SELF-STUDY WORKBOOK
LABORATORY DIAGNOSIS OF INTESTINAL CRYPTOSPORIDIOSIS*
Course No. 8008-C

Sandra L. Bullock-Iacullo, Dr.P.H.
Laboratory Methods Development Branch
Division of Training
Training and Laboratory Program Office

*Note: Although originally designed to be used with specimens provided for the exercises, the information provided in this workbook will prove useful even without study specimens. Students may work through the procedures using controls from their own laboratory or commercial supply companies.

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Sandra L. Bullock-Iacullo, Dr.P.H.

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LABORATORY DIAGNOSIS OF INTESTINAL CRYPTOSPORIDIOSIS

INTENDED INSTRUCTION:
Self-study. Intermediate level. Instruction is limited to laboratory diagnosis; references for clinical information are listed in the last section of this workbook. Instruction is intended for use in a practicing parasitology laboratory environment.

INTENDED PARTICIPANT:
Laboratorian with sufficient experience in diagnostic parasitology to correctly identify *Giardia lamblia* (syn. *Giardia intestinalis*) cysts in saline and iodine stained wet preparations, and in trichrome-stained preparations. Given written instructions and all equipment and materials, laboratorian must be able to perform an acid-fast type stain and the formalin-ethyl acetate concentration technique.

TIME REQUIRED TO COMPLETE STUDY PACKAGE:
Approximately 6 hours, provided all equipment and materials are ready for use.

MATERIALS SUPPLIED WITH STUDY PACKAGE:
1. Workbook
2. Six vials of formalinized feces (one positive control and five "unknowns")**

MATERIALS AND EQUIPMENT NEEDED:
1. Binocular microscope equipped with the equivalent of a 20-watt halogen lamp, 40 X and 100X oil-immersion objectives, a blue daylight filter, and a calibrated ocular micrometer
2. Immersion oil
3. Glass microscope slides, 3 x 2 inch, approximately 20
4. Coverslips, #1 thickness, 22 x 22 mm, approximately 20
5. Formalin, 10% neutral buffered, approximately 100 ml (same as for concentration technique; see instructions for preparation on page 14)
6. Dobell and O'Connor's iodine, approximately 10 ml, fresh (see instructions for preparation on page 14)
7. Wooden applicator sticks, approximately 40
8. Pasteur pipets with rubber bulbs, approximately 20
9. Materials and solutions to perform the formalin-ethyl acetate concentration technique (see instructions on page 15)
10. Materials and solutions to perform either the modified Kinyoun or the auramine O acid-fast stain, or both (see instructions on pages 18 and 21)

**Not supplied with the CD-ROM version of this workbook.
OBJECTIVES:
Upon completion of this study program and given all necessary materials and this workbook, the participant will be able to:

1. Describe the morphology of Cryptosporidium species oocysts in saline and iodine-stained wet preparations, and in trichrome and acid-fast stained preparations.

2. Describe which types of stool specimens are most likely to contain oocysts and which should be routinely tested for the presence of oocysts.

3. Perform the formalin-ethyl acetate concentration technique on a stool specimen that contains oocysts and microscopically demonstrate the oocysts in an iodine-stained wet preparation.

4. Using the concentrate from #3 above, perform the modified Kinyoun or the auramine O acid-fast stain, or both, and microscopically demonstrate the oocysts.

To evaluate fulfillment of the above objectives, the participant will perform tests for Cryptosporidium species oocysts on the five "unknowns" supplied in the study package**. The participant must report the presence or absence of oocysts in each sample. Correct reports for four of the five (80%) samples are considered satisfactory performance.

IMPORTANT NOTE ABOUT ISOSPORA BELLI:

Although this study program specifically addresses the laboratory diagnosis of Cryptosporidium species oocysts, the procedures given, especially the acid-fast stains may be used for diagnosing Isospora belli oocysts. Additional information about Isospora belli oocysts in acid-fast stains is given with each stain technique in the last section of this workbook. References for clinical information are included with those for Cryptosporidium, also in the last section of this workbook.

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SAFETY PRECAUTIONS:

The stools in this study program are preserved in 10% neutral buffered formalin and most likely are not infectious**. However, **ALL SPECIMENS**, especially unpreserved specimens, should be treated as infectious and capable of transmitting serious infection. Stools can contain viruses, bacteria, protozoa, and even some helminths, which are immediately infectious. Please adhere closely to the safety policies of your laboratory in the handling and testing of medical specimens.

The following references address the safe handling and testing of medical specimens and may be helpful:


National Committee for Clinical Laboratory Standards. Protection of laboratory workers from infectious disease transmitted by blood and tissue; proposed guideline. Villanova, PA: NCCLS, 1987; NCCLS Document M29-P.

