PROVIDENCE
Sacred Heart
Medical Center &
Children's Hospital

Ova & Parasite Exam Procedure

Department of Microbiology

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1.0 Principle

When present in small numbers, parasites may be missed in a direct wet mount. A concentration procedure should be employed on all stools, whether the direct smear is positive or not. Because the preserved organisms will no longer exhibit motility, the direct wet smear is no longer considered a mandatory part of the routine ova and parasite examination. However, if fresh liquid specimens are delivered to the laboratory immediately, the direct wet smear, particularly on liquid stools should be performed.

A modification of the formalin-ether method of Ritchie, 1948, is used to recover protozoan cysts and helminth eggs and larvae. Less distortion of cysts and ova occurs with this technique than with the zinc sulfate flotation method. It also gives a higher recovery rate since some of the ova do not float in zinc sulfate.

The Gomori-Trichrome stain is used for making permanent stains. It provides contrasting colors for both the background debris and parasites present. It is designed to allow examination and recognition of detailed organism morphology under oil immersion examination.

It is important to note that initial steps in the smear preparation and staining process vary depending on whether the stool is received fresh or preserved. DMSO (Dimenthyl Sulfoxide) is used to fix wet unpreserved stools. Dried smears made from specimens received in Unifix™ or Total-Fix™ vials require no initial steps prior to the trichrome stain.

2.0 Clinical Significance

An ova and parasite (O&P) exam is a microscopic evaluation that is commonly used to look for parasites that have infected the lower digestive tract.

3.0 Scope

This procedure is classified under CLIA as Highly Complex. It should be carried out by technical personnel familiarized and trained to process samples and perform microscopic examination. Testing includes but is not limited to: macroscopic and microscopic specimen examination, QC checks, technical proficiency testing and competency assessment.

4.0 Safety - Personal Protective Equipment

Performance of this procedure will expose testing personnel to biohazardous material. All specimens must be handled as potentially infectious material as outlined in the Providence Sacred Heart Microbiology Safety Guidelines.

The reagents and/or chemicals that are used in this procedure may be hazardous to your health if handled incorrectly. A brief listing of precautions for each chemical hazard is included in the reagent section of this procedure.

More extensive information concerning the safe handling of the reagents and/or chemicals used in this procedure, as well as other important safety information may be obtained by consulting the Material Safety Data Sheet (MSDS). Before performing any part of this procedure, the technologist must take any and all precautions and adhere to all prescribed policies.

This procedure may expose you to:

- Enteric pathogens
- Bloodborne pathogens
- Hazardous reagents

To perform this procedure, you must use:

- Gloves – must be worn when handling specimens and reagents.
- Laboratory Coat – must be worn when handling specimens and reagents.
- Fume hood – must be used when preparing permanent smears and concentrates.

Disinfectant following procedure:

- Bleach dilution sprayers or wipes can be used for on demand disinfectant.

Reference for spill/decontamination:

- MSDS
- Chemical hygiene plan

5.0 Specimen Information

1. Duodenal contents or sigmoidoscopy material, refer to Technical Note 1.
2. Sputum, bronchoalveolar lavage, transtracheal aspirates for *Ascaris lumbricoides* larvae, *Strongyloides filariform* larvae, hookworm larvae, *Paragonimus westermani* ova, and *Echinococcus granulosus* hooklets, *Entamoeba histolytica* and *Cryptosporidium* spp.
3. Urine for *Schistosoma* spp. Peak egg excretion occurs between noon and 3 PM. Samples collected during this time or during a 24-h urine collection without preservatives, may be used for examination.
4. Freshly collected stool
   - Pass the stool into any clean, dry container
   - Select a walnut sized portion of the stool from areas that are watery or bloody and place it into the clean, leak-proof container.
   - Label the container with the patient name, physician, and date and time of collection.
   - Transport the specimen to the laboratory immediately, (within 30 min) if stool is watery/liquid of collection.
   - A total of 3 specimens should be collected and submitted on separate days or collected and submitted every other day.
5. Preservative
   - If there is an expected delay in transport to the laboratory, a stool preservative should be used.
   - Pass the stool into a clean, dry container.
   - Using the spoon attached to the fixative cap, collect small amounts of stool from areas that are mucoid, watery, or bloody, and place them into the vial. Use only one vial of preservative per specimen collection. Each vial must be filled with enough specimen so that the liquid reaches the Fill Line located on the label.
   - Tighten the cap securely to avoid leakage.
   - Shake the sample thoroughly to mix the specimen and preservative.
   - Label each container with the patient name, physician, and date and time of collection.
   - Place the vial(s) back into the bag, and seal.
   - Three specimens should be collected, collecting 24 h apart or every other day.

