mg of fecal material (about the size of a small pea) in 1 to 2 mL sterile 0.85% saline solution, and add approximately 0.5 mL into each tube of complete medium.

(5) Incubate the tubes in an upright position at 37 °C for 48 hours with caps tightly closed.

12.3.2.2 Examination of Culture

(1) Gently invert the tubes several times to mix.

(2) Place the tubes in the incubator at a slightly inclined angle (5°) for one hour.

(3) Place each of the tubes on an inverted microscope and examine the length of the tube using a 10x objective for the presence of amoebae. If present, amoebae can usually be found attached to the glass or bacterial masses.

(4) If few amoebae are found and enhancement of culture is desired, centrifuge tubes at 270 x g for three minutes. Carefully remove supernatant and discard. Add more medium and rice supplement and incubate as described above.

12.4 Quality Control/Assurance

• Media should be checked weekly. New lots of media should be checked for the support of growth using Entameoba histolytica ATCC® 30015. (This is the L.S. Diamond strain HK-9.)

• Before routinely using new lot numbers of media components in the medium, each lot number should be checked to see that it supports the growth of organisms.

12.4.1 Inoculation

Tubes of medium containing the Entameoba histolytica HK-9 should be placed in an ice bath for 10 to 15 minutes, inverted several times to distribute the amoebae, and then 0.1 mL of medium removed with a sterile pipet. Aseptically, inoculate each tube of medium with 0.1 mL of sediment from the bottom of the slant or 0.1 mL of liquid from the tube.

12.4.2 Incubation

Each tube should be incubated on a slant at 35 °C. Subcultures should be made every three to four days to insure maximum viability of organisms.
12.4.3 QC Data

Record all QC data.

12.5 Media Examination

Aliquots of all inoculated media should be examined every other day for the presence of amoebae for a period of six days before the culture is discarded.

12.5.1 Direct Wet

(1) Remove a drop of sediment aseptically with a sterile pipet and place in the center of a glass slide (1 inch x 3 inch).

(2) Place a No. 1 coverslip (22 x 22 mm) on top of the drop of sediment.

(3) Examine the slide under 100x and 400x, looking for amoebic motility.

**NOTE:** Do not allow the slide to remain at room temperature or below for prolonged periods of time before examining. Cold temperature will inhibit normal motility of the organism.

(4) If necessary, warm the slide by placing it in the palm of the hand for one minute in an incubator or on a hot plate (warm temperature only, not hot) briefly. Also, a heated stage could be used.

12.5.2 Stained Slide

(1) Remove a drop of sediment aseptically with a sterile pipet and place in the center of a glass slide (1 inch x 3 inch).

(2) Add a drop of PVA or SAF to the slide (with albumin for SAF).

(3) Mix the drops over an area the size of a 22 x 22 mm coverslip.

(4) Allow the slides to air dry for at least 30 minutes.

(5) Stain the slides in trichrome or iron-hematoxylin.

(6) Examine under oil immersion (1000x) for the presence of *Entamoeba histolytica* trophozoites.

12.6 Reporting Results

An example of a positive report would be: *Entamoeba histolytica* confirmed by culture.

An example of a negative report would be: no amoebae recovered from culture.

12.7 Limitations of the Procedure

12.7.1 Quality Control Strains

It is mandatory that appropriate quality control strains be cultured every time a patient specimen is cultured. Without this type of control, a negative patient culture may represent a false negative. If the control strain culture demonstrates adequate growth, then any patient results will be acceptable using this control system.
12.7.2 Contamination of Stock Cultures

In the presence of contaminating bacteria and/or yeast, amebic stock control strain cultures will not survive. This type of contamination overgrowth may even occur in the presence of antibiotics. If the control culture becomes contaminated, the final results are subject to error. Both cultures should be reset before accepting any results obtained using the culture system.

13 Identification Aids: Stool Artifacts

13.1 Sources

Gross and microscopic examination of stool may be complicated by the presence of artifacts resembling parasitic trophozoites, cysts, oocysts, eggs, larvae, and adult worms. Table 2 provides a summary of artifacts that resemble parasites, which can be found in clinical fecal specimens. 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, such as free-living protozoa or helminths.

13.1.1 Protozoa

- **Amoebae:** Improper collection and preservation of feces may result in contamination with free-living amoebae from the soil or water. Most species have nuclei with large karyosomes, large cytoplasmic contractile vacuoles, and thick cyst walls. Inflammatory cells are often present in a variety of infectious and noninfectious enteric syndromes; they include PMNs, eosinophils, lymphocytes, and macrophages. Careful evaluation should be made to prevent their being mistaken for intestinal parasites, especially *Entamoeba histolytica*. Any occurrence of inflammatory cells should be reported, and a quantitative assessment should be made. Also, amoebae must be differentiated from intestinal epithelial cells (squamous or columnar), yeasts, plant cells, and other protozoans.

