COMBINATION MODIFIED ACID-FAST, MODIFIED TRICHROME STAIN (Coccidia, Microsporidia)

Preanalytical Considerations

I. PRINCIPLE

The detection of Cryptosporidium parvum and the microsporidia from stool specimens has depended on two separate stains. However, a method is not available that will stain both organisms, an important improvement since dual infections have been demonstrated in AIDS patients. This acid-fast trichrome stain yields results comparable to those obtained by the Kinyoun and modified trichrome methods and considerably reduces the time necessary for microscopic examination. Also, it appears that modified trichrome stains and staining with fluorochromes are equally useful in the diagnosis of microsporidiosis; however, a combination of both methods may be more sensitive in cases where the number of spores is very few (1-3). The diagnosis of intestinal microsporidiosis (*Brachiola* spp., *Enterocytozoon bieneusi*, *Encephalitozoon* spp., *Nosema* spp., *Vittaforma* spp., *Pleistophora* spp., *Trachipleistophora* sp., and *Microsporidium* spp.) has depended on the use of invasive procedures and subsequent examination of biopsy specimens, often using electron microscopy methods. However, additional methods are now available. Slides prepared from fresh or formalin-fixed stool specimens can be stained by chromotrope-based techniques and can be examined using light microscopy. Stain penetration of the microsporidial spore is quite difficult; therefore, the dye content of the chromotrope 2R in the stain formula is 10 times that normally used in the Wheatley's modification of Gomori's trichrome stain for intestinal protozoa. Also, the staining time is much longer (90 min). Modified acid-fast stains are recommended for demonstrating the coccidia (*Cryptosporidium, Cyclospora, Isospora*). Unlike the Ziehl-Neelsen modified acid-fast stain, the modified Kinyoun acid-fast stain does not require heating the reagents used for staining

II. SPECIMENS

The specimen can be fresh stool or stool that has been preserved in 5 or 10% formalin, SAF, or some of the newer single-vial system fixatives. Actually, any specimen other than tissue could be stained using this method. PVA-preserved fecal material is not recommended.

III. MATERIALS

A. Reagents: Reagents may be purchased commercially or prepared in the laboratory.

a. Modified trichrome (Ryan-Blue formulation)
b. Trichrome decolorizer
c. Carbol fuchsin solution
d. Acid-alcohol decolorizer

B. Supplies

a. Glass slides (25 by 75 mm), frosted ends desirable
b. Coverslips (22 by 22 mm; no. 1)
c. Glass or plastic centrifuge tubes
d. Pasteur pipettes
e. Coplin jars or other suitable staining containers
f. Immersion oil

C. Equipment: Optional materials, depending on specimen source of laboratory protocol

a. Binocular microscope with 10X, 40X, and 100X objectives (or the approximate magnifications for low power, high dry power, and oil immersion examination).
b. Oculars should be 10X. Some workers prefer 5X; however, overall smaller magnification may make final organism identifications more difficult.
c. Tabletop centrifuge

Analytical Considerations

IV. QUALITY CONTROL

A. Unfortunately, the only way to perform acceptable QC procedures for this method is to use actual microsporidial spores and coccidian oocysts as the control organisms. Obtaining these positive controls may be somewhat difficult. It is particularly important to use the actual organisms because the spores are difficult to stain and the size is very small (spores: 1 to 1.5 µm)(oocysts: 4 to 10 µm).

B. A QC slide should be included with each run of stained slides, particularly if the staining setup is used infrequently.

C. All staining dishes should be covered to prevent evaporation of reagents (screw-cap Coplin jars or glass lids).

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

E. When the smear is thoroughly fixed and the stain is performed correctly, the spores will be ovoid and refractile, with the spore wall being bright pinkish red. Occasionally, the polar tube can be seen either as a stripe or as a diagonal line across the spore. The majority of the bacteria and other debris will tend to stain blue. However, there will still be some bacteria and debris that will stain red. The coccidia (*Cryptosporidium, Cyclospora, Isospora*) will stain as with any modified acid-fast stain: from pink to violet (some *Cyclospora* may not take the stain – acid-fast variable).

F. The specimen is also checked for adherence to the slide (macroscopically).

G. 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 pasted on the body of the microscope). Although recalibration every 12 months may not be necessary, this will vary from laboratory to laboratory, depending on equipment care and use. Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not recommended on a yearly basis.

H. Known positive microscope slides, Kodachrome 2 x 2 projection slides, and photographs (reference books) should be available at the work station.