Richardson JH, Barkley WE, eds. Biosafety in microbiological and biomedical laboratories. 1st ed. Atlanta, GA: Centers for Disease Control-National Institutes of Health, PHS, HHS, 1984; HHS publication no. (CDC) 86-8395.

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CRYPTOSPORIDIUM: MORPHOLOGY AND DETECTION

The laboratory diagnosis of Cryptosporidiosis usually requires more effort than that for other intestinal parasites. The oocyst, the diagnostic stage of this coccidian, is very small and colorless and is not easily detected in routine examinations. Most often, special procedures (either meticulous microscopic examination on higher magnification or a separate stain technique) are required to make a positive identification of these tiny oocysts. This self-study program will provide instructions and practice in gaining the visual experience to detect few or more oocysts in any given specimen and will also provide the protocols and instructions for the special stains‡.

You should now STUDY the information on the following pages about the morphology and detection of the oocysts. AFTER you have a clear mental image of what these oocysts look like and in which specimens you are most likely to find them, go to the next section of the workbook, follow the instructions, and PRACTICE, PRACTICE, PRACTICE. Through PRACTICE you will gain the competence and confidence to readily detect oocysts in stool specimens.

‡Many published procedures are used for the recovery and detection of oocysts. Those chosen for presentation here seem to be the most successful and cost-effective for most laboratories.
MORPHOLOGY OF THE OOCYST:

Size range is 4-6 µm; most are 5-6 µm.

Shape is usually spherical, rarely oval.

Nonmotile.

Oocyst wall is very thin and delicate in appearance.

Mature oocyst contains 4 "naked" sporozoites (no sporocyst is present) and a single dark "residual" body. The sporozoites are highly refractive to light.

Variations Observed Under Bright-field Microscopy:

In acute infections, large numbers of what appear to be immature oocysts are frequently passed. These forms exhibit the characteristics above except that individual sporozoites are not seen. Instead, the sporozoite material appears undivided and clings to the oocyst wall, thus yielding the appearance of tiny, highly refractile "donuts" or "crescents." Residual bodies are usually large and quite visible in these forms. The oocyst wall can be seen but is very thin and frequently collapses on one side.

In resolving infections and chronic infections, the oocysts usually exhibit typical morphology, although all sporozoites are not visible in all oocysts. In addition, there are usually significantly fewer oocysts present.

Appearance of Oocysts in Different Types of Preparations:

SALINE: Morphology is like that described above. The most notable feature at 400 X magnification is that the oocysts are more refractile than surrounding structures of similar size and shape.

At 1000 X oil-immersion magnification, most but not all of the described structures can be seen. With very careful observation, oocysts can be differentiated from common fecal yeasts on the bases of shape, thickness of the cell wall, and the appearance of the internal contents. Yeasts are oval, have thick cell walls, and are filled with cytoplasm and dark granules.
DOBELL AND O'CONNOR'S IODINE: Oocysts, typically, do not stain with this iodine solution but remain clear and highly refractive to light, even if left in this solution for a long time. Yeasts rapidly stain brownish-yellow and are therefore easily distinguished from oocysts. Magnification at 400 X is usually sufficient to see this difference.

NOTE 1: A degenerated yeast (yeast "ghost") consisting of a thick cell wall and scanty internal contents will not take up this iodine stain. Examination at 1000 X magnification will reveal the thick wall of an unstained yeast.

NOTE 2: Some oocysts uncharacteristically take up the iodine stain in a relatively short time; some take up the stain within a few minutes of contact. The reason for this is unknown and, therefore, the specimens should be examined as soon as possible after the iodine preparation is made.

TRICHRoME STAIN: Usually oocysts do not stain with trichrome and appear as "holes" in the fecal background. Occasionally, however, some strains of oocysts do take up a pink to red color but USE OF THE TRICHRoME STAIN IS NOT RECOMMENDED FOR THE DIAGNOSIS OF CRYPTOSPORiDIUM SPECIES OOCYSTS.

MODIFIED KINYOUN ACID-FAST STAIN: Cryptosporidium species oocysts are acid-fast and retain a bright to dark-red color with this stain. Most appear as tiny, solid, red balls. Sometimes, only the sporozoites within mature oocysts retain the stain and thus produce a picture of four pink to red tiny sausages close together. Finding these forms containing sporozoites confirms that the structures are oocysts. Most yeasts and other structures take up the color of the counterstain, usually green or blue.