- **Flagellates:** Free-living aquatic flagellates may be recovered from feces contaminated with water or saline, and are difficult to differentiate from the enteric flagellates. Stool samples contaminated with urine may also contain *Trichomonas vaginalis*.

- **Ciliates:** As with amoebae and flagellates, free-living ciliates are also commonly found in standing water, and they may contaminate improperly collected stool specimens. Some species appear similar to *Balantidium coli*, and may require differentiation by an expert.

- **Coccidia:** The identification of *Cryptosporidium* spp. poses significant challenges because of the small size 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 significantly increases specificity, and this method may be used either as a primary or confirmatory method. Certain pollen grains are known to mimic *Isospora belli* or *Sarcocystis* spp. oocysts, and they need to be differentiated on the basis of size. Cyanobacteria-like or coccidia-like bodies (CLBs) (now identified as coccidia in the genus *Cyclospora*) also need to be differentiated from *Cryptosporidium* oocyst organisms on the basis of size and internal morphology.

- **Microsporidia:** The identification of microsporidial spores using modified trichrome procedures can be very difficult because of the small size of the organism and lack of specificity of stains currently being used. Many of the spores can resemble bacteria or very small yeast cells. Use of optical
brightening agents (calcofluor white) are helpful, but are also nonspecific in terms of identifying microsporidial spores; the spores can resemble yeast cells and both will fluoresce.\textsuperscript{76}

### 13.1.2 Helminths

- **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 for subsequent identification.

- **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 human parasites. 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. Such reported infections have included *Fasciola hepatica*, *Dicrocoelium dendriticum*, and *Capillaria hepatica*, among others. True infections may be ruled out with subsequent stool examinations.

- **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 which lack both symmetry and identifiable internal organs. Unlike plant hairs, true larvae retain iodine when stained. Also, free-living larval stages could be seen in a stool specimen which had been picked up off the ground.

### 13.2 Corrective Action

Misidentification of artifacts as parasites may be minimized by 1) providing appropriate training for the parasitologist; 2) performing preservation and staining procedures according to established protocols; 3) stressing the importance of correct collection procedures to patients or staff; and 4) strictly adhering to established morphologic criteria when performing the microscopic examinations.

### 13.3 Reporting of Stool Artifacts

Most parasites are not quantitated on the laboratory report form. Several exceptions would include *Blastocystis hominis* and helminth eggs (some laboratories quantitate *Trichuris* and the trematode eggs only; some quantitate all helminth eggs seen). Certain artifacts (including host cells) or other structures (such as Charcot-Leyden crystals) are quantitated and reported for the following reasons:

- **Human cells**—Macrophages, PMNs, eosinophils, and RBCs: These cells may suggest certain etiologic agents and are often requested by the clinician.

- **Charcot-Leyden crystals**—These crystals are formed from the breakdown products of eosinophils and may suggest the presence of an immune response which may or may not be related to a parasitic infection.

- **Yeast cells and/or pseudohyphae**—If large numbers of yeast cells and/or pseudohyphae are present, they should be reported. However, when reporting these findings, make sure the specimen was fresh or preserved immediately after passage.
Multiplication of yeast cells and formation of pseudohyphae will occur in unpreserved stool; in such cases, the information on the laboratory report may be misleading and may not be an indication of invasive organisms.