I. Record all QC results; the laboratory should also have an action plan for ``out of control'' results.

V. PROCEDURE

A. Using a 10-µl aliquot of concentrated (formalin ethyl-acetate sedimentation concentration; 500 X g for 10 min centrifugation), preserved liquid stool (5 or 10% formalin or SAF), prepare the smear by spreading the material over an area of 45 by 25 mm.

B. Allow the smear to air dry.

C. Place the smear in absolute methanol for 5 or 10 min.

D. Allow the smear to air dry.

E. Place in carbol-fuchsin solution for 10 min (no heat required).

F. Briefly rinse with tap water.

G. Decolorize with 0.5% acid-alcohol.

H. Briefly rinse with tap water.

I. Place in trichrome stain for 30 min at 37°C.

J. Rinse in acid-alcohol for no more than 10 s (1 to 3 s).

K. Briefly rinse; dip slides several times in 95% alcohol. Use this step as a rinse (no more than 10 s).

L. Place in 95% alcohol for 30 s.

M. Allow slides to air dry.

N. Examine smears under oil immersion (1,000 x) and read at least 100 fields; the examination time will probably be at least 10 min per slide.

VI. RESULTS

A. Microsporidia spores might be seen. The spore wall should stain pinkish to red, with the interior of the spore being clear or perhaps showing a horizontal or diagonal stripe that represents the polar tube. The background will appear blue (Ryan Stain). A vacuole may also be visible in some spores. The coccidian oocysts will stain bright pink or

violet. The background will appear green. If *Cyclospora* oocysts are present (uncommon), they tend to be approximately 10 µm, they resemble *C. parvum* but are larger, and they have no definite internal morphology; the acid-fast staining will tend to be more variable than that seen with *Cryptosporidium* or *Isospora* spp. Modified acid-fast stains stain the *Cyclospora* oocysts from light pink to deep red, some of which will contain granules or have a bubbly appearance, often being described as looking like "wrinkled cellophane."

B. Other bacteria, some yeast cells, and some debris will stain pink to red; the shapes and sizes of the various components may be helpful in differentiating the spores from other structures.

C. The results from this staining procedure should be reported only if the positive control smears are acceptable. The production of immunoassay reagents should provide a more specific and sensitive approach to the identification of the microsporidia in fecal specimens.

Postanalytical Considerations

VII. REPORTING RESULTS

A. Report the organism and stage (do not use abbreviations

Examples (Stool Specimens): Microsporidia spores present *Enterocytozoon bieneusi* or *Encephalitozoon (Septata) intestinalis* present (if from fecal specimen); the two organisms cannot be differentiated on the basis of size or morphology.

Example from urine: *Encephalitozoon (Septata) intestinalis* present (identification to species highly likely); generally this organism is involved in disseminated cases from GI tract to kidneys and will be found in urine.

Example from stool: *Cryptosporidium parvum* oocysts present.

(Dual Infection) Microsporidia spores present *Enterocytozoon bieneusi* or *Encephalitozoon (Septata) intestinalis* present (if from fecal specimen); the two organisms cannot be differentiated on the basis of size or morphology.

B. Quantitate the number of spores and oocysts seen (rare, few, moderate, many).

VIII. PROCEDURE NOTES

A. It is mandatory that positive control smears be stained and examined each time patient specimens are stained and examined.

B. Because of the difficulty in getting stain penetration through the spore wall, prepare thin smears and do not reduce the staining time in trichrome. Also, make sure the slides are not left too long in the decolorizing agent (acid-alcohol). If the control organisms are too light, leave them in the trichrome longer and shorten the time to two dips in the acid-alcohol solution. Also, remember that the 95% alcohol rinse after the acid-alcohol should be performed quickly to prevent additional destaining from the acid alcohol reagent.

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

D. Polyvinyl alcohol-preserved specimens are not acceptable for staining with the modified acid-fast stain. However, specimens preserved in SAF are perfectly acceptable.

E. Avoid the use of wet gauze filtration (an old, standardized method of filtering stool prior to centrifugation) with too many layers of gauze that may trap organisms and allow them to flow into the fluid to be concentrated. It is recommended that no more than two layers of woven (not pressed) gauze be used; another option is to use the commercially available concentrators that use no gauze but instead use plastic or metal screens.

F. Other organisms, such as acid-fast bacteria and some *Nocardia* spp., stain positive.

G. It is very important that smears not be too thick. Thick smears may not adequately destain.

H. Concentration of the specimen is essential for demonstrating organisms. The number of organisms seen in the specimens may vary from numerous to very few.