AURAMINE O ACID-FAST STAIN: This is a fluorescent acid-fast stain and requires the use of a fluorescence microscope. Here, the acid-fast oocysts stain a brilliant orange-yellow and appear solid, crescent, or crater-like. Sometimes individual sporozoites can be seen. Nonacid-fast or partial acid-fast fluorescing material is pale in color. Most yeasts do not stain.
CRYPTOSPORIDIUM SPECIES OOCYSTS

IMMATURE – TRAPPED IN MUCUS
UNSTAINED – 1000 x

MATURE – NOTE
SPOROZOITES AND BUDDING YEAST
UNSTAINED – 1000 x

IMMATURE - NOTE LACK OF STAINING
COMPARED TO YEASTS
IODINE STAINED – 800 x

MATURE – NOTE
SPOROZOITES
IODINE STAINED – 1000 x

MODIFIED KINYOUn’S
ACID-FAST STAIN
800 X

AURAMINE O
ACID-FAST STAIN
800 X
DETECTION OF OOCYSTS:

Types of Stools:

Recall that stools are classified on the basis of consistency:

- **FORMED**
- **SOFT**
- **LOOSE**
- **WATERY**

In acute Cryptosporidiosis (frequent in immunocompetent children), generally, the more liquid the stool, the greater the number of oocysts found. Watery and loose stools containing very large numbers of oocysts frequently are light in color (i.e., light yellow, brown, or green) and usually appear homogenized. Soft stools usually contain fewer oocysts. Formed stools rarely contain more than a few oocysts.

In chronic infections (usually in immunocompromised persons, such as those with acquired immunodeficiency syndrome), oocysts range from very many to scanty, even in liquid stools. All stool specimens from known or suspected immunocompromised persons should be tested for *Cryptosporidium* oocysts.

Suggested Regimen for Recovery and Detection of Oocysts:

Oocysts can be recovered from fresh, unpreserved stools or from stools preserved in 10% neutral buffered formalin (buffering is important for effective concentration of oocysts), MIF-fixative, SAF-fixative, or PVA-fixative.

Because formalin preservation of oocysts allows the most effective subsequent staining with both iodine and the acid-fast stains, this preservative is recommended for routine use. In addition, because *Cryptosporidium* oocysts are infectious when passed, preservation with formalin before testing probably reduces the possibility of transmission of the infection. All of the stool samples in this study program** are preserved in 10% neutral buffered formalin.

When a stool specimen is received with a request for a routine examination for parasites, the first step is to determine the consistency of the stool when it was passed. If the specimen is unpreserved, this is easily done by looking at the stool and comparing it to the pictures above. If the specimen has been preserved, the container or request form should be marked with the original specimen consistency. Although knowledge of specimen consistency is not imperative to testing, it does increase the cost-effectiveness of the work. It is unnecessary and impractical to do special procedures on every specimen. As stated earlier, the yield of oocysts from formed specimens is usually quite small.

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**Not supplied with the CD-ROM version of this workbook.**
Routinely, all loose and watery specimens, and those from known or suspected immunocompromised persons, should be checked for Cryptosporidium oocysts. In addition, all specimens with special requests for Cryptosporidium, regardless of specimen consistency, should be tested.

To what extent or how these specimens are tested depends primarily on the expertise of those doing the testing. Some laboratories visually scrutinize with iodine all candidate specimens and use the acid-fast stain(s) to confirm questionable specimens and/or to provide permanent records of positive specimens. Other laboratories routinely perform an acid-fast stain on all candidate specimens. This, of course, requires more time and energy but reduces the chance of missing specimens containing few oocysts. Each laboratory must evaluate its own capabilities and resources, and its usual patient population, and decide on the most appropriate system to use.

Regardless of the type of detection system selected by the laboratory, all candidate specimens should be concentrated before they are examined microscopically. Although special techniques are available for concentrating oocysts, it has been shown that the most popular technique used for concentrating stool parasites, the formalin-ethyl acetate (FEA) technique, also concentrates Cryptosporidium oocysts. Therefore, the formalin-ethyl acetate technique is recommended and used for this purpose in this study program.

After concentration, the resulting sediment can be examined by one, two, or a combination of three different techniques. These are:

1. Meticulous microscopic examination with high magnification, using Dobell and O'Connor's iodine
2. The modified Kinyoun acid-fast stain
3. The auramine O acid-fast stain

Each of these techniques is more useful than the others in certain situations.

For routine purposes, i.e., for loose and watery routine stools, examination with iodine is the easiest, most cost-effective method, but it requires greater expertise and is not as sensitive as the others. Laboratorians with limited experience should run an acid-fast stain on these specimens to be able to detect rare to few oocysts. For other than routine specimens, an acid-fast stain is needed. The modified Kinyoun is easy and provides a permanent record, but it is not as reliable as the auramine O in staining oocysts. In addition, rare oocysts are more difficult to detect in Kinyoun preparations than in auramine O preparations.
The auramine O is quite sensitive and is easy to perform, but a fluorescence microscope must be used to examine these samples, and occasionally small debris resembles an oocyst. To decrease the risk of misidentifying debris as an oocyst in this stain, the laboratorian should check, by another method, any specimen found to have rare to few "oocyst-like structures" that lack internal sporozoites. The other technique should reveal sufficient characteristics of the oocysts to enable positive identification.

