Auramine-Phenol vs. Modified Kinyoun’s Acid-Fast Stains for Detection of Coccidia Parasites
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ABSTRACT

Objective: Auramine-phenol stain was compared with Kinyoun’s acid-fast stain to detect coccidia parasites in fecal samples from immunocompromised patients. The comparison was based on the number of detected cases, sensitivity, specificity, time required for the procedure, ease of use, interpretation, and cost.

Methods: A total of 112 fecal specimens were examined by conventional methods: Direct wet saline smear, iodine smear, and formol ether sedimentation technique. Duplicate smears of the fecal concentrates were stained by both procedures.

Results: Kinyoun's and auramine-phenol stains detected 22 and 24 positive coccidia specimens respectively. The control group (27 immunocompetent relatives) showed a high incidence of Giardia lamblia infection. Kinyoun smears were difficult to interpret, while auramine smears were extremely easy to read, thus requiring less time. Artifacts were readily recognizable. The cost of auramine-phenol reagents was much higher than Kinyoun's acid-fast stain.

Conclusion: Although the overall ranking of both staining techniques was high, the auramine-phenol stain was a more desirable test despite its higher cost.

Keywords: coccidia, feces, auramine-phenol, Kinyoun, interpretation, cost

The increased number of documented human coccidia infections that are often indistinguishable from other forms of community-acquired diarrhea, together with the possibility of treating some of them, suggests a need for proper diagnostic techniques to recover and identify the organism.¹⁴

Human intestinal coccidia, including Cryptosporidium parvum (C. parvum), C. cayetanensis (C. cayetanensis), Isospora belli, and Sarcocystis spp., are significant causes of gastrointestinal symptoms. Most of these parasites cause major problems in the presence of acquired immunodeficiency syndrome (AIDS) and other causes of immunodeficiency. These organisms also cause disease in immunocompetent individuals.¹²

These coccidia are intracellular cyst-forming apicomplexan protozoa that predominantly infect intestinal epithelial cells. Most are transmitted by the fecal-oral route or via contaminated water or food, although Sarcocystis spp is transmitted by improperly cooked meat. Infection is associated with intestinal inflammation, pathological lesions such as villus blunting, and abnormal function such as malabsorption. Mild to moderate self-limiting diarrhea is common in healthy individuals during the infective stage of these organisms; however, asymptomatic infection can occur. Patients with immune dysfunction can have severe intestinal injury, prolonged diarrhea, extreme weight loss, and generalized wasting.²⁵,⁶

Both C. parvum and Sarcocystis spp are zoonotic coccidia. Their oocysts are immediately infective. Ingesting cryptosporidial oocysts can lead to intestinal cryptosporidiosis. However, the lifecycle of Sarcocystis spp requires an alternating infection between intermediate hosts—cattle or pigs—and definitive hosts, which can be carnivores or omnivores, including humans.

Ingesting Sarcocystis spp oocysts causes cysts to form in muscles. Intestinal Sarcocystis spp. occurs after ingesting these cysts in raw or undercooked beef or pork. C. cayetanensis and Isospora belli infect humans only when their oocysts are mature. Both infections are acquired by

Abbreviations
C. parvum, Cryptosporidium parvum; C. cayetanensis, Cyclospora cayetanensis; AIDS, acquired immunodeficiency syndrome

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ingesting oocysts, which require moisture and moderate temperature to sporulate and become infective.\textsuperscript{2-4}

Fluid replacement and supportive care are essential in the treatment of coccidial diarrhea. Nitazoxanide has been used for treatment of diarrhea caused by \textit{C. parvum} in people with healthy immune systems. However, its effectiveness in immunosuppressed individuals is unclear. Moreover, atovaquone, azithromycin, and metronidazole often help for a short time, but the infection usually returns.\textsuperscript{7} Cyclosporiasis can be treated successfully with a 7-day course of oral trimethoprim-sulfamethoxazole in both immunocompetent and immunosuppressed patients.\textsuperscript{8} Isosporiasis responds to initial therapy with trimethoprim-sulfamethoxazole, followed by prophylaxis with pyrimethamine.\textsuperscript{9} For intestinal sarcocystosis, there is no known prophylaxis or effective therapy. The efficacy of co-trimoxazole or furazolidone and albendazole in sarcocystosis has not been demonstrated.\textsuperscript{10}

