PROVIDENCE
Sacred Heart
Medical Center &
Children's Hospital

Modified Kinyoun's Stain for Coccidia Procedure

Department of Microbiology

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1.0 Clinical Significance

Cryptosporidium is a coccidian parasite that has been implicated in intestinal disease, primarily in immunosuppressed or immunocompromised patients, but it can cause diarrhea in the immunocompetent host. *Cryptosporidium* is not host specific and is transmitted by the fecal-oral route. Clinical symptoms include nausea, low-grade fever, abdominal cramps, anorexia and 5-10 watery, frothy bowel movements a day, which may be followed by constipation.

Cystoisospora belli (formerly *Isospora belli*) is also a coccidian parasite. *C. belli* infections are becoming increasingly important as a cause of diarrhea in immunosuppressed patients. Infections are often asymptomatic. Clinical symptoms include chronic diarrhea, vague or crampy abdominal pain, weight loss, malaise, and anorexia.

Another coccidian parasite *Cyclospora caytanensis* has recently been associated with intestinal disease in both healthy and immunocompromised hosts. The symptoms of *Cyclospora* infection are non-specific. There is generally one day of malaise, low-grade fever and diarrhea. There also may be fatigue, anorexia, vomiting, myalgia, and weight loss. Diarrhea is self-limiting in 3 to 4 days but may be followed by relapses for up to 4 weeks.

2.0 Principle

Coccidia may be visible in a routine ova and parasite saline or iodine mount. However, the oocysts are difficult to detect. Staining with a modified acid-fast stain enhances recognition of the parasites. *Cryptosporidium* and *Cystoisospora* retain the pink carbol fuchsin with the modified Kinyoun's staining. *Cyclospora* stains more variably. The organisms are differentiated by their size and shape.

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. 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.

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. Freshly collected stool (for PSHMC inpatients only)
   - 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.
2. Preserved stools
   - If there is an expected delay in transport to the laboratory beyond 30 min, 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.
3. Sputum, bronchoalveolar lavage, transtracheal aspirates for *Cryptosporidium* spp.

6.0 Materials

6.1 Preservatives Acceptable for Specimen Submission

- Unifix™
- Total-Fix™
- 10% Formalin

6.2 Equipment

- Centrifuge
- Light microscope with 10 and 100 X objectives and a calibrated ocular micrometer

6.3 Consumables

- Parasep® concentrator devices without reagent (for preserved fecal specimens) or Parasep® devices prefilled with preservative and Triton X-100 (for fresh specimens)
- Transfer pipettes with a wide bore (Samco part no. 993)
- Glass slide

6.4 Reagents

- 0.85% Saline
- 5% Solution of Triton X-100 Surfact-Amps® Detergent
  Prepare by making a 1:2 dilution of Triton X-100 (Thermo Scientific prod. # 85111) with saline or deionized water. Store at room temperature for up to one year.
- Carbol fuchsin stain, store at room temperature in the dark
- Decolorizer: 1.0% sulfuric acid
- 50% ethanol (reagent alcohol)
- Methylene blue, 0.3% methylene blue in demineralized water, store at room temperature.
- 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 Fecal Specimens

8.1 Concentration of Specimens

Refer to the Ova & Parasite Procedure for concentration protocol using the Parasep™ device for preserved or fresh stool specimens. Lower respiratory specimens should be processed the same way as a stool sample.

8.2 Smear Preparation

1. Re-suspend the sediment.
2. Prepare a slide for permanent staining by adding a small sample of the suspended sediment to the slide. Avoid making the smear too thick.
3. 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.

8.3 Staining Smears

1. Place one smear and controls on the staining rack in the sink.
2. Flood the smears with Kinyoun's carbol-fuchsin, and let it stain for 5 min.
3. Rinse slide briefly (3 to 5 s) with 50% ethanol.
4. Rinse slide with running tap water.
5. Decolorize the slides with 1% sulfuric acid for 2 min or until no more color runs from the slide.
6. Rinse the slide with water and drain.
7. Counterstain by flooding smears with methylene blue for 30 to 60 s.
8. Rinse slide with water and air dry.