PRACTICE WITH EACH OF THESE TECHNIQUES WILL HELP YOU DECIDE WHICH TO USE IN YOUR LABORATORY, SO GO ON TO THE NEXT SECTION OF THIS STUDY PROGRAM AND PRACTICE, PRACTICE, PRACTICE***.

***Note: This self-study exercise was published before the use of FA an antigen techniques for diagnosing Cryptosporidium.
STUDY EXERCISES

BE SURE YOU HAVE ALL MATERIALS AND EQUIPMENT READY TO USE. DON’T FORGET THE MATERIALS FOR THE CONCENTRATION AND STAINING EXERCISES.

1. Prepare unstained and iodine-stained preparations of the positive specimen, using the following procedure:

   a. Label appropriately a 3 x 2-inch microscope slide.

   b. Using a Pasteur pipet, place 1/3-1/2 drop (a full drop is too much) of saline on one side of the slide and the same amount of iodine on the other.

   ![Saline and iodine drops](image)

   c. Shake the specimen vial thoroughly and, using a clean Pasteur pipet, place 1/3-1/2 drop of positive specimen beside each area of diluent.

   ![Specimen and diluent](image)

   d. Using the corner of a coverslip, mix the saline and specimen together and then place that coverslip on top of the mixture.

   e. Using a second coverslip, repeated for the iodine-specimen side.

   These preparations should be fairly thin. You should be able to turn the entire slide upside down and the coverslip will stay in place. If you cannot do this, try again, using smaller quantities of diluent and specimen.

   f. These preparations can be sealed around the edges of the coverslips with a melted 1:1 Vaseline-paraffin mixture to prevent their drying for a few hours. Sealing may also help stabilize the material during oil immersion examination. However, because you will be looking at this slide soon after preparation and it will be thin, sealing is not essential.
2. Observe carefully the iodine-specimen preparation under 400 X magnification. During observation, you should rotate the fine adjustment knob almost continuously. By doing this, you will be able to see clear, highly refractile objects of about 5 µm, which stand out against the yellow-colored background. Focus carefully to locate one, place it in the center of the field, and change to the 100X oil immersion objective. At this magnification (1000 X), you should be able to see the internal details. If you can see a thin cell wall, sporozoites, and a residual body, you're looking at a Cryptosporidium oocyst. Compare it with the photograph in the previous section of this workbook. Look at several oocysts to acquire a range of variation.

3. After observing several oocysts in the iodine-stained preparation, look at the saline preparation. Although the oocysts won't be as easy to detect in saline, the purpose of this exercise is to Train your eyes to search for refractivity. When you have mastered detection by observing refractivity, you will be successful in detecting oocysts in wet preparations. Be sure to look for organisms on both 400 X and 1000 X magnifications.

4. Now return to the iodine-stained preparation and PRACTICE, PRACTICE, PRACTICE.

5. Following the instructions on page 17, perform the formalin-ethyl acetate concentration technique on the positive specimen. At the end of the procedure, be sure to drain all fluid from the sediment and remove all traces of ethyl acetate from the wall of the tube. Any ethyl acetate residue will cause the material to spread on the slide and result in a poor preparation. After all fluid has been removed, add 2-4 drops of 10% neutral formalin to the sediment and mix well with a clean Pasteur pipet. To prevent evaporation, keep the tube stoppered. Finally, prepare saline and iodine-stained preparations and observe microscopically under 400 X and 1000 X magnifications for oocysts. Note that more oocysts are present per microscope field than in the direct examination of the specimen; hence, the formalin-ethyl acetate technique has concentrated the organisms.

6. Using the same sediment from the formalin-ethyl acetate technique, perform either or both of the acid-fast stains given on pages 18 and 21. You select which technique(s) is/are appropriate for your laboratory. Compare the oocysts in your stain(s) with the pictures on page 7.

7. After you have completed the above exercises and feel confident in detecting oocysts, test the five "unknowns" supplied with the study package. Use any technique or combination of techniques you would use in your laboratory.
8. Record your results for each specimen on the report form located in the back of this workbook. Please follow the instructions on the form when quantitating organisms; also follow the instructions for folding and mailing the self-addressed form.

Successful completion of this study program, i.e., four of the five "unknowns" correct, is awarded 0.6 CDC Continuing Education Units†.