Table 2. Clinical Specimens: Summary of Artifacts Resembling Parasites

<table>
<thead>
<tr>
<th>Stool Artifacts</th>
<th>Resemblance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CELLS OF HOST ORIGIN</strong></td>
<td></td>
</tr>
<tr>
<td>Polymorphonuclear neutrophils (PMNs)</td>
<td>Entamoeba histolytica/E. dispar cysts</td>
</tr>
<tr>
<td>Macrophages</td>
<td>Entamoeba histolytica/E. dispar trophozoites</td>
</tr>
<tr>
<td>Red blood cells (RBCs)</td>
<td>Yeast cells or small homogeneous cysts with no</td>
</tr>
<tr>
<td></td>
<td>nuclei, Cryptosporidium spp., Cyclospora cayetanensis</td>
</tr>
<tr>
<td>Columnar epithelial cells</td>
<td>Amebic trophozoites</td>
</tr>
<tr>
<td>Squamous epithelial cells</td>
<td>Amebic trophozoites</td>
</tr>
<tr>
<td><strong>COPROZOIC (FREE-LIVING) PROTOZOA</strong></td>
<td></td>
</tr>
<tr>
<td>Amoebae</td>
<td>Amebic trophozoites or cysts</td>
</tr>
<tr>
<td>Flagellates</td>
<td>Any of the intestinal flagellates</td>
</tr>
<tr>
<td>Ciliates</td>
<td>Balantidium coli trophozoites</td>
</tr>
<tr>
<td><strong>YEASTS</strong></td>
<td>Protozoan cysts (particularly Endolimax nana)</td>
</tr>
<tr>
<td></td>
<td>Blastocystis hominis (central body form)</td>
</tr>
<tr>
<td></td>
<td>Cryptosporidium oocysts</td>
</tr>
<tr>
<td></td>
<td>Red blood cells</td>
</tr>
<tr>
<td></td>
<td>Microsporidial spores</td>
</tr>
<tr>
<td><strong>BACTERIA</strong></td>
<td>Microsporidial spores</td>
</tr>
<tr>
<td><strong>FUNGAL CONIDIA OR SPORES</strong></td>
<td>Helminth eggs</td>
</tr>
<tr>
<td><strong>PLANT CELLS</strong></td>
<td>Protozoan cysts, Helminth eggs</td>
</tr>
<tr>
<td><strong>PLANT HAIRS</strong></td>
<td>Nematode larvae</td>
</tr>
<tr>
<td><strong>POLLEN GRAINS</strong></td>
<td>Helminth eggs (particularly Ascaris lumbricoides or Taenia)</td>
</tr>
<tr>
<td></td>
<td>Protozoan cysts</td>
</tr>
<tr>
<td><strong>DIATOMS</strong></td>
<td>Helminth eggs</td>
</tr>
<tr>
<td></td>
<td>Protozoan cysts</td>
</tr>
<tr>
<td><strong>STARCH GRANULES, FAT GLOBULES, AIR BUBBLES, MUCUS</strong></td>
<td>Protozoan cysts</td>
</tr>
<tr>
<td><strong>INGESTED MITE EGGS</strong></td>
<td>Helminth eggs</td>
</tr>
<tr>
<td><strong>INGESTED PLANT NEMATODE EGGS, LARVAE</strong></td>
<td>Helminth eggs, nematode larvae</td>
</tr>
</tbody>
</table>
References


55 Little MD. Comparative morphology of six species of *Strongyloides* (Nematoda) and redefinition of the genus. *J Parasitol.* 1966;52:69-84.


**Additional Reference**

Helpful Websites

The websites listed below have excellent postings of pictures of parasites, as well as other information relevant to diagnostic medical parasitology. Also, one can search the Internet using the key term “parasitology websites” for additional entries that may be helpful.

http://www.ksu.edu/parasitology/
Human parasitology laboratory image tutorial is excellent because images are presented along with a question which asks one to identify what is seen (Quiz style)

http://www.dpd.cdc.gov/dpdx/
Website of the Centers for Disease Control and Prevention in Atlanta, Georgia

http://www.life.sci.qut.edu.au
Large image list of medically important parasites.

http://www.biosci.ohio-state.edu/~parasite/home.html
Some of the images contained herein are labeled so the user can identify internal structures.

http://www.cdfound.to.it/HTML/atlas.htm
Good atlas with other useful diagnostic information

http://www.medicine.cmu.ac.th/dept/parasite
Image listing from Thailand

http://dir.yahoo.com/science/biology/parasitology/
Listing of many sites

http://as.p.unl.edu/
American Society of Parasitologists site

http://facstaff.uwa.edu/rbuckner/PARASITOLOGY/LinksPara.htm
Various other links

http://med-chem.com (Under construction)
Diagnostic medical parasitology (parasite), images, case histories, downloadable protocols, etc.

http://www.scientificdevice.com
Scientific Device Laboratory

http://www.iata.org
International Air Transportation Association (IATA)

http://www.myregs.com/dotrspa
http://www.rspa.dot.gov
http://hazmat.dot.gov
Department of Transportation, Research and Innovative Technology Administration
Appendix A. How to Use the Relative Centrifugal Force Nomograph

Because models and sizes of centrifuges vary considerably, the use of gravity (g) forces instead of revolutions per minutes (rpm) is suggested. A nomograph for calculating centrifugal speed is provided. The rotating radius of the centrifuge head is the basis for the calculation and it must be carefully determined. Information on this procedure is also provided.

Figure reprinted with permission from Thermo Electron Corporation, Milford, MA 01757, U.S.A. Tel: (866) 9THERMO; Fax: (508) 634-2199; Email: info.sampleprep@thermo.com.
Appendix B. OSHA Regulations on the Use of Formaldehyde

OSHA requires all laboratorians to be protected from dangerous levels of vapors and dust. Formaldehyde vapor is the most likely air contaminant 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-minute, 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 eight-hour and 15-minute exposure must have been documented.1,2

If each measurement is below the permissible exposure limit and the eight-hour 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 (Example: 20 to >100), change in location of reagents from inside a fume hood to a location outside of the fume hood (on the open laboratory bench), or the introduction of new procedures which require the use of formalin. If either the 0.5 ppm, eight-hour TWA or the 2.0 ppm, 15-minute level is exceeded, monitoring must be repeated every six months. If either the 0.75 ppm, eight-hour TWA, or the 2.0 ppm, 15-minute level is exceeded (unlikely in a clinical laboratory setting), employees must be required to wear respirators. Accidental skin contact with aqueous formaldehyde must be prevented with the use of proper clothing and equipment (gloves, laboratory coats).

