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Rapid Dimethyl Sulfoxide-Modified Acid-Fast Stain of *Cryptosporidium* Oocysts in Stool Specimens

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A rapid dimethyl sulfoxide modification of an acid-fast technique was applied to direct fecal smears to monitor cryptosporidiosis in nonhuman primates. Brilliantly stained pink oocysts against a pale green background demonstrated well-preserved internal morphology and facilitated rapid, simple, noninvasive diagnosis without fluorescent or phase-contrast microscopy.

Protozoa of the genus *Cryptosporidium* can cause severe, life-threatening enteritis in immunocompromised humans (4, 10, 12, 17); they can also cause diarrhea in immunocompetent humans (3, 5) and in several animal species (16). A recent outbreak of cryptosporidiosis in the primate colony at the Regional Primate Research Center, Seattle, Wash., led to a search for a single method that is noninvasive, rapid, and differential and that could be applied to a variety of extant preparations, as well as to fresh fecal specimens, and that would provide a permanent record for review and documentation.

Cryptosporidiosis has been diagnosed by identification of characteristic oocysts in sections stained with hematoxylin and eosin and by sugar cover slip flotation (15). Other methods have included phase-contrast microscopy (2); Giemsa-stained fecal smear (1); modified Ziehl-Neelson acid-fast stain (7, 9); a three-step procedure involving an iodine wet mount, acid-fast staining, and sugar flotation (11); and a nonpermanent negative stain technique with a Kinyoun carbol-fuchsin-feces emulsion overlaid with oil to demonstrate refractile oocysts by bright-field and phase-contrast microscopy (8). Auramine staining (7, 13), commonly used in clinical laboratories for identification of *Mycobacterium* spp., now is used widely to detect *Cryptosporidium* spp., but it is beyond the capacity of small laboratories equipped with only a bright-field microscope.

When more than 100 potential cases of cryptosporidiosis occurred over a short period of time, the direct saline or iodine wet mounts and classic flotation and sedimentation procedures were considered to be too time consuming and impractical for this small laboratory. Most specimens were from infant primates and thus were very small, leaving little or no pellet after sedimentation or providing only enough material for a smear. Several factors interfered with staining, including sugar from flotation, KOH digestion of mucus, the Formalin used to preserve the feces, polyvinyl alcohol fixative, and Schaudinn's fixative. Small numbers of oocysts were lost completely in flotation. We tried the dimethyl sulfoxide (DMSO) acid-fast technique (14) and found that this fast, simple, clean technique yielded excellent staining and identification of oocysts.

Solutions. Staining solutions were prepared by following the original recipe (14). For the carbol fuchsin-DMSO stain, 4 g of basic fuchsin crystals (Color Index no. 42500, certified, 99% dye content; Fisher Scientific Co., Fair Lawn, N.J.) was dissolved in 25 ml of 99% ethyl alcohol. Twelve grams of phenol crystals liquefied in a water bath (or 12 ml of liquefied phenol; Mallinckrodt, Inc., Paris, Ky.) was added and mixed well with a glass stirring rod. Then, 25 ml of glycerol, chemically pure, 25 ml of DMSO (Sigma Chemical Co., St. Louis, Mo.), and 75 ml of distilled water were added and mixed well. The solution was allowed to stand for 30 min and then filtered. The stain may be used immediately or kept indefinitely at room temperature in an amber glass bottle. For the decolorizer-counterstain solution, 220 ml of a 2% aqueous solution of malachite green (Color Index no. 42000, certified, 99% dye content; Harleco, Philadelphia, Pa.) was prepared; 30 ml of glacial acetic acid (99.5%) and 50 ml of glycerol, chemically pure, were added and mixed well. Filtration was unnecessary. This solution keeps indefinitely in a closed container at room temperature.

Procedure. Rectal swabs were collected in Culturettes (Marion Scientific Corp., Kansas City, Mo.). Fecal material was smeared over a 2.5- by 3.0-cm area of a clean, flamed-glass slide and air dried on a warming plate. The slides were prefixed in a Coplin jar of absolute methanol for 5 to 10 s, stained in carbol fuchsin-DMSO solution in a Coplin jar for 5 min, and rinsed individually in gently running tap water until excess solution no longer ran off each slide (10 to 30 s per slide). Slides were then placed in the decolorizer-counterstain for 1 min or until a green background appeared and then were rinsed individually under running tap water for 10 s, drained, blotted, and placed on a warming plate until thoroughly dry (5 or 10 min). A thin film of immersion oil was applied over each smear with an applicator stick. Slides were examined under bright-field low power (×10).

This procedure yielded oocysts that were brilliant pink to fuchsia against a pale green background. Organisms seen on low-power screening were checked under oil immersion at ×100 for the *Cryptosporidium*-typical internal vacuole and material clumped to one side of the 4- to 5-μm cyst. In contrast to the results of conventional acid-fast stains, the internal morphology of the resulting material in this study was well preserved.

Empty cyst walls (ghosts) continued to be shed during resolving infections but did not stain well. They appeared as an empty space of the typical size and shape of the cyst, with no remaining internal structures. Disintegrating partially acid-fast particles could be seen within some oocysts.

Other particles that could be confused with *Cryptosporidium* oocysts did not stain with the same clarity and brilliance. Yeast cells and leukocytes were not acid fast, and stained blue-green. Erythrocytes did not stain. Seeds, spores, and other vegetative cells that might have stained partially acid fast had a brown or black overcast or a distinctive rough geometric shell. Sperm capita were a pale, smooth, homogeneous pink and were smaller than cryptosporidial oocysts.

This DMSO method is versatile and widely applicable. It is a compatible overstain with previous Gram stains and methanol-decolorized Giemsa and Wright stains. Strict adherence to the times specified for direct thin fecal smears and mucus is not necessary. Long staining times (up to several hours in carbol fuchsin) have no detrimental effects. The superior penetrating qualities of DMSO added to carbol fuchsin eliminate the need for heat or steam and promote rapid staining. The milder acetic acid incorporated in the counterstain solution simplifies the decolorization-counterstain process. Stained smears may be dehydrated and mounted with a cover slip for permanent documentation (6).

Rapid diagnosis is facilitated by the DMSO method. The pink-green color contrast is easier to read than the pink-blue-purple range of the modified Kinyoun or Ziehl-Neelson methods. An experienced technologist can screen a smear on low power (×10) and easily identify the pink oocysts. By this method, 160 simians were screened and 69 cases of cryptosporidiosis were confirmed; one case in human personnel was also confirmed. Positive fecal specimens obtained by this method have correlated well with onset and demise of clinical symptoms in natural and experimental infections in nonhuman primates.

For comparison, an independent laboratory stained 133 duplicate slides with auramine. The DMSO method demonstrated 34 cases of cryptosporidiosis (25.4%), and auramine yielded 38 (28.6%). The 3% that were positive by auramine and negative by DMSO–acid-fast bacillus all contained fewer than 10 oocysts per 2.5- by 3.0-cm smear. The results suggest that the auramine stain may be slightly more sensitive for the detection of very small numbers of oocysts. This may indicate simply that it is easier for the human eye to recognize a fluorescence-flagged spot. Uneven or infrequent distribution of oocysts could also account for a 3% difference, and multiple specimens would be indicated by clinical evaluation.

From these results it is concluded that the DMSO method is as reliable and consistent in detecting cryptosporidiosis as more complicated procedures, and it offers two important advantages. (i) It simplifies diagnosis and hastens initiation of supportive therapy, and (ii) it reduces the health risks inherent in prolonged handling of contaminated material.

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