† Not applicable for the CD-ROM version of this workbook.
I. Preparation of Dobell and O'Connor's Iodine Solution

Prepare fresh each month:

- Iodine (powdered crystals) 1 gm
- Potassium iodide 2 gm
- Distilled water 100 ml

Dissolve the potassium iodide in the water. Add the iodine crystals and shake thoroughly. The crystals may not dissolve completely, but the solution should be a definite reddish-brown or the color of strong tea. Decant into a dropping bottle or other type of dispenser.

Because most clinical laboratories don't use this amount of solution in a month, the following procedure is suggested.

1. Prepare 500 ml of 2% potassium iodide (10 gm in 500 ml distilled water).

2. Store this stock potassium iodide solution in a glass-stoppered bottle in the dark. It will keep for several months.

   Pour a small amount (20-30 ml) into a small beaker or flask and add some iodine crystals (about 0.2-0.3 gm; the exact amount is not important) and shake until the solution is the proper color. Replace each month.

II. Preparation of 10% Neutral Buffered Formalin

Commercial formaldehyde is approximately 40% concentration by volume, which means that 10% formalin is actually about 4% formaldehyde.

- Na$_2$HPO$_4$ 6.10 gm
- NaH$_2$PO$_4$ 0.15 gm
- Formaldehyde 800.0 ml
- Water (tap or distilled) 7,200.0 ml

Mix the formaldehyde with the water. Add the buffer salts and mix thoroughly. This makes 8 liters of neutral 10% formalin. This solution will keep indefinitely.
III. Formalin-Ethyl Acetate Concentration Technique

A. Materials and solutions needed:

1. Small (3-4 oz) paper cups (FOR FRESH SPECIMENS ONLY)
2. Wooden applicator sticks
3. Conical paper cups that have had the tips cut off, OR small funnels
4. Surgical gauze, OR #50 mesh cheesecloth
5. Centrifuge tubes (15 ml, glass or polypropylene)
6. Stoppers or caps to fit centrifuge tubes
7. Centrifuge
8. Squeeze bottles for dispensing tap water, formalin, ethyl acetate.
   Bottle for ethyl acetate needs a cap or stopper.
9. Timer or clock
10. Cotton-tipped applicators (nonsterile)
11. Pasteur pipets fitted with bulbs
12. Glass microscope slides, 3 x 2 inch
13. Coverslips, #1 thickness, 22 x 22 mm
14. Tap water
15. Ethyl acetate, certified grade
16. Formalin, 10% neutral buffered. See formula for preparation on page 14.

B. Technique for Fresh Specimens

1. Thoroughly mix a portion of stool about the size of a large marble in sufficient water so that 10 ml of the suspension will yield about 1 ml of sediment upon centrifugation. The suspension can be prepared in a small paper cup.

2. Strain about 9 ml of the suspension through a small funnel containing either two single layers of surgical gauze OR a single layer of cheesecloth. To conserve glassware, a conical paper cup with the point cut off can be substituted for the funnel.

3. Centrifuge at 500 x g for 10 minutes. Decant supernatant. About 1 ml of sediment should be present. If the amount is much larger or smaller, adjust to the proper quantity in the following manner:

   a. Amount too large.

   Resuspend the sediment in water and pour out a portion. For example, if the amount is twice the desired quantity, pour out about half of the suspension. Add water to bring the fluid level to about 10 ml and centrifuge again.
b. Amount too small.

Pour off the supernatant and strain a second portion of the original fecal suspension into the tube. The amount to be strained can be determined from the sediment; that is, if about half of the quantity necessary is obtained with the first centrifugation, strain another 10 ml into the tube. Centrifuge again.

It is not necessary to have an exact quantity of sediment in the tube, but the quantity should approximate the amounts indicated above. Too much or too little sediment will result in an ineffective concentration.

4. Resuspend the sediment in fresh water, centrifuge, and decant as before.
5. Add about 9 ml of 10% neutral formalin to the sediment, mix thoroughly, and allow to stand for 5 minutes or longer. At this point, the mixture may be stoppered and saved until a later time.
6. Add 4 ml of ethyl acetate, stopper the tube, and shake vigorously in an inverted position for at least 30 seconds. Remove the stopper with care.
7. Centrifuge at 500 x g for 10 minutes. Four layers should result as follows: (1) layer of ethyl acetate, (2) plug of debris, (3) layer of formalin, and (4) sediment.
8. Free the plug of debris from the sides of the tube by ringing with an applicator stick and carefully decant the top three layers. Before returning the tube to the upright position, use a cotton swab to clean debris and all traces of ethyl acetate from the walls of the tube and prevent them from settling down into the sediment. The presence of ethyl acetate in the final sediment will cause great difficulty in making a satisfactory wet preparation.
9. With a pipet, add twice the amount of 10% neutral formalin as the amount of final sediment. For example, if there is 0.2 ml of final sediment, add 0.4 ml of formalin. Be careful not to add too much, otherwise the specimen will be too thin for satisfactory preparations.