NOTE: The use of monitoring badges is not a sensitive enough method to correctly measure the 15-minute exposure level. Contact the Occupational Health and Safety Office within the institution for monitoring options. Usually, the accepted method involves monitoring airflow in the specific area(s) within the laboratory where formaldehyde vapors are found.

B.1 Procedure for Monitoring Formalin Exposure

1. Obtain the badge and air-sampling device from the individual at the Occupational Health and Safety Office who will be monitoring formalin exposure within the laboratory setting. The laboratory would normally not be required to purchase these items.

2. Perform the 0.5 ppm, eight-hour TWA exposure measurement while the employee is wearing a badge during the performance of his/her normal job functions involving the use of formalin. Monitor over an eight-hour time frame, and then return the badge for determination of total exposure findings.

3. Perform the 2.0 ppm, 15-minute exposure measurement through the use of a small air-sampling device which is attached to the employee’s lapel with a corresponding battery pack attached to the belt or in the laboratory coat pocket. The battery pack serves as a power source for the air, which is drawn from the room and bubbled through a liquid used to trap the formalin vapors (for evaluation at a later time at the end of the monitoring session). This apparatus is used twice for the 15-minute exposure measurements, each time with new, unused fluid within the chamber. The individual from Occupational Health and Safety will provide the fluid and will empty and refill the air sampling container prior to the second 15-minute reading. At the end of the day, both fluid samples and the badge will be taken from the laboratory by this same individual.

4. The badge and fluid from the air-sampling device will then be evaluated for overall formalin exposure. Once the findings are received, consultation will determine whether any additional steps need to be taken or whether the laboratory is in compliance with formalin exposure guideline.
Appendix B. (Continued)

5. Record these results (date; name of person monitored; job functions performed during monitoring; names and telephone numbers of the company and the individual performing monitoring; status of further action, if necessary), and maintain the information as a part of the laboratory safety records.

B.2 Chemical Hygiene Plan

OSHA also requires each laboratory to develop a comprehensive, written chemical hygiene plan (CHP).\(^2\) Regardless of type of risk, volume, or concentration, every hazardous chemical in the laboratory must be included in the chemical hygiene plan. The plan should include storage requirements, handling procedures, location of material, safety data sheets, employee information and training, 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.

References for Appendix B


### Appendix C. Stool Specimen Collection and Testing Options: Fecal Specimens for Parasites – Options for Collection

<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>Data suggests that patients who begin to have diarrhea after they have been inpatients for a few days are not symptomatic from parasitic infections, but generally other causes.</td>
<td>There is always the chance that the problem is related to a nosocomial parasitic infection (rare), but Cryptosporidium 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. Two O&amp;P exams (concentration, permanent stained smear) are acceptable, but not always as good as three specimens (may be relatively cost-effective approach); any patient remaining symptomatic would require additional testing.</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 may be positive for different organisms.</td>
</tr>
<tr>
<td>Examine a second stool only after the first is negative and the patient is still symptomatic.</td>
<td>With additional examinations, yield of protozoa increases (Entamoeba histolytica, 22.7%; Giardia lamblia, 11.3%; and Dientamoeba fragilis, 31.1%)</td>
<td>Assumes 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>Pool three specimens for examination; perform one concentration and one permanent stain</td>
<td>Three specimens are collected by the patient (three separate collection vials) over 7-10 days and pooling by the laboratory may save time and expense.</td>
<td>Organisms present in low numbers may be missed due to the dilution factor once the specimens are pooled.</td>
</tr>
<tr>
<td>Pool three specimens for examination; perform a single concentrate and three permanent stained smears.</td>
<td>Three specimens are collected by the patient (three separate collection vials) over 7-10 days; pooling by the laboratory for the concentration would probably be sufficient for the identification of helminth eggs. Examination of the three separate permanent stained smears (one from each vial) would maximize recovery of intestinal 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>The patient collects three stools, but puts a sample of stool from all three specimens into a single vial (patient given a single vial only)</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>Perform immunoassays on selected patients using methods for Giardia lamblia, Cryptosporidium spp, and/or the Entamoeba histolytica/E. dispar group or Entamoeba histolytica.</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. This approach assumes the physicians have guidance in terms of correct ordering options (See Appendix D. Ordering Recommendations: Routine O&amp;P Examinations or Fecal Immunoassays)</td>
<td>Laboratories rarely receive information that would allow them to place a patient in a particular risk group: children ≤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 positive rate will be low unless an outbreak situation is involved.</td>
</tr>
</tbody>
</table>
**Appendix C. (Continued)**