6.0 Materials

6.1 Preservatives Acceptable for Specimen Submission

- Unifix™
- Total-Fix™

6.2 Equipment

- Light microscope with 10, 40, and 100 X objectives and a calibrated ocular micrometer
- Coplin jars labeled with contents and procedural time

6.3 Consumables

- Plastic vials with caps (for fresh stool and formalin mixing)
- 15-mL conical tubes
- Plastic funnel
- Gauze squares (large enough to line funnel)
- Centrifuge

- Wooden applicator sticks
- Plastic pipettes
- Glass slide
- Paper towels

6.4 Reagents

- 10% Formalin
- Ethyl acetate
- 0.85% Saline
- D'Antoni's Iodine
- 100% Dimethyl Sulfoxide (DMSO) (fresh stools only)
- 70% ETOH for DMSO fresh slide. (fresh stools-holding jar)
- Gomori-trichrome Stain Solution
- Ethyl Alcohol (Reagent Alcohol)
- Citrisolv
- Microscope immersion oil

7.0 Interfering Substances

1. Fecal specimens should be collected prior to the administration of antibiotics or antidiarrheal agents.
2. Mineral oil, bismuth, and barium may interfere with the detection or identification of intestinal parasites.

8.0 Procedure for Stool Specimens

8.1 Direct Mount of Fresh, Unpreserved Stool

1. Examination must be carried out within 30 min of passage for liquid stools.
2. Place 1 drop of 0.85% saline on a glass slide. Add 1 drop of stool, mix, and cover slip. Examine the entire coverslip for motile trophozoites, larvae, and eggs.

8.2 Permanent Smears of Stool Specimens

8.2.1 Preparation of Smears from Fresh Stool

1. Apply stool material onto a glass slide and spread with wooden applicator stick so that the material varies in thickness. There should be visible material on the slide. However, you should be able to read newsprint through the thinner areas of the smear. DO NOT ALLOW TO AIR DRY.
2. IMMEDIATELY immerse the slide while it is still moist into 100% DMSO for 2 min in the fume hood; drain excess.
3. Place in 70% ethyl alcohol for 1 min: drain excess. Slides can be held in 70% ethyl alcohol until ready for a batch of smears to stain.

8.2.2 Preparation of Smears from Preserved Stool (either Unifix™ or Total-Fix™)

1. Stir the mixture well using wooden applicator sticks.
2. Depending on the amount and viscosity of the specimen, strain a sufficient quantity through 1 or 2 layers of gauze into a conical 15-mL centrifuge tube to yield the desired amount of sediment (0.5 to 1 mL). Typically 8 to 10 mL of stool mixture will be sufficient. If the specimen is too viscous to flow through the gauze, use 0.85% saline to flush the specimen through.
3. Add 0.85% saline almost to the top of the tube. **Do not add formalin.** Centrifuge for 10 min at 500 x *g*. The amount of sediment obtained should be approximately 0.5 to 1 mL.
4. Decant. Mix the remaining preserved sediment with an applicator stick.
5. Prepare a slide for permanent staining by adding a small sample of the suspended sediment to the slide. The sediment can also be used to prepare smears for modified acid-fast staining for coccidia.

6. Spread the sample over the slide to prepare a thin smear which varies in thickness. Allow to dry for a minimum of 30 min (60 min if slide is thicker) in a 37°C incubator or slide warmer. Smear will appear opaque when dry. Do not use a heating block. The higher temperature will be detrimental to any organisms present.

8.2.3 Staining Permanent Smears

1. Place slide in Trichrome stain jar for 5 min.
2. Rinse in 95% ethyl alcohol jar for 10 s.
3. Place in 95% ethyl alcohol jar for 4 min.
4. Place in Citrisolv/95% Ethyl alcohol jar for 4 min.
5. Place in Citrisolv jar for 5 min.
6. Remove Slide, drain excess, and allow to air dry.