Once formalin has been added, the tube may be stoppered or capped and kept for some time (months). Of course, the sediment cannot be allowed to dry.
10. With a Pasteur pipet, mix the sediment and formalin together and prepare iodine and unstained mounts in the usual manner for microscopic examination.

C. Technique for Formalin-Preserved Specimens

1. Thoroughly shake or stir the formalinized specimen.

2. Depending on the density of the specimen, strain a sufficient quantity through either two single layers of surgical gauze OR a single layer of cheesecloth into a centrifuge tube to yield the desired amount of sediment indicated below. Usually 2 to 3 ml is sufficient unless the fecal suspension is thin.

3. Add tap water to make 9 ml of suspension, mix thoroughly, and centrifuge at 500 x g for 10 minutes.

4. Decant supernatant. The amount of sediment should be about 0.5 ml. If too much or too little is present, adjust the quantity by the same methods described in step 3 for fresh specimens. In formalin preserved specimens, the formalin has already clarified the feces to some extent, and further clarification is done primarily by the ethyl acetate. Therefore, the 11 reduction of the sediment is less than for fresh feces, and the initial quantity must be less.

5. Add 9 ml of 10% neutral formalin to the sediment and mix thoroughly.

6. Add 4 ml of ethyl acetate, stopper the tube, and shake in an inverted position for 30 seconds. Remove the stopper with care.

7. Centrifuge at 500 x g for 10 minutes. Four layers should result. See description in step 7 for fresh specimens.

8. Free the plug of debris from the sides of the tube by ringing with an applicator stick, and carefully decant the top three layers. Before returning the tube to the upright position, use a cotton swab to clean debris and all traces of ethyl acetate from the walls of the tube and prevent them from settling down into the sediment. The presence of ethyl acetate in the final sediment will cause great difficulty in making a satisfactory wet preparation.
9. With a pipet, add twice the amount of 10% neutral formalin as the amount of final sediment. For example, if there is 0.2 ml of final sediment, add 0.4 ml of formalin. Be careful not to add too much, otherwise the specimen will be too thin for satisfactory preparations.

Once formalin has been added, the tube may be stoppered or capped and kept for some time (months). Of course, the sediment cannot be allowed to dry.

10. With a Pasteur pipet, mix the sediment and formalin together and prepare iodine and unstained mounts in the usual manner for microscopic examination. See page 11.

IV. Modified Kinyoun Acid-Fast Stain for Cryptosporidium* Oocysts in Stool Specimens

Acid-fast stains are helpful in distinguishing Cryptosporidium oocysts from other organisms commonly found in stools, particularly yeasts. Oocysts are acid-fast and retain the red color of the carbol fuchsin dye even after acid decolorization. Since most yeasts are not acid-fast, they are decolorized by the acid destain and, subsequently, take on the color of the counterstain.

The technique presented here is a modified cold stain method adapted by Marilyn S. Bartlett, Department of Microbiology/Pathology, Indiana University Medical Center, from the Kinyoun's modified acid-fast stain for Nocardia.

*This technique can also be used to stain Isospora belli oocysts.

A. Materials and solutions needed:
1. Glass microscope slides, 3 x 1 inch
2. Pasteur pipets fitted with bulbs
3. Coplin jars, 50-ml capacity
4. Forceps for handling slides
5. Timer or clock
6. Blotting paper
7. Mounting medium
8. Coverslips, #1 thickness, 22 x 22 mm
9. Microscope (description on page 1)
10. Running tap water (gentle stream)
11. Absolute methyl alcohol
12. Acid-fast stain: Kinyoun Modified Carbol Fuchsin

a. Commercial stain: Carbol Fuchsin Modified Kinyoun Formula, EMDS/Harleco #7645X

b. Preparation:
   Basic Fuchsin, Color Index No.42510  4 gm
   Ethyl alcohol, 95%       20 ml
   Phenol, liquid           8 ml
   Distilled water          100 ml

   Place basic fuchsin dye into a 500 - or 1,000-ml Erlenmeyer flask. Slowly add the alcohol, stirring continuously to dissolve the powder. (Basic fuchsin tends to "boil up" when alcohol is added; slowly adding the alcohol with constant stirring is important.)

   Add the phenol and the water to the dye solution. Mix thoroughly. Store in a screw-capped bottle.

13. 50% Ethyl Alcohol
   Ethyl alcohol  50 ml
   Distilled water  45 ml
   Mix thoroughly. Store in glass-stoppered bottle.