| Perform immunoassays and O&P examinations ON REQUEST* | Using this approach, will limit number of stools on which immunoassay procedures are performed for parasites. Immunoassay results do not have to be confirmed by any other testing (such as O&P examinations or modified acid-fast stains). However, if specific kit performance problems have been identified, individual laboratories may prefer to do additional testing. HOWEVER, the fecal immunoassays are more sensitive than the O&P examination and special stains (modified acid-fast stains). Also, this may be considered duplicate testing and may not be approved for reimbursement unless specifically ordered by the physician. | 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&P exams) is required. Remember, a single O&P may not reveal all organisms present. Present plan to physicians for approval: Immunoassays or O&P examinations, procedure discussion, report formats, clinical relevance, and limitations on each approach. |
| **Giardia lamblia**, **Cryptosporidium spp.** and/or **Entamoeba histolytica/E. dispar** group or **Entamoeba histolytica** | *A number of variables will determine the approach to immunoassay testing and the O&P examination (geography, organisms recovered, positive rate, physician requests). Immunoassays and/or O&P examinations should be separately ordered, reported, and billed.** |

**NOTE:** It is difficult to know when one may be in an early outbreak situation where screening of all specimens for either **Giardia lamblia**, **Cryptosporidium spp.**, or both, may be relevant. Extensive efforts are underway to finalize guidelines for handling potential or actual outbreaks, and identification and use of appropriate communications channels.

---

**References for Appendix C**

## Appendix D. Ordering Recommendations for Routine O&P Examination and Fecal Immunoassays¹⁴

<table>
<thead>
<tr>
<th>Patient and/or Situation</th>
<th>Test Ordered¹</th>
<th>Follow-up Test Ordered</th>
</tr>
</thead>
<tbody>
<tr>
<td>● Patient with diarrhea and AIDS or other cause of immune deficiency</td>
<td>Cryptosporidium or Giardia/Cryptosporidium 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></td>
<td>Microsporidia if AIDS</td>
<td></td>
</tr>
<tr>
<td>● Potential waterborne outbreak (municipal water supply swimming pool)</td>
<td>Giardia or Giardia/Cryptosporidium 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></td>
<td>NOTE: More than one fecal specimen may be required to demonstrate a positive Giardia immunoassay.</td>
<td></td>
</tr>
<tr>
<td>● Patient with diarrhea (nursery school, day care center, camper, backpacker)</td>
<td>O&amp;P exam, Entamoeba histolytica/E. dispar immunoassay; immunoassay for confirmation of E. histolytica; various tests for Strongyloides may be relevant (eosinophilia) Giardia, Cryptosporidium, Cyclospora</td>
<td>If exams are negative and symptoms continue, special tests for coccidia and microsporidia should be performed.</td>
</tr>
<tr>
<td>● Patient with diarrhea and relevant travel history</td>
<td>Consider agar plate culture for Strongyloides stercoralis</td>
<td>If test is negative and symptoms continue, O&amp;P exam and special tests for microsporidia and other coccidia should be performed; consider serologic testing for helminthes.</td>
</tr>
<tr>
<td>● Patient with unexplained eosinophilia</td>
<td>Test for Cyclospora cayetanensis (modified acid-fast stains, autofluorescence)</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>
<tr>
<td>● Patient with diarrhea (suspected food-borne outbreak)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹Depending 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.

### References for Appendix D

Appendix E. Permanent Stained Smears From MIF-Preserved Material

E.1 Specimen

Any fecal specimen submitted in MIF fixative can be used. Fresh fecal specimens after fixation in MIF for 30 minutes can also be used.

E.2 Reagents

- Mayer’s albumin
  
  See Section 8.4.2 in the main text.

- Trichrome stain
  
  See Section 8.2.2 in the main text.

E.3 Quality Control

See Section 8.3.3 in the main text.

E.4 Procedure

NOTE: Wear gloves when performing this procedure.

E.4.1 Slide Preparation

1. Using a cotton-tipped applicator, spread a thin film of Mayer’s albumin-glycerin on a clean slide. Allow to dry overnight.

2. If preparing a smear, carefully draw a portion of the MIF specimen from approximately 2/3 down in the vial, using a disposable pipet. If Dientamoeba fragilis is suspected, also draw first from the top layer if the specimen can be observed to settle in layers.

3. Place the specimen on the prepared glass slide. If excess fluid exists, tilt the slide gently on a paper towel to drain.