I. Because of their mucoid consistency, some specimens require treatment with 10% KOH. Add 10 drops of 10% KOH to the sediment, and vortex until homogeneous. Rinse with 10% formalin, and centrifuge (500 x *g* for 10 min). Without decanting supernatant, take 1 drop of the sediment and smear it thinly on a slide.

J. Commercial concentrators and reagents are available

K. Weak concentrations of sulfuric or hydrochloric acid (1.0 to 3.0%) are normally used. Stronger concentrations will remove too much stain.

L. There is some debate about whether organisms lose their abilities to take up the acid-fast stain after long-term storage in 10% formalin. Use of the hot modified acid-fast method might eliminate this problem.

M. Centrifuge specimens in capped tubes, and *wear gloves during all phases of specimen processing*.

IX. LIMITATIONS OF THE PROCEDURE

A. Although this staining method will stain the microsporidia, the range of stain intensity and the small size of the spores will cause some difficulty in identifying these organisms. Since this procedure will result in many other organisms or objects staining in stool specimens, differentiation of the microsporidia from surrounding material will still be very difficult. There also tends to be some slight size variation among the spores.

B. If the patient has severe watery diarrhea, there will be less artifact material in the stool to confuse with the microsporidial spores; however, if the stool is semiformed or formed, the amount of artifact material will be much greater; thus, the spores will be much harder to detect and identify. Also, remember that the number of spores will vary according to the stool consistency (the more diarrhetic, the more spores that will be present).

C. Those who developed some of these procedures feel that concentration procedures result in an actual loss of microsporidial spores; thus there is a strong recommendation to use unconcentrated, formalinized stool. However, there are no data indicating what centrifugation speeds, etc., were used in the study.

D. In the UCLA Clinical Microbiology Laboratory, we have generated data (unpublished) to indicate that centrifugation at 500 X g for 10 min increases dramatically the number of microsporidial spores available for staining (from the concentrate sediment). This is the same protocol we use for centrifugation of all stool specimens, regardless of the suspected organism.

E. Avoid the use of wet gauze filtration (an old, standardized method of filtering stool prior to centrifugation) with too many layers of gauze that may trap organisms and allow them to flow into the fluid to be concentrated. It is recommended that no more than two layers of gauze be used. Another option is to use the commercially available concentration systems that use metal or plastic screens for filtration.

F. Light infections (low number of oocysts) may be missed. Immunoassay methods for *Cryptosporidium parvum* are more sensitive.

G. Multiple specimens must 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.

REFERENCES

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APPENDIX

Acid-fast trichrome stain reagents available from Medical Chemical Corporation are as follows:

REAGENT	CATALOG NUMBER	SIZE AND CATALOG NUMBER	
Ryan modification of Trichrome-Blue	601A	601A	16 oz
Kinyoun Carbol Fuchsin	483A	483A-8oz 483A-1gl	8 oz 1 gallon
Acid Alcohol Decolorizer	311A	311A-8oz 311A-1gl	8 oz 1 gallon
Methylene Blue 1%	675A	675A-8oz 675A-1gl	8 oz 1 gallon
Brilliant Green 1%	460B	460A-8oz 460A-1gl	8oz 1 gallon
SED-CONNECT (Closed Concentration System)	693A	693A 693A-E	15 ml conc kit With Ethyl-acetate 50 kits/cs
PARA-SED (Closed Concentration System)	695A	695A	50 ml conc kit 50 kits/cs
MICRO-SED (Open Concentration System)	694A	694A 694A-E	15 ml conc kit With Ethyl-acetate 50 kits/cs
SAF Vials	574-05	574-05	10 vials/pk 100 vials/cs
Formalin 5% Vials	5753-05	5753-05	10 vials/pk 100 vials/cs
Formalin 10% Vials	575-05	575-05	10 vials/pk 100 vials/cs
Ethyl Acetate	4992	4992-16oz 4992-1gl	16 oz 1 gallon
95% Reagent Alcohol	3719A	3719A	1 gal
Reagent Alcohol 90% ethyl alcohol 5% methyl alcohol 5% isopropyl alcohol	374B	374B-16 oz 374B-1gal	16 oz 1 gal
Trichrome Decolorizer	3720A	3720A-32 oz 3720-A1 gal	32 oz 1 gal
Xylene	134B	134B-16 oz 134B-1 gal	16 oz 1 gal
Xylene Substitute (d-limonene)	930E	930E	1 gal