This study was undertaken to compare auramine-phenol stain with Kinyoun’s acid-fast stain to detect coccidia parasites in human fecal samples. The comparison included the number of detected cases, the sensitivity, the specificity, the average time required for the technique including the procedure and the screening time, the ease of performance and interpretation, and the cost.

Materials and Methods

Specimens

Fecal samples were collected from 112 immunocompromised patients (37 females and 75 males), ranging in age from 2 to 50 years, from different departments of Alexandria University hospitals from July to September 2011. The majority of these patients suffered from malignant diseases such as lymphoma and leukemia (67 patients) and the rest had renal failure. Among the 112 patients, 71 had diarrhea.

Controls

Family members of patients with the same environmental background who were immunocompetent and had diarrhea were chosen as controls. We collected fecal samples from 27 such subjects. The study was approved by the Alexandria University ethical committee, and informed consent was obtained from all participants prior to enrollment in the study.

Fecal samples were collected in wide-mouth disposable containers. If they were not processed immediately, the samples were preserved in 2.5% potassium dichromate at 4°C. The samples were divided into 2 parts. The first part was subjected to a direct wet saline smear and iodine smear. With the help of an applicator stick, fecal samples were emulsified in a drop of saline on a clean dry slide and in a drop of Lugol’s iodine on another slide. They were covered with cover slips and observed under the microscope at ×400 magnification to detect ova and cysts.\textsuperscript{11}

The second aliquot of each sample was concentrated by a formalin ethyl acetate sedimentation technique. One gram of each fecal specimen was emulsified in 10% formalin in water. An additional 4 mL of 10% formalin was added and the mixture was passed through double layered gauze into calibrated centrifuge tubes. Then, 4 mL of ethyl acetate was added. The tubes were stoppered, shaken vigorously for 30 seconds, and centrifuged at 3000 rpm for 1 minute. There were 4 layers; sediment was at the bottom. The supernatant was discarded and the sediment was screened microscopically.\textsuperscript{11}

Stains

Thin smears of the fecal concentrates prepared with formalin ethyl acetate were air dried on 2 different slides. The first slide was stained with Kinyoun’s acid-fast stain (Sigma-Aldrich, St. Louis, MO, USA) and the second was stained by auramine-phenol stain (Sigma-Aldrich, St. Louis, MO, USA).

\textbf{Kinyoun’s Acid-Fast Stain}

The smear was fixed with absolute methanol for 1 minute and stained with Kinyoun’s carbol fuchsin for 5 minutes. Then, it was rinsed briefly (3 to 5 seconds) with 50% ethanol and immediately rinsed with tap water. Stained mounts were decolorized with 1% sulfuric acid for 2 minutes. The slides were rinsed thoroughly with water and counterstained by methylene blue for 1 minute. Slides were rinsed with water and allowed to air dry. Stained slides were initially scanned at a magnification of ×100 and ×400, with confirmation at a magnification of ×1000 using Leitz Dialux HM Lux-3 light microscope (Ernst Leitz, Rockleigh, N.J.).\textsuperscript{12-16}

\textbf{Auramine-Phenol Stain}

Smears were stained with auramine-phenol for 10 minutes. The smears were then rinsed with water and
decolorized with 3% acid alcohol (0.5% HCl in 70% ethanol) for 2 minutes. Slides were then rinsed in tap water and counterstained with 0.1% aqueous potassium permanganate for 2 minutes. Then, slides were rinsed with tap water and air dried. Stained slides were initially scanned at a magnification of ×100 and ×400, with confirmation at a magnification of ×1000 using Zeiss HBO 50 fluorescent microscope (Carl Zeiss, New York, NY).15–17

Positive and negative control materials were processed with each batch of stained patient slides. For both staining methods, at least 100 fields were examined under oil immersion for each slide. The detected parasites were measured by an ocular micrometer calibrated with a stage micrometer slide.18

Quality Control
A control slide of *C. parvum* from a 10% formalin preserved specimen was included with each staining batch and both staining techniques. If the *Cryptosporidium* stains well, it was assumed that any other coccidia would also take up the stain.