4. Allow these slides to dry for exactly five minutes.

5. Immerse in 95% alcohol overnight. The slides will keep for two months.

E.4.2 Staining Smears

1. Place in 95% alcohol for five to ten minutes

2. Place in 70% alcohol for five minutes.

3. Place in 70% alcohol for five minutes.

4. Trichrome stain for ten minutes.

5. Dip slide once in 90% acid-alcohol.
Appendix E. (Continued)

(6) Dip slide once in 95% alcohol.
(7) Place in two changes 95% alcohol for five minutes each.
(8) Place slide in xylene for five to ten minutes.
(9) Place slide in xylene for ten minutes.
(10) Mount with No. 1 coverslip using mounting medium.

E.5 Results
See Section 8.2.5 in the main text.

E.6 Reporting Results
See Section 8.2.6 in the main text.

E.7 Procedure Notes

- The cytoplasm of thoroughly fixed and well-stained cysts and trophozoites is blue-green, tinged with purple. Occasionally, Entamoeba coli cysts may stain slightly more purplish than cysts of other species. The nuclear chromatin, chromatoidal bodies, and ingested red cells and particles, such as yeasts or molds, generally stain green, but variations frequently occur in the color reactions of ingested particles. Nonstaining cysts and those staining predominantly red are most frequently associated with incomplete fixation.

- Eggs and larvae stain red and contrast strongly with the green background. Thin-shelled eggs often collapse when placed in mounting medium, although some diagnostic features may be retained.

- Mononuclear and polymorphonuclear leukocytes and Blastocystis hominis present the same diagnostic problems that they present with hematoxylin. Like the protozoa, the nuclei of pus cells and tissue cells stain red and the cytoplasm green.

- Stained slides should be saved at least one month after reading, so they can be reviewed, if necessary.

E.8 Limitations of the Procedure
See Section 8.2.8 in the main text.

Reference for Appendix E

### Appendix F. Reporting Recommendations

<table>
<thead>
<tr>
<th>ORGANISMS</th>
<th>STAGE</th>
<th>QUANTITATION</th>
<th>COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestinal artifacts</td>
<td>Yes</td>
<td>Yes</td>
<td>Reports related to presence of yeast should be based on the fact that the specimen was examined and/or placed in fixative immediately after passage.</td>
</tr>
<tr>
<td>Human cells</td>
<td>Yes</td>
<td>WBCs, RBCs,</td>
<td>Yeast reports related to presence of yeast should be based on the fact that the specimen was examined and/or placed in fixative immediately after passage.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>eosinophils</td>
<td></td>
</tr>
<tr>
<td>Charcot-Leyden crystals</td>
<td>Yes</td>
<td>Yes</td>
<td>Reports related to presence of yeast should be based on the fact that the specimen was examined and/or placed in fixative immediately after passage.</td>
</tr>
<tr>
<td>Yeast</td>
<td>Yes</td>
<td>Yes</td>
<td>Reports related to presence of yeast should be based on the fact that the specimen was examined and/or placed in fixative immediately after passage.</td>
</tr>
<tr>
<td>Intestinal amoebae</td>
<td>Yes</td>
<td>No</td>
<td>Exception to quantitation would be <em>Blastocystis hominis</em> (rare, few, moderate, many, packed).</td>
</tr>
<tr>
<td>Flagellates, ciliates</td>
<td>Yes</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Intestinal coccidia</td>
<td>Yes</td>
<td>No</td>
<td>Not identified in routine O&amp;P.</td>
</tr>
<tr>
<td>Microsporidia</td>
<td>Yes</td>
<td>No</td>
<td>Not identified in routine O&amp;P.</td>
</tr>
<tr>
<td>Intestinal helminths</td>
<td>Yes</td>
<td>No</td>
<td>Exception to quantitation would be helminthes such as <em>Trichuris trichiura</em> and <em>Clonorchis sinensis</em>; also report viability of schistosome eggs.</td>
</tr>
</tbody>
</table>

*Refers to budding yeast, pseudohyphae, trophozoite, cyst, oocyst, spore, eggs, larvae, malarial stages.
†Refers to categories: Rare, few, moderate, many, packed
### Appendix F. (Continued)

