Department of Microbiology
Cryptosporidium, Isospora, and Cyclospora
Smear Procedure

I. Principle and 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. Oocysts in clinical specimens are difficult to detect but can be identified by the modified acid fast (Kinyoun) stain.

Isospora belli is also a coccidian parasite. *I. 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. *Isospora* may be visible in a routine ova and parasite saline or iodine mount. Staining with a modified acid-fast stain enhances recognition of the parasite.

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. *Cyclospora* can be detected using a modified acid fast stain. It can be differentiated from *Cryptosporidium* because of its larger size.

II. Specimen

A. Fresh stool or specimen preserved in 10% formalin or Unifix.
B. Stool preserved in PVA is <u>not</u> acceptable, because PVA distorts *Cryptosporidium*.
C. Specimens on in-patients who have been in-house greater than 5 days should generate a request for consultation.

III. Materials and Reagents

A. Filtering devices (plastic funnels, gauze squares)
B. 15 mL centrifuge tube with caps
C. Glass slides
D. Light microscope with calibrated ocular micrometer and 10X and 100X objectives
E. Immersion oil
F. Pipettes
G. 10% Formalin
H. Staining rack
I. Reagents for modified acid fast (Kinyoun) stain procedure. Stock supplies can be found in the processing area

1. Kinyoun carbol fuchsin stain, store at room temperature in the dark.
2. Decolorizer: 1.0% sulfuric acid.
3. 50% ethanol (reagent alcohol)
4. Methylene blue, 0.3% methylene blue in demineralized water, store at room temperature.

IV. Safety

A. Minimum personal protective equipment are gloves and lab coat.
B. All concentration procedures are performed under the fume hood.

V. Quality Control

A. Positive and negative controls are included each time the staining procedure is performed.
   1. Positive controls are made from a positive formalin vial containing *Cryptosporidium* oocysts. Negative control slides are made from a known negative patient stool sample.
   2. Label a slide with "*Cryptosporidium* Control" and another slide with “Negative Control”.
   3. Place a small drop of fecal suspension from each of the controls to a separate slide.
   4. Spread the material to make a thin uneven smear about the size of a nickel.
   5. Heat fix on a slide warmer.
B. Following staining, *Cryptosporidium* oocysts stain red with the Kinyoun acid fast stain. The negative control slide should have no typical red staining items.
C. If the control does not show characteristic staining, the second slide on each patient should be stained with a new control slide.
D. Notify the supervisor of any discrepancies and record the information in the QC section for *Cryptosporidium*.

VI. Procedure

A. Preparation of Smears
   1. Stool specimens should be received in formalin. If a freshly collected specimen is received, mix a 5 g portion with 10% formalin in a 15 mL plastic centrifuge tube. The preserved specimens are kept at room temperature until processed for *Cryptosporidium/ Isospora*/*Cyclospora.*
   2. Specimens must be fixed in formalin for at least 30 min prior to testing.
   3. Check the Misys accession number. Label the vial with a Misys label. Label a 15 mL plastic centrifuge tube with a Misys label.
   4. Mix the contents of the formalin vial thoroughly by shaking the vial several times.
   5. Remove the cap and insert a plastic funnel into the top of the disposable 15 mL centrifuge tube.

6. Open a gauze square to a one layer thickness. Fold the square in half, and place it into the plastic funnel. Moisten the gauze by squirting a small amount of 10% formalin onto the gauze into the 15 mL centrifuge tube.
7. Pour approximately 3 mL (formed, soft) or 5-6 mL (watery) of the fecal suspension through the gauze and funnel into the centrifuge tube.
8. Remove the gauze and funnel, and discard it into a plastic autoclave bag. Add 10% formalin to the top of the tube, cap, and centrifuge for 10 min at 1725 rpm (500 X *g).*
    a) NOTE: The centrifuge should come up to speed and then centrifuge for 10 min.
9. Decant supernatant fluids into the plastic waste container, retaining the sediment.
    a) Resuspend the sediment in 9 ml of 10% formalin. Use a wood applicator stick to loosen the sediment.
    b) Add approximately 3 ml of ethyl acetate, and cap tube. Invert and shake for 30 sec. IMPORTANT: If there is only a small amount of sediment or stool, do not add ethyl acetate, just recentrifuge the specimen.
    c) Centrifuge the tube for 10 min (see 8.a. above) at 1725 rpm (500X g, old AFB centrifuge). Decant the 3 layers, wipe the tube with a cotton swab.
    d) Mix the sediment, and smear 1 or 2 drops of specimen on the slide, and allow it to air dry. Do not make the smears too thick (you should be able to see through the wet material before it dries). Prepare two smears.
    e) Fix with absolute methanol for 1 min. Allow to air dry.

B. Staining
1. Place one smear and controls on the staining rack in the sink (the second smear is not routinely stained).
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 the slides by flooding them with methylene blue for 30 to 60 s.
8. Rinse slide with water and air dry.
9. 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.

VII. Interpretation

A. *Cryptosporidium* oocysts stain red and are ovoid or spherical and measure 4-8 μm. The background will be blue.
   1. Unstained "ghost" cells do occur.
   2. Specimens containing "ghost" cells should be brought to the attention of the supervisor or the Microbiology director.

B. *Isospora* oocyst internal structures stain bright red and measure 20-33 μm x 10-19 μm.
   1. Immature cysts containing one sporoblast are usually seen. The central portion of the oocyst or sporoblast stains deep red.
      a) The outside wall of the oocyst does not stain but may be surrounded by stain precipitate.
      b) It is possible for the oocysts to lose the outer shell completely.
   2. Mature oocysts containing 2 sporocysts may be found. The internal structures, the 2 sporocysts, stain bright red.
      a) The outside wall of the oocyst does not stain but may be surrounded by stain precipitate.
      b) It is possible for the oocysts to completely lose the outer shell.

C. *Cyclospora* organisms are variably acid-fast with some organisms staining deep red with a mottled appearance and no internal structure.
   1. Unstained organisms appear as glassy, wrinkled spheres.
   2. Organisms are generally round, 8-9 μm in diameter.
   3. Organisms can be seen on a wet mount as non-refractile spheres.
   4. On a wet mount, *Cyclospora* will autofluoresce in the presence of UV light.

VIII. Result reporting

A. Positive Results
   1. If oocysts consistent with *Cryptosporidium* are seen, report: "Oocysts of *Cryptosporidium* seen."
      a) If *Cryptosporidium* is found, hold the slide for the supervisor to review.
   2. If oocysts consistent with *Isospora* are seen, hold the slide for the supervisor to review.
   3. If oocysts consistent with *Cyclospora* are seen, hold the slide for the supervisor to review.

B. Negative Results
   1. If no *Cryptosporidium*, *Isospora* or *Cyclospora* are seen, report: “No *Cryptosporidium*, *Isospora* or *Cyclospora* seen”.

IX. References

A. Baron, EJ, Peterson, LR, and Finegold SM. 1994. Diagnostic Microbiology, 9th edition. Mosby, St. Louis, MO.

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