8.4 Examination & Interpretation of Permanent Smears

1. Examine the slides using the 10X objective of a light microscope for the presence of oocysts and sporocysts. After scanning on low power, review the smear with the oil immersion (100X) objective.
2. *Cryptosporidium* oocysts stain red and are ovoid or spherical and measure 4-8 μm. The background will be blue. Unstained "ghost" cells do occur. Specimens containing "ghost" cells should be brought to the attention of the supervisor or the Microbiology director.
3. *Cyclospora* organisms are variably acid-fast with some organisms staining deep red with a mottled appearance and no internal structure. Unstained organisms appear as glassy, wrinkled spheres. Organisms are generally round, 8-9 μm in diameter.
4. *Cystoisospora* oocyst internal structures stain bright red. The organisms measure 20-33 μm x 10-19 μm. Immature cysts containing one sporoblast are usually seen. The central portion of the oocyst or sporoblast stains deep red. The outside wall of the oocyst does not stain but may be surrounded by stain precipitate. It is possible for the oocysts to lose the outer shell completely. Mature oocysts containing 2 sporocysts may be found.

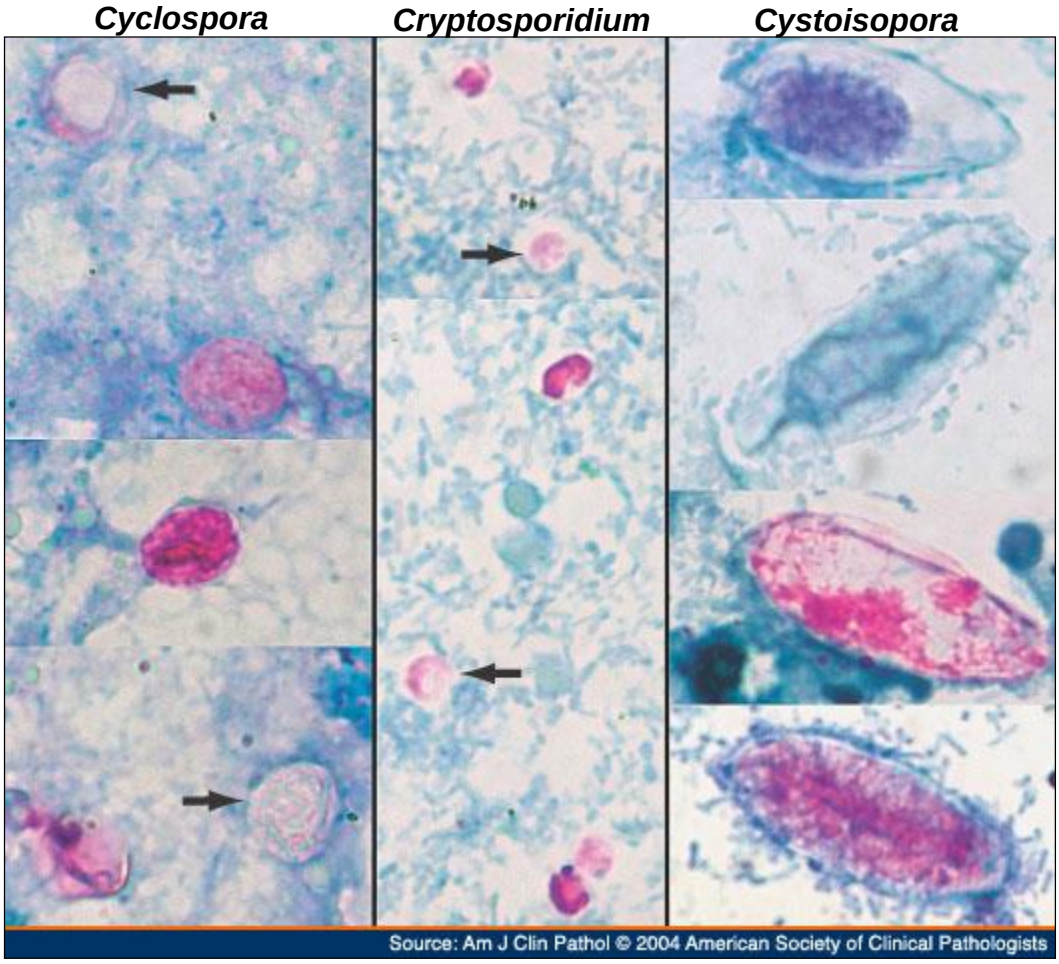
9.0 Reporting Results

9.1 Positive Results

1. If oocysts consistent with *Cryptosporidium* are seen, report: "*Cryptosporidium* seen." Hold the slide for the supervisor to review.
2. If oocysts consistent with *Cystoisospora* are seen, hold the slide for Rounds review. After Rounds confirmation, report "*Cystoisopora belli* seen (formerly *Isospora belli*)."
3. If oocysts consistent with *Cyclospora* are seen, hold the slide for Rounds review. After Rounds confirmation, report "*Cyclospora* seen."

9.2 Negative Results

If no Coccidia are seen, report: "Negative for *Cyclospora*, *Cryptosporidium*, and *Cystoisospora* by Acid Fast Stain."



10.0 Quality Control & Quality Assurance

Positive and negative controls are included each time the staining procedure is performed. Controls can be purchased commercially. Following staining, *Cryptosporidium* oocysts stain red with the Kinyoun acid fast stain. The negative control slide should have no typical red staining items. If the controls do not show characteristic staining, notify the supervisor and enter QC results in LIS.

11.0 Limitations

1. Light infections (low number of oocysts) may be missed.
2. Multiple specimens should be examined, since the numbers of oocysts in the stool will vary from day to day. A series of three specimens submitted on alternate days is recommended.