**Reporting Specific Test Format Results**

<table>
<thead>
<tr>
<th>TEST NAME</th>
<th>REPORT</th>
<th>COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ova and parasite examination</td>
<td>Report genus, species, and stage when appropriate.  Examples:</td>
<td>Indicate in the report that the O&amp;P is NOT designed to detect the presence of the coccidia and/or microsporidia.</td>
</tr>
<tr>
<td></td>
<td><em>Giardia lamblia</em> cysts</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Entamoeba histolytica/E. dispar</em> trophozoites</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Ascaris lumbricoides eggs</em></td>
<td></td>
</tr>
<tr>
<td>Special stains for the coccidia</td>
<td>Report genus, species, stage or general name when appropriate.</td>
<td>If performing the modified acid-fast stains for coccidia, indicate in a negative report:  No coccidia seen.</td>
</tr>
<tr>
<td>and/or microsporidia</td>
<td><em>Cryptosporidium</em> spp. oocysts</td>
<td>If performing the modified trichrome stains for microsporidia, indicate in a negative report:  No microsporidia seen.</td>
</tr>
<tr>
<td></td>
<td><em>Cyclospora cayetanensis</em> oocysts</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Microsporidian spores</em></td>
<td></td>
</tr>
<tr>
<td>Fecal immunoassays</td>
<td>Report the actual organism name (genus, species) for either a</td>
<td>It is important to name and emphasize exactly which organism(s) are relevant for each particular kit.</td>
</tr>
<tr>
<td></td>
<td>positive or negative report.</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Giardia lamblia</em> positive</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Cryptosporidium</em> spp. negative</td>
<td></td>
</tr>
</tbody>
</table>

### Reference for Appendix F

Summary of Comments and Working Group Responses

M28-A: Procedures for the Recovery and Identification of Parasites from the Intestinal Tract; Approved Guideline

Section 7.2, Concentration Procedures (Formerly Section 4.2)

1. Under the concentration procedure, mention the quick way to detect cyclospora through auto fluorescence using fluorescence microscopy.

- The following note has been added to the end of Section 7.2: “Concentrated stool sediment can be used for the detection of Cyclospora cayetanensis oocysts using UV epifluorescence. On the wet mount examination of concentrated stool sediment, oocysts measure approximately 8 to 10 μm and will autofluoresce green (450 to 490 DM excitation filter) or blue (365 DM excitation filter) under UV epifluorescence.”

Section 8.8.2, Reagents for Weber-Green (Formerly Section 5.6.2)

2. Section 5.6.2 recommends preparing a fresh mixture of chromotype 2R stain every month. In Section 5.2.2 (4) and again in the Summary of Comments, question/response number 6, the shelf life of trichrome stain is given at 24 months. Why the difference?

- The following note has been added to the end of Section 8.8.2: “Since Wheatley’s trichrome, and the two formulas for chromotrope stain for the microsporidia use different amounts of various ingredients, the shelf life for each stain is stated according to the recommendation of the developer of that particular stain formula. Thus, the Weber-Green modified trichrome stain shelf life is stated as one month, while the Ryan-Blue modified trichrome stain shelf life is 24 months. (There is no reason why a fresh mixture of chromotrope 2R stain would need to be prepared monthly, unless it becomes contaminated or was giving unacceptable staining characteristics. Generally, this stain has a long shelf life that should be consistent with the 24 months indicated in Section 8.2.2.”)

Appendix D (D2.6), Ordering Recommendations: Routine O & D Examinations Fecal Immunoassays

3. We do not recommend using picric acid; there are safer and better stain procedures that reduce time.

- The selection of any particular staining method is certainly at the discretion of the user. The Modified Iron–Hematoxylin Stain Incorporating the Carbol Fuchsin Step method (see Section 8.4) lists picric acid as one of the reagents. Common dyes used in solution with picric acid include indigo-carmine, aniline blue, methyl blue, and acid fuchsin; the picric acid provides the acidic pH necessary for selective staining and also acts as a dye in itself in counterstaining muscle, cytoplasm, etc. A laboratory may select another staining method that does not contain the picric acid component (other hematoxylin stains and/or modified acid-fast stains). In the Procedure Notes (see Section 8.4.7), a comment indicates the following: 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 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.

Appendix F. Reporting Recommendations (Formerly Appendix I)

4. In the guideline for the recovery and identification of parasites from the intestinal tract, Appendix I recommendations quantitation of amoebae (except Blastocystis hominis), flagellates, ciliates, and helminthes (except Trichuris trichiura and Clonorchis sinensis) is not recommended. Can you please explain why some are recommended and others are not?

- The majority of the intestinal protozoa pass sporadically; thus, one day the specimen might be negative and another day positive. The presence of pathogens is significant regardless of the numbers present. However, there may be some relationship between the numbers of Blastocystis hominis present and patient symptoms. Although the pathogenicity of this organism is still relatively controversial, it may be helpful to quantitate in order to assist the physician in assessing the clinical relevance of the presence of this parasite. In the case of a few of the helminths,
having some idea of the helminth load can determine the necessity for therapy. It is also important to understand why proficiency testing challenges are quantitated, while the majority of patient reports do not contain this information. This approach (indicating few, moderate, many) provides the proficiency testing agency some idea of how many organisms were in that particular challenge; thus, it serves as an internal quality control check for the vials and slides that are sent to participants.
Summary of Delegate Comments and Working Group Responses


Section 11.3.4, Procedure

1. The document is very comprehensive and the new enhancements are welcome. There is a brief reference to Auramine-O stain in Section 11.3.4, but no procedure. Is this because not many labs are using it? It appears to be a very rapid and economical screen for Cryptosporidium and other coccidia and may be included in the routine o and p test.