To avoid exposure to the toxic effects of phenol, great care and safety measures were applied during the preparation of both staining techniques, owing to the presence of phenol crystals as a reagent in both stains.16,19

The 2 methods were assessed and compared for their ability to detect coccidia parasites in fecal specimens. Since there is not a reference standard for detecting these parasites, a specimen was considered to be truly positive or truly negative when concordant results were obtained with Kinyoun’s acid-fast and Auramine-phenol methods, or agreed upon by 2 observers in a single slide, or when found on different slides of the same sample. If discrepant results were obtained, testing was repeated. Only the original histological result was used in calculating the performance characteristics of the methods.

For the comparative study of the diagnostic tests the relevant parameters were: number of detected cases, sensitivity, specificity, average time required for the technique (including the procedure and the screening time), ease of performance and interpretation, and cost.14,15 Ease of performance and interpretation were subject to an evaluation based on the number of steps in each staining technique and the ease of getting positive and negative results. The cost included the reagent costs; we did not include the cost of any equipment such as a fluorescent microscope.14,15

The diagnostic procedures were evaluated and ranked based on the Multiattribute utility theory and analytical hierarchy process, which identifies, characterizes, and combines different parameters to rank diagnostic tests in any particular setting. Each test was compared by using a linear ranking scale from 1 to 6 for each parameter (1 was used for the least preferable characteristic and 6 for the most preferred one).15,20

Statistics
The percentage of positive cases detected by the Kinyoun’s acid-fast stain and the auramine-phenol fluorescent stain were calculated. Furthermore, sensitivity, specificity, negative predictive value, and positive predictive value of both techniques were calculated for in accordance with the procedure described by Knapp and Miller.21

Results
Out of the 112 fecal samples collected, 38 were positive for intestinal parasites. Coccidia parasites were detected in 24 specimens, *Cryptosporidium* spp was detected in 17 samples (15.17%), followed by 4 samples with *Cyclospora* spp (3.57%), 2 samples with *Sarcocystis* spp (1.78%), and 1 sample with *Isospora belli* (0.89%). Both Kinyoun’s acid-fast and Auramine-phenol stains identified 22 of the coccidia specimens; 2 were identified as positive with the Auramine-phenol fluorescent stain (1 case of *Sarcocystis* spp and 1 case of *Isospora belli*). Repeated Kinyoun’s acid-fast stain of both specimens and measuring with the ocular micrometer resulted in detecting organisms in 1 specimen (*Isospora belli*). Both of these specimens were considered false negative by Kinyoun’s acid-fast stain results. Sensitivity, specificity, negative predictive value, and positive predictive value were calculated for each method on the basis of the results of the first test applied to the specimen (Table 1).

Other intestinal parasites were diagnosed by microscopic examination of concentrated specimens from 14 samples: *Giardia lamblia* (n=7); *Giardia lamblia*, *Iodamoeba butschili* and *Blastocystis hominis* (n=3); *Giardia lamblia* and *Blastocystis hominis* (n=2); *Ascaris egg* (n=1) and *Hymenolepis nana egg* (n=1). Thus, the total number of negative specimens was 74.