3. It is very important that the smears not be too thick. Thick smears may not adequately destain.

12.0 Verification

The Parasep device was evaluated in June and July of 2015. Preserved specimens that were positive for a variety of parasites using conventional concentration methods were concentrated with the Parasep device. The Parasep concentrates were used to prepare modified Kinyoun's stained smears. The coccidia examined include *Cyclospora cayetanensis* (1) and

Cryptosporidium (1). The parasites recovered using the Parasep device were equivalent or better in morphology and quantity to those observed in the preparations from conventional concentration methods.

13.0 References

1. Package insert: Total-Fix™, Medical Chemical Corporation, Revision date: June 3, 2014.
2. Package insert: Unifix™, Medical Chemical Corporation, Revision date: April 23, 2009.
3. Garcia, Lynne, Diagnostic Medical Parasitology. 2007. 5th Ed.
4. Clinical Microbiology Procedures Handbook, 3rd ed. and 2007 update, Vol. 2. Garcia, L.S., editor in chief. ASM Press, Washington, D.C.
5. Couturier, B.A., Jensen, R., Arias, N., Heffron, M., Gubler, E., Case, K., Gowans, J., and Couturier, M.R.: Clinical and analytical evaluation of a single vial stool collection device with formalin free 2 fixative for improved processing and comprehensive detection of gastrointestinal parasites. J. Clin. Microbiol. Accepted Manuscript posted online May 27, 2015
6. Murray, P.R., E.J. Baron, J.H. Jorgensen, M.L Landry, M.A. Pfaller. 2007. Manual of Clinical Microbiology, 9th ed., Vol. 1, ASM Press, Washington, D.C.
7. Kehl, S.E., Cicirello, Havens, PL. 1995. Comparison of four different methods for detection of *Cryptosporidium* species. J. Clin. Micro. 33: 416-418.

14.0 Document Control

Microbiology Director Approval: Dr. Ann Robinson 06/2001

Microbiology Supervisor Reviews: Jerry Claridge 04/05/2002, 04/04/2003, 03/18/2004, 05/05/2004, 11/2005, 01/18/2006, 03/2007, 10/2008, 02/2009, 02/2010, 06/2011, 03/2013, Jason Ammons 07/2015

Revisions: 09/04/2010 Updated acceptable preservatives to include Unifix. 08/22/2012 Updated staining procedure to reflect Clin Micro Proc Handbook and Diagnostic Parasit. To include ethanol wash and change decolorizer recipe. 09/08/2012 Added images to interpretation section. Added use of saline w/Unifix. 07/22/2015 Added Total Fix as an acceptable fixative. Removed concentration procedure (refer to O&P procedure). Added verification information for Parasep device. Added limitations section.