8.2.4 Examination of Permanent Smears

1. The trichrome stain should be read under oil immersion. Examine for presence of parasite egg, ova, or larvae after reviewing 300 oil/fields.
2. The use of a calibrated ocular objective will aid in definitive identification.
3. The cytoplasm of cysts and trophozoites is blue-green, tinged with purple. Occasionally, *E. coli* cysts may stain slightly more purplish than cyst of other species. The nuclear chromatin, chromatoid bodies, and ingested red cells, and bacteria stain red or purplish red. Other ingested particles, such as yeast or molds, generally stain green, but variations frequently occur in the color reaction of ingested particles. Background usually stains red and contrasts strongly with the green background. The cytoplasm of pus and tissue cells, however, does stain more greenish than that of the protozoa.
4. Thick and thin areas should be observed. Organisms are more easily located in the thin areas, but in some preparations these portions may be poorly stained or the organisms poorly differentiated. In these cases, the thicker portions would be of greater diagnostic value.

8.3 Concentration of Stool Specimens

8.3.1 Concentrate Preparation from Fresh Stool

1. Mix a half-teaspoon (about 4 g) of stool into approximately 10 mL of 10% formalin. Mix the stool and the formalin thoroughly. Let the mixture stand for a minimum of 30 min for fixation.
2. Depending on the amount and viscosity of the specimen, strain a sufficient quantity through 1 or 2 layers of gauze into a conical 15-mL centrifuge tube to yield the desired amount of sediment (0.5 to 1 mL). Typically 8 to 10 mL of stool mixture will be sufficient. If the specimen is too viscous to flow through the gauze, use 0.85% saline or formalin to flush the specimen through.
3. Add 0.85% saline or formalin almost to the top of the tube. Centrifuge for 10 min at 500 x *g.* The amount of sediment obtained should be approximately 0.5 to 1 mL.
4. Decant and proceed to step 2 below.

8.3.2 Concentrate Preparation from Preserved Stool (either Unifix™ or Total-Fix™)

1. After the permanent smear has been prepared from the sediment described above, the amount of sediment obtained should be approximately 0.5 to 1 mL.
   - Amount too large: Suspend sediment in 10% Formalin and pour out a portion. Add 10% formalin to bring level to 10 ml and centrifuge again.
   - Amount too small: Strain a second fecal suspension into the tube and centrifuge again.
2. Suspend the sediment in 10% formalin. Fill the tube about 2/3 full only (10 mL mark on tube). **Note:** If the amount of sediment left on the bottom is very small or if the original specimen contained a lot of mucus, do not add ethyl acetate; merely add the formalin, spin, decant, and examine the remaining sediment.
3. Add 4 to 5 mL of ethyl acetate. Stopper the tube, shake it vigorously for at least 30 s, exerting pressure on the stopper throughout. Hold the tube so that the stopper is directed away from your face.

4. Centrifuge for 10 min 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 a layer of ethyl acetate at the top.
5. Carefully uncap the tube and free the plug of debris by ringing the plug with an applicator stick; decant all of the supernatant fluid. After proper decanting, a few drops of fluid remaining on the side of the tube may run down into the sediment. Mix this fluid with the sediment.
6. Cap tube to prevent drying.

8.3.3 Examination of Stool Concentrates

1. If 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. Mix with 1 drop of D'Antoni's Iodine, add a coverslip, and examine under reduced light.
2. Systematically scan entire 22 x 22-mm area with the low 10x objective looking for eggs and larvae. If something suspicious is seen, the 40x objective can be used to study detail. At least one-third of the coverslip should be examined under high dry power even if nothing suspicious is seen.
3. Consult charts in appendix and appropriate reference books for definitive identification. Refer to supervisor or director if questionable identification.

9.0 Procedure for Lower Respiratory Specimens

Sputum should be examined as a wet mount (saline or iodine), using low and high dry power (10X and 40X objectives). The specimen should not be concentrated before preparation of the wet mount. For thick sputum samples, an equal amount of 3% sodium hydroxide (or undiluted chlorine bleach) can be added; the specimen is thoroughly mixed and then centrifuged. NaOH should not be used for sample if *Entamoeba histolytica* is suspected. After centrifugation, the supernatant fluid is discarded and the sediment can be examined as a wet mount with saline or iodine.

Lower respiratory samples submitted for O & P should be examined for *Ascaris lumbricoides* larvae, *Strongyloides filariform* larvae, hookworm larvae, *Paragonimus westermani* ova, and *Echinococcus granulosus* hooklets, *Entamoeba histolytica. Cryptosporidium* spp. are rarely found in lower respiratory samples. If requested, *Cryptosporidium* may be ruled out by preparing a Modified Kinyouns smear.