14. Acid destain
   Sulfuric acid, concentrated  10 ml
   Ethyl alcohol, 95%          90 ml

   Mix the acid with the alcohol. Store in glass-stoppered bottle.

15. Counterstain: Malachite Green
   Malachite Green, Color Index No.42000.  3 gm
   Distilled water             100 ml

   Dissolve the dye in the water. Store in screw-capped bottle.

B. Technique:

1. Make a thin smear of stool specimen on a 3 x 1-inch slide. The smear can be made either from fresh, unpreserved feces or from feces preserved in 5% or 10% neutral formalin. Smears made from the sediment after formalin-ethyl acetate concentration may yield a higher number of oocysts.
2. Let the smear air dry at room temperature, OR speed-dry in a 37-42 °C incubator.

3. Fix the smear with absolute methyl alcohol for 30 seconds. Hold the slide in a slanted position, and using a dropping bottle or pipet, allow the alcohol to run over the smear, OR dip the slide into a Coplin jar of methyl alcohol. Let dry.

4. Place slide in carbol fuchsin stain (in Coplin jar) for 5 minutes at room temperature.

5. Rinse briefly in running tap water.

6. Rinse in 50% ethyl alcohol (in Coplin jar) for about 5 seconds with gentle agitation.

7. Rinse briefly in running tap water.

8. Decolorize with 10% alcoholic H₂SO₄ (in Coplin jar) for 1-2 minutes. Adjust time, depending on the thickness of the smear.

9. Rinse in running tap water.

10. Counterstain with malachite green for 2 minutes.

11. Rinse very briefly in running tap water.

12. Dry thoroughly by wiping the back of the slide and gently blotting the front.

13. Cover with #1 thickness coverslip, using suitable mounting medium.

14. Examine with a bright-field microscope at 400 X and 1000 X magnifications.

*Cryptosporidium* oocysts stain red. Occasionally, individual pink to red stained sporozoites can be seen, either inside an oocyst wall or in groups of four without an oocyst wall.

*Isospora belli* oocysts also stain, but not entirely. The germinal mass within the oocyst stains red, and although the wall does not stain, it is sometimes surrounded by precipitated stain that outlines the overall structure. Frequently, the oocyst wall will collapse but, in the presence of other oocysts, the parasite usually remains recognizable.
V. Auramine O Acid-Fast stain for Cryptosporidium* Oocysts in Stool Specimens

Since the demonstration of the acid-fast nature of Cryptosporidium oocysts, a variety of acid-fast stains have been used to distinguish these coccidia from yeasts, other protozoa, and normal fecal material. This technique employs auramine O, a dye for general staining and fluorescence microscopy procedures. This is a true acid-fast stain and should not be confused with fluorescent antibody (FA) procedures. It is an adaptation of the auramine O procedure used for detecting Mycobacterium spp. in some clinical laboratories.

*This stain is also effective in staining Isospora belli oocysts.

A. Materials and solutions needed:
1. Glass microscope slides, 3 x 1 inch
2. Pasteur pipets fitted with bulbs
3. Electric slide warmer, 65 °C (FOR FRESH SPECIMENS)
4. Incubator, 37- 42 °C (FOR FORMALINIZED SPECIMENS)
5. Staining rack
6. Forceps for handling slides
7. Timer or clock
8. Fluorescence microscope outfitted with a BG-12 exciter filter and an OG-I barrier filter, or some other equivalent system.
9. Tap water
10. Absolute methyl alcohol
11. Acid-fast stain

   Auramine O, Color Index No.41000 0.1 gm
   Ethyl alcohol, 95% 10.0 ml
   Phenol crystals 3.0 gm
   Distilled water 87.0 ml

   Dissolve the auramine O in the ethyl alcohol. Dissolve the phenol crystals in the water. Mix the two solutions together. Store in a dark bottle.

12. Acid destain

   Hydrochloric acid, concentrated 0.5 ml
   Ethyl alcohol, 70% 100.0 ml

   Carefully add the acid to the alcohol.

13. Counterstain

   Potassium permanganate 0.5 gm
   Distilled water 100.0 ml

   Dissolve the potassium permanganate in the water.
B. Technique:

1. Prepare a thin smear of stool specimen on a 3 x 1 inch slide. The smear can be made either from fresh, unpreserved feces or from feces preserved in 5% or 10% neutral formalin. Smears made from the sediment after formalin-ethyl acetate concentration may yield a higher number of oocysts.

Smears made from unpreserved stool specimens should be heat fixed for at least 1 hour at 65 °C on an electric slide warmer. Formalin-preserved smears may be heat fixed at 37-42 °C until the smears dry or may be air dried for 2-3 hours.

2. Fix all smears with absolute methyl alcohol for 30 seconds. Let dry.

3. Place slide on a staining rack and flood with auramine O for 15-20 minutes at room temperature. Make certain that the staining solution covers the smear.