Samples collected from the control group showed a high prevalence of *Giardia lamblia*, which was detected in 4
controls (14.8%), followed by 2 cases of Cryptosporidium spp (7%) and 1 case of Hymenolepis nana egg (3%). In the quality control by Kinyoun’s acid-fast stain, Cryptosporidium oocysts stained pink-red. They were 4-6 µm in diameter. Four sporozoites were sometimes visible internally. The background stained uniformly blue. With auramine-phenol stain, the stained Cryptosporidium spp fluoresced yellowish-green against a dark background. The internal structures and the staining of the oocyst wall varied according to each type of coccidia. In contrast, with the auramine-phenol fluorescent stain, the organisms appeared yellowish-green against a dark background with staining of the oocyst wall under fluorescence microscopy. There was no difference in the size of each parasite by both stains.

Cryptosporidium spp oocysts in fresh smears were round and 4-6 µm in diameter (Image 1). With Kinyoun’s acid-fast stain, the oocysts were similar in size and shape to those detected in the fresh smear. Some of the 4

<table>
<thead>
<tr>
<th>Method</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Positive Predictive Value</th>
<th>Negative Predictive Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kinyoun</td>
<td>91.6%</td>
<td>100%</td>
<td>100%</td>
<td>97.3%</td>
</tr>
<tr>
<td>Auramine-phenol</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

*aCalculated as follows: (number of true positives/number of true positives + number of false negatives) ×100.

*bCalculated as follows: (number of true negatives/number of true negatives + number of false positives) ×100.

*cCalculated as follows: (number of true positives/number of true positives + number of false positives) ×100.

*dCalculated as follows: (number of true negatives/number of true negatives + number of false negatives) ×100.

Table 1. Sensitivity, Specificity, and Predictive Values for Kinyoun and Auramine-Phenol Methods

Image 1
Fresh smear shows round oocysts of Cryptosporidium spp, 4-6 µm in diameter (×400).

Image 2
Kinyoun’s acid-fast stain shows oocysts of Cryptosporidium spp, 4-6 µm with 4 dots (×1000).
sporozoites were visible as dots inside the oocysts, but usually they were difficult to see (Image 2). Stained Cryptosporidium spp oocysts fluoresced brightly by Auramine-phenol, and were easily differentiated from other fecal contents by their uniform small size and morphology (Image 3).

Cyclospora oocysts in fresh smears were round and approximately 8-10 µm in diameter (Image 4). Kinyoun’s acid-fast staining of the oocysts showed a similar size and shape with a fresh smear. However, great variability in the staining pattern was noticeable. Many oocysts were stained deep red with a mottled appearance, but the internal structures weren’t visible. Some were stained pink, some were faint in color, and others remained unstained and appeared as glassy membranous cysts (Image 5). When auramine-phenol was used, the parasite appeared round with an apple green fluorescence, consisting of

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**Image 3**

Auramine-phenol stain shows fluorescent oocyst of Cryptosporidium spp with dots (×1000).

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**Image 4**

Fresh smear shows round oocyst of Cyclospora cayetanensis, 8-10 µm in diameter (×1000).

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**Image 5**

Kinyoun’s acid-fast stain shows oocyst of Cyclospora cayetanensis, 8-10 µm with ill-defined contents (×1000).
a single ill-defined mass or several globular structures (Image 6).

In fresh smears, a few oval oocysts of *Sarcocystis* spp with round edges containing 2 sporocysts were seen. A large number of free sporocysts were also detected. The free sporocysts were 7.5-9.5 µm in diameter, and were fairly round (Image 7). Using Kinyoun’s acid-fast stain, the Sarcocystis sporocysts were also 7.5-9.5 µm in diameter, mature, and round. They contained 4 well-defined crescent-shaped sporozoites and a prominent residual body. The cyst walls remained unstained and many empty sporocysts were detected (Image 8). With the auramine-phenol stain, the sporocyst wall fluoresced brightly. The internal structures appeared as 4 falciform fluorescent sporozoites with a residual body (Image 9).
Isospora oocysts were ellipsoidal and 23-33 µm × 12-15 µm in diameter. With Kinyoun’s acid-fast stain, oocysts were pink to purple with stained sporoblast and oocyst walls (Image 10), and they revealed intense fluorescence with the auramine-phenol stain (Image 11).