10.0 Procedure for Urine

Urine submitted for examination to rule out *Schistosoma* species should be concentrated prior to microscopic examination. Ideally, at least 15 mL should be centrifuged at 500 x *g* for 5-10 min. Decant supernatant and examine a drop of sediment microscopically on low power (10X objective) for the presence of ova.

11.0 Reporting Results

11.1 Reporting in LIS

Since there are two results for each stool specimen, one for the concentrate and one for the trichrome, the first results should be entered and placed in "Hold." When the second results are available, the report can be modified, if necessary, and released as a final report. This prevents multiple reports from being sent to the clinician. It also allows for one consolidated report rather than duplicating findings and comments under two separate headers. The final report will contain only one set of results and no headers for the trichrome or concentrate.

Observations ☐ Suppress test

#	S	H	O	B	SIG	HLD	SUP	Result	QA	Description
1.	☐	☐	☐	☐	☐	☑	☐	NOPS		No Ova or Parasites seen

11.2 Commensal vs. Pathogenic Organisms

Report the presence of all parasites. Commensal organisms and *Giardia* should be confirmed by another tech with $\geq$ 5 years of experience. Other pathogenic organisms should be confirmed on Rounds with the supervisor, director, or designee.

1. Commensal organisms of the gastrointestinal tract include the following:
   - *Endolimax nana*
   - *Entamoeba coli*
   - *Entamoeba hartmanni*
   - *E. polecki,*
   - *Iodamoeba bütschlii*
2. For commensal organisms, add the comment **"This parasite is considered to be non-pathogenic but indicates the patient has Ingested fecally contaminated food or water. Additional samples should be submitted for Ova and Parasite examination." [NPATOP]**

11.3 Non-Parasites

1. For organisms submitted that are not human parasites report, **"Specimen is negative for human parasites." [NOTPAR]**
2. For items submitted that do not contain an organism to identify report, **"No parasites present." [NOPAR]**

11.4 Fecal Leukocytes

Report and quantitate fecal leukocytes when seen. When fecal leukocytes are specifically requested also report absence of fecal leukocytes if applicable.

11.5 *Blastocystis* species

1. If an average of < 5 organisms/oif are seen, report as "Few."
2. If $\geq$ 5 organisms/oif are seen, report as "Abundant."
3. Add the comment, **"**There is controversy regarding the pathogenicity of *Blastocystis* species. It is commonly found in the intestinal tract but has been found to cause a self-limiting diarrhea. Studies suggest a correlation between infection density and symptoms. It is questionable whether treatment is necessary, although metronidazole has been reported to be effective.**" [BLHOC]**

12.0 Quality Control & Quality Assurance

1. All staining dishes should remain covered to prevent evaporation of reagents.
2. All jars should be cleaned and refilled with new reagents as needed depending on the volume of slides stained. When the smear is thoroughly fixed and the staining procedure is performed correctly, the cytoplasm of protozoan trophozoites is blue-green, sometimes with a tinge of purple. Cysts tend to be slightly more purple. Nuclei and inclusions (chromatoidal bars, RBCs, bacteria, and Charcot Leyden crystals) are red, sometimes tinged with purple. The background material usually stains green, providing a nice color contrast with the protozoa.
3. The positive control smear is made from known positive stool samples containing protozoan cysts or trophozoites. A control slide should be stained and examined weekly. The smear should be examined for proper staining. If staining is inadequate, the reagents should be replaced and a new positive control slide should be stained and read. Results of the control smear should be documented in the LIS.

13.0 Limitations

1. Inadequate Specimens
   Insufficient quantity for concentration procedure: Watery or scant specimens received in preservative may yield very little sediment after centrifugation. In these instances, the supernatant should be decanted and the specimen should not proceed through the formalin/ethyl acetate step. Instead the sediment can be used to prepare a trichrome smear and wet mount exam. Add the following comment to the report: **A suboptimal amount of**

specimen was submitted and might lead to a false negative result. [QNSS] If no material is observed after centrifugation, consult with the supervisor or charge tech.