- Due to space constraints, we elected not to include every possible procedure. Certainly, the Auramine O procedure is relatively easy to perform and can be used for the detection of the intestinal coccidia (Cryptosporidium spp., Cyclospora cayetanensis, Isospora belli). However, remember that when a test is called an O&P, proper coding and billing do not include the Auramine O test within the O&P name. If you decide to offer an Auramine O procedure, it should be named accordingly. Reference: Isenberg HD, ed. Clinical Microbiology Procedures Handbook. 2nd ed. Washington, DC: ASM Press; 2004:7.2.1-7.2.4.
The Quality System Approach

Clinical and Laboratory Standards Institute (CLSI) subscribes to a quality management system approach in the development of standards and guidelines, which facilitates project management; defines a document structure via a template; and provides a process to identify needed documents. The approach is based on the model presented in the most current edition of CLSI/NCCLS document HS1—A Quality Management System Model for Health Care. The quality management system approach applies a core set of “quality system essentials” (QSEs), basic to any organization, to all operations in any healthcare service’s path of workflow (i.e., operational aspects that define how a particular product or service is provided). The QSEs provide the framework for delivery of any type of product or service, serving as a manager’s guide. The quality system essentials (QSEs) are:

- Documents & Records
- Organization
- Personnel
- Equipment
- Purchasing & Inventory
- Process Control
- Information Management
- Occurrence Management
- Assessment
- Process Improvement
- Service & Satisfaction
- Facilities & Safety

M28-A2 addresses the quality system essentials (QSEs) indicated by an “X.” For a description of the other documents listed in the grid, please refer to the Related CLSI/NCCLS Publications section on the following page.

Adapted from CLSI/NCCLS document HS1—A Quality Management System Model for Health Care.

Path of Workflow

A path of workflow is the description of the necessary steps to deliver the particular product or service that the organization or entity provides. For example, CLSI/NCCLS document GP26—Application of a Quality Management System Model for Laboratory Services defines a clinical laboratory path of workflow which consists of three sequential processes: preexamination, examination, and postexamination. All clinical laboratories follow these processes to deliver the laboratory’s services, namely quality laboratory information.

M28-A2 addresses the clinical laboratory path of workflow steps indicated by an “X.” For a description of the other documents listed in the grid, please refer to the Related CLSI/NCCLS Publications section on the following page.

Adapted from CLSI/NCCLS document HS1—A Quality Management System Model for Health Care.
Related CLSI/NCCLS Publications*

GP2-A4  Clinical Laboratory Technical Procedure Manuals; Approved Guideline—Fourth Edition (2002). This document provides guidance on development, review, approval, management, and use of policy, process, and procedure documents in the laboratory testing community.

GP5-A2  Clinical Laboratory Waste Management; Approved Guideline—Second Edition (2002). Based on U.S. regulations, this document provides guidance on safe handling and disposal of chemical, infectious, radioactive, and multihazardous wastes generated in the clinical laboratory.


M15-A  Laboratory Diagnosis of Blood-borne Parasitic Diseases; Approved Guideline (2000). This document contains guidelines for specimen collection, blood film preparation, and staining procedures. Recommendations for optimum timing of specimen collection to assist laboratories in detecting and identifying certain parasites are also included.

M29-A3  Protection of Laboratory Workers From Occupationally Acquired Infections; Approved Guideline—Third Edition (2005). Based on U.S. regulations, this document provides guidance on the risk of transmission of hepatitis viruses and human immunodeficiency viruses in any laboratory setting; specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials; and recommendations for the management of blood-borne exposure.

* Proposed-level documents are being advanced through the Clinical and Laboratory Standards Institute consensus process; therefore, readers should refer to the most recent editions.
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UZ-KUL, Medical Center (Belgium)

VA (Taekege) Medical Center (AL)

Virginia Beach General Hospital (VA)

Virginia Department of Health

Washington Adventist Hospital (MD)

Washoe Medical Center Laboratory (NV)

Waterford Regional Hospital (Ireland)

Weilcrest Health Systems (GA)

West China Second University Hospital, Sichuan University (P.R. China)

West Jefferson Medical Center (LA)

Willford Hall Medical Center (TX)

William Beaumont Army Medical Center (TX)

William Beaumont Hospital (MI)

Winn Army Community Hospital (GA)

Winnipeg Regional Health Authority (Winnipeg, Canada)

Wishard Memorial Hospital (IN)

York Hospital (PA)