In our laboratory, the preparation of the slide and the performance of Kinyoun’s acid-fast stain procedure required about 7 minutes, but did not exceed 10 minutes. Screening and interpretation required an additional 5 to 6 minutes per slide. The auramine-phenol stain procedure required a total time of 15 to 17 minutes. Interpretation of the fluorescent smears was straightforward and required less than 1 minute per specimen. Both stains were easy to perform and had few steps.
The ease of interpretation varied with each assay. The Kinyoun's acid-fast stained smears were difficult to interpret, requiring examination of frequent fields at ×1000 oil magnification to identify the organisms. Misdiagnosis due to artifacts commonly found in fecal samples was possible. The auramine-phenol stain was extremely easy to read as fluorescent organisms could be detected at ×100, with confirmation at a magnification of ×400, thus requiring much less time. However, characterizing the morphology required ×1000 oil magnification. Artifacts were recognizable by their often irregular shape and homogenous staining without any defined internal structures.

The auramine-phenol required a fluorescent microscope, while the Kinyoun's acid-fast did not. The Kinyoun's carbol-fuchsin reagent was approximately $25 for 140 fecal samples, while the auramine-phenol stain was $79.16 for the same number of fecal samples.

Each method was ranked for the number of detected cases, sensitivity, specificity, average time required for the procedure and screening, ease of performance and interpretation, and cost. The results are listed in Table 2.

### Discussion

Human intestinal coccidians are easy to acquire and difficult to treat, and are an increasing problem in immunodeficient patients. These parasites also cause disease in immunocompetent patients.22

Detecting these protozoa is a diagnostic challenge. Beginning from examining a small bowel biopsy material to different staining techniques and their modifications, several methods have been described. Many of these techniques are cumbersome and time consuming. Therefore, rapid and sensitive techniques are needed to give an early diagnosis of these protozoal infections because the results can influence therapeutic intervention.23

Despite the development of various detection methods over the past few years, diagnosis of intestinal parasites still depends on microscopic examination of fresh, unpreserved fecal specimens.24 Most laboratories diagnose intestinal parasites with the combination of a formalin-ethyl acetate concentrate and a trichrome stain. These techniques do not adequately detect coccidia organisms, so special staining techniques are needed.1,25

Kinyoun's acid fast stain is used to detect the developmental stages of all coccidia organisms. Their size, together with their overall morphological features, permits differentiation of these organisms.4,8,26 Other detection methods appear to be more sensitive, such as antigen detection assays and nucleic acid amplification methods. However, these methods are expensive.27

Acid-fast staining is a physical property of certain bacteria, and less commonly, protozoa. This stain distinguishes organisms with waxy cell walls which repel stain, and once stained, resist decolorization with acid-alcohol.28,29

Auramine-phenol is a fluorescent dye used in the microscopic examination of acid-fast microorganisms. In mycobacteriology, auramine-phenol has mostly replaced Kinyoun's acid fast stain because of the ease of interpretation.27 However, Kinyoun's acid fast stain was considered the best method for diagnosing coccidia in fecal samples, as auramine-phenol stain has been reported to yield ambiguous results when used to screen for coccidia.17

In this study, auramine-phenol stain was compared with Kinyoun's acid-fast stain for detecting coccidia parasites in human fecal samples. Ranking of tests for each attribute studied allowed for a simple comparative evaluation of methods.15

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**Table 2. Ranking of Modified Kinyoun's Acid-Fast Stain and Auramine-Phenol Methods in the Detection of Coccidia Parasites**

<table>
<thead>
<tr>
<th>Methods</th>
<th>Number of Detected Cases</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Time Required for the Procedure (min)</th>
<th>Time Required for the Screening (min)</th>
<th>Ease of Performance</th>
<th>Ease of Interpretation</th>
<th>Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modified Kinyoun's acid-fast method</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>3</td>
<td>5</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Auramine-phenol method</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>4</td>
<td>6</td>
<td>5</td>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>