4. Rinse with tap water and drain.

5. Cover smear with acid alcohol and destain for 2 minutes.
6. Rinse smear and drain.

7. Flood the slide with potassium permanganate counterstain for 2 minutes.

8. Rinse smear, drain, and air dry.

9. Examine smear with fluorescence microscope by scanning with a 20-25X objective and critically observing with a 40-63X objective.

*Cryptosporidium* oocysts stain a bright orange-yellow, whereas the nonacid-fast and partial acid-fast fluorescing material exhibits a pale, less brilliant color.

*Isospora belli* oocysts stain similar to *Cryptosporidium* oocysts in color and intensity, but, of course, exhibit typical species morphology with regard to size, shape, and internal contents.
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LABORATORY DIAGNOSIS OF INTESTINAL CRYPTOSPORIDIOSIS

UPDATE - JANUARY 1991*

*Updated from the original printed version in 2001.
ABOUT THE ORGANISM

1. Since the printing of this workbook, the species name *parvum* has been generally accepted for identifying those cryptosporidia recovered from humans, i.e., *Cryptosporidium parvum*.

2. A diagram of the life cycle of *Cryptosporidium* is attached. The following definitions may help you understand the cycle:

   a. Oocyst- the encysted product of fertilization of macrogamete by a microgamete.

   b. Sporozoite- the slender, spindle shaped organism that is the infective stage of sporozoan parasites.

   c. Sporogony- the sexual portion of the life cycle of sporozoan parasites in which the encysted zygote (oocyst) undergoes multiple divisions, giving rise to sporozoites.

   d. Schizont- the stage in the development of a sporozoan parasite following the trophozoite in which the nucleus divides into many smaller nuclei.

   e. Schizogony- the asexual portion of the life cycle of sporozoan parasites that results in the formation of merozoites.

   f. Merozoite- the end stage of schizogony (asexual reproduction), which is capable of infecting other cells of the same host.

   g. Gametogenesis- the development of the male and female sex cells, or gametes.

   h. Microgametocyte- the male gametocyte that produces microgametes.

   i. Macrogametocyte- the female gametocyte that matures into a macrogamete.

   j. Zygote- the cell resulting from the union of a male and a female gamete; the fertilized ovum.

3. The upper right photograph on page 7 needs some help. The structure in the upper half slightly right of center is the mature oocyst, and the sporozoites are discernible by careful examination. The budding yeast is in the lower half barely left of center.
ABOUT THE TECHNIQUES

1. Although most yeasts are not acid-fast and take up the color of the counterstain in the acid-fast staining procedures, you may occasionally encounter strains of yeasts that are acid-fast. Without careful observation, they could be confused with *Cryptosporidium parvum* oocysts. The differential characteristic between the two is that *Cryptosporidium* oocysts contain sporozoites whereas yeasts do not. In acid-fast stained preparations, look carefully for the presence of sporozoites ("pink sausages") in the suspected oocysts. If you find sporozoites, even if in only a few of the oocysts, you can make a positive identification of *Cryptosporidium*.

2. Directions for the acid-fast stain techniques instruct you to make "thin" preparations from the concentrated stool. "Thin" is a matter of judgment, but generally this means slightly thinner than the wet preparation made for the examination of a concentrated stool. It does not mean so thin as to question where the preparation was placed on the slide. Fecal material should be quite visible.

3. Other stains which are useful for the detecting *Cryptosporidium* oocysts include the Ziehl-Neelsen acid-fast technique, the auramine-rhodamine fluorescence stain, and the safranin-methylene blue technique. Any of these could be used with this training workbook if such use simplifies or accelerates the learning process. The techniques presented in this workbook were chosen on the basis of reliability, ease of use, and cost-effectiveness. Whatever the technique, be sure that you can detect individual sporozoites in at least some of the stained oocysts.

4. A commercial monoclonal antibody/direct fluorescence test is available for detecting *Cryptosporidium* oocysts and is reported to be 100% sensitive and 100% specific. However, it does require the use of a fluorescence microscope and the cost per test is quite high relative to the methods given here.

5. *Cryptosporidium* oocysts have been recovered from specimens other than feces --lung biopsy, sputum, gall bladder, duodenal aspirate, and other specimens directly and indirectly associated with the gastro-intestinal tract. Detection of oocysts in these specimens can be accomplished with the techniques similar to those given in this package, but instruction for such techniques is beyond the intended scope of the package.

6. Commercial antigen detection kits are also now available for detecting *Cryptosporidium* oocysts.
LIFE CYCLE OF CRYPTOSPORIDIUM

*Probable development (based on studies in animals, and electron microscope examinations of biopsied human intestinal tissue).