2. Alternative Fixatives
   Fixatives other than Unifix™ and Total-Fix™ have not been validated for use. If specimens are received in an alternate preservative, a CRM case should be created. If the client does not want to recollect, PAML may be able to send the specimens to another reference lab for testing.
3. Pseudoparasites
   There are many items which may be encountered in fecal specimens that resemble or mimic parasites. Care should be taken to differentiate these items from pathogenic organisms. References that provide illustrations of some of the common pseudoparasites are available.

14.0 Technical Notes

1. Duodenal contents or sigmoidoscopy material must be examined by direct wet mount method when received within 30 min. Specifically look for motile *Giardia lamblia* trophozoites while searching for parasites. A concentration procedure can be performed if quantity is sufficient. A permanent trichrome stain must be prepared and reported. Usually, parasites such as *Giardia* will stick to the mucous portions. If mucous is present, it should be included in the direct mount examination and permanent trichrome stain.
2. ***Strongyloides stercoralis*** **rhabditiform (first stage) larvae:** The primary diagnostic stage found in the feces measuring 180 to 380 µm long by 14-20 µm wide. They can be differentiated from hookworm rhabditiform larvae by the short buccal chamber and a prominent cluster of cells, the genital primordium, which is located at mid-body between the intestine and the ventral body wall.
3. ***Strongyloides stercoralis*** **filariform (third stage) larvae**: May be seen in sputum (and other lower respiratory specimens) and feces particularly in cases of hyperinfection. These larvae are approximately 500 to 600 µm long by 16 µm wide. The ratio of esophagus length to intestinal length is 1:1 and the tail of the larva is notched.
4. **Hookworm (*Ancylostoma* and *Necator*)**: Although eggs are the diagnostic stage in feces, rhabditoid larvae may be seen if there has been a prolonged delay in examination of the fecal specimen (usually more than a day). These first stage larvae measure 250 to 350 µm long by 17 µm wide. These must be differentiated from *Strongyloides* rhabditiform larvae.

15.0 Verification

Total-Fix preservative was evaluated in 2014. The manufacturer indicated that Unifix will be phased out and replaced with Total-Fix. Total-Fix is similar to Unifix but lacks PVA, making it compatible with EIA and molecular assays. Since fresh specimens are difficult to obtain, a limited verification study was performed to evaluate the performance of Total-Fix.

In the initial evaluation, live *Trichomonas vaginalis* was grown in culture and then preserved in both Unifix and Total-Fix. Smears were prepared from both samples and stained with trichrome staining reagents. When the smears were examined microscopically, no morphologic differences were observed.

Additional material was provided by the vendor for evaluation. Unstained smears prepared from specimens preserved in Total-Fix were obtained from Lynne Garcia's lab. These smears contained *Dientamoeba fragilis.* The smears were stained using our own staining reagents. The trophozoites observed in these smears presented a classic morphology and were easily recognizable.

16.0 References

1. Package insert: Total-Fix™, Medical Chemical Corporation, Revision date: June 3, 2014.
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17.0 Document Control

Medical Director Approval: Reviewed by Dr. Schappert 03/10/2010

Microbiology Director Approval: Dr. Ann Robinson 10/14/2011

Microbiology Supervisor Reviews: Jerry Claridge 05/02/2002, 04/18/2003, 03/2004, 12/2005, 2/28/2006, 09/2006, 03/2007, 10/2008, 02/2009, 06/2011, 10/17/2011, 03/2013

Revisions:
10/28/2010 Updated concentration procedure for Unifix-preserved specimens to avoid formalin-Unifix precipitation.
10/14/2011 Added details for urine specimen collected for Schistosomes, added details for preparing trichrome smears with the appropriate thickness, added procedure for processing and examining lower respiratory and urine specimens, added details for reporting commensal organisms with comment, added verbiage for reporting items submitted that are not parasites, changed Blastocystis hominis to Blastocystis spp., added criteria for quantitating Blastocystis, modified clinical significance comment for Blastocystis, added detail for determining integrity of staining reagents and examining weekly control smear, and added limitations for scant specimens and potential for pseudoparasites.
10/18/2011 Removed PVA as an acceptable fixative.
11/16/2011 Added *Giardia* to list of parasites that can be confirmed by a senior tech.
3/21/14 Added comment: A suboptimal amount of specimen was submitted and might lead to a false negative result. [QNSS]

9/2/2014 Protocol was updated for Total-Fix. Permanent smears made from sediment after first spin. Reporting protocol updated to eliminate the use of headers for trichrome and concentrate. Preliminary results are entered and held. Results entered once and released after both tests have been performed.