1 meaning for the least preferable characteristic and 6 for the most preferred one.28
By both techniques, Cryptosporidium spp (15.17%) was the most commonly isolated protozoa, followed by Cyclospora spp (3.57%), and then Sarcocystis spp (1.78%). The least detected coccidian was Isospora belli (0.89%). Similar results were obtained by Abou El-Naga and colleagues, 1998, Rezk and colleagues, 2001, and Hanscheid and colleagues, 2008. However, Sarcocystis spp was not detected as often as the other coccidia parasites, and was not detected at all in some studies. With the increasing incidence of immune suppression, and the appearance of intestinal sarcocystosis, we may consider intestinal sarcocystosis as an emerging zoonotic disease.

In this study, Kinyoun’s acid-fast staining produced somewhat lower recovery of organisms when compared with the auramine-phenol stain. This could be attributed to the similarity of oocysts of Isospora belli or Sarcocystis spp to certain stool artifacts as pollen grains. Isospora belli oocysts can be differentiated from fecal artifacts based on their size. In this study, these measurements helped to differentiate between the coccidia oocysts and artifacts. Measurement of the parasites was also advised by Awadalla and colleagues, 1995, and Abou El-Naga and colleagues, 1998. In addition, in our study Kinyoun’s acid-fast stain showed great variability in the staining pattern of Cyclospora oocysts, and some of the oocysts remained unstained. This may result in misidentification especially in mild coccidial infections. This was also reported by Negm, 1998, and Abou El-Naga, 1999. The present results showed that by auramine-phenol stain, Cyclospora spp was homogenously stained. Furthermore, the Sarcocystis sporocyst wall could not be easily visualized with Kinyoun’s acid-fast stain fluoresced by auramine-phenol stain, in addition to the evident appearance of its internal structures. Also, this fluorescent stain could easily differentiate the artifacts from the coccidial parasites, thus yielding better results than Kinyoun’s acid-fast stain. The advantage of the auramine-phenol over Kinyoun’s acid-fast stain was reported by Abou El-Naga and colleagues in 1998, and by Hanscheid and colleagues in 2008. Since the sensitivity and specificity of both staining methods were comparable, the choice of method must be based on other criteria. The time required for the performance of each stain procedure and for the screening and interpretation of each slide was calculated; they showed more or less equal time with the 2 staining techniques we used. Furthermore, both stains were easy to perform and had few steps. However, the ease of interpretation varied considerably between the 2 methods. Kinyoun’s acid-fast stained smears were difficult to interpret, requiring frequent examination at oil magnification to identify the organisms, which was tedious and time consuming, whereas detection and identification of the coccidia parasites with the auramine-phenol test was easy and rapid. By this technique, the parasite could be detected at ×100 magnification and could be easily identified at ×400 magnification, thus requiring much less interpretive time. However, the morphology required ×1000 magnification under oil immersion. The ease of this test was noted by Kehl and colleagues in 1995, Ash and Orihel in 1997, and by Mansfield and Gajadhar in 2004, all of whom concluded that the difficulty of interpreting Kinyoun’s acid-fast stain smears made the fluorescent stain a more desirable test.

The reagent cost of the Kinyoun’s acid-fast stain was less than the auramine-phenol stain. Also, the auramine-phenol required a fluorescent microscope. MacPherson and McQueen (1993), and Kehl and colleagues (1995) also observed that the cost of fluorescent staining and the availability of the fluorescent microscope are major obstacles to the use of the auramine-phenol stain. If fluorescent microscopy resources are not available, acid-fast staining as Kinyoun’s stain should be used for all diarrheal stool samples when coccidial infection is suspected.

Conclusion

We concluded that auramine-phenol stain was better and more rapid than Kinyoun’s acid-fast stain for identifying coccidia parasites. Although the overall ranking of both staining techniques was high, the auramine-phenol was a more desirable test despite its higher cost. LM

References

Available in the full-length version of this article, which can be accessed at http://labmed.ascpjournals.org/content/45/1.toc.