Detection of Helminth Ova and Larvae in Trichrome-Stained Stool Smears

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The detection and identification of intestinal helminths were studied retrospectively by comparing the Formalin concentration technique with the trichrome-stained smear technique. A total of 3,997 stool samples from 1,570 patients were examined by both methods. Of the 3,997 samples, 31% (1,239 of 3,997) contained helminths or protozoans or both. A total of 11% (434 of 3,997) of the samples representing 14% (221 of 1,570) of the patients were positive for one or more helminth species. A total of 570 separate identifications of helminth ova/larvae were made. Among the helminth ova/larvae identified, 14.6% (83 of 570) were detected only in the trichrome-stained smear, representing 6.3% (14 of 221) of the patients. From these data, it can be concluded that unless a diligent search of the stained smear for helminths is made, a significant number of helminth infections may be missed. It is, therefore, recommended that stained stool smears be used to aid the detection of helminth ova/larvae in conjunction with Formalin concentration. The appearance of the most common helminth ova/larvae in trichrome-stained smears is described, along with specific characteristics that may be used for their identification. In addition, the advantages and disadvantages of the trichrome staining technique for helminth identification are discussed.

The diagnosis of intestinal parasitic infections requires three basic types of procedures for optimal recovery and detection. These include the direct wet preparation of a fresh stool sample, the preparation of a concentrate of a Formalin-preserved specimen, and the preparation of a stained smear of a polyvinyl alcohol (PVA)-preserved specimen. The uses and limitations of each technique have been delineated in previous publications (1, 2, 5, 8, 9). A Formalin-ethyl acetate concentration (12) can be performed on a Formalin-preserved specimen, and a smear of a PVA-preserved specimen can be stained by the Wheatley (11) modification of the Gomori trichrome stain technique (6). Both of these techniques are widely used and allow the greatest recovery of intestinal parasites (2, 8).

It is well known that the Formalin concentration technique is the most effective procedure for the recovery and identification of helminth ova/larvae (1, 2, 8–10) and that the preparation of a trichrome-stained smear is mandatory for the detection of all protozoan trophozoites (3, 5). It has also been reported that helminth ova/larvae can be seen in trichrome-stained smears (2, 4, 7, 8). In this laboratory the observation was made that a routine search of the stained smears for helminths revealed that a significant number of helminth ova/larvae that were missed in the wet preparation of the concentrate of the same specimen were detected. Based on that observation, a retrospective study was conducted to compare the recovery of helminth ova/larvae by the Formalin concentration and the trichrome-stained smear techniques. The first purpose of this discussion is to elucidate the results of this study, and the second purpose is to demonstrate how helminth ova and larvae may be recognized and identified from trichrome-stained smears and used in conjunction with the Formalin concentrate to increase the detection rate of helminths in specimens from patients.

MATERIALS AND METHODS

The retrospective analysis of stool specimens was performed between 11 February 1980 and 5 May 1981 at the medical microbiology laboratory of the University of California Irvine Medical Center, Orange, where a large population of immigrants is seen. Specimens were collected by the two-vial method (8). Outpatients or nurses on the wards were instructed to emulsify one portion of stool in the vial containing PVA fixative and another portion in the vial containing 10% Formalin. When received, specimens were processed by modifications of the Formalin-ethyl acetate concentration technique (12) and the trichrome-stained smear technique as follows.
TABLE 1. Helminth species identified in individual stool specimens by the Formalin concentration technique, stained smear technique, or both

<table>
<thead>
<tr>
<th>Helminth</th>
<th>Concentrate only</th>
<th>Concentrate and stained smear</th>
<th>Stained smear only</th>
<th>Total no. identified</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. lumbricoides</em></td>
<td>40 (41.7)</td>
<td>49 (51.0)</td>
<td>7 (7.3)</td>
<td>96 (16.9)</td>
</tr>
<tr>
<td><em>S. stercoralis</em></td>
<td>23 (41.1)</td>
<td>24 (42.8)</td>
<td>9 (16.1)</td>
<td>56 (9.8)</td>
</tr>
<tr>
<td>Hookworm</td>
<td>91 (46.0)</td>
<td>92 (46.4)</td>
<td>15 (7.6)</td>
<td>198 (34.7)</td>
</tr>
<tr>
<td><em>T. trichiura</em></td>
<td>45 (38.8)</td>
<td>43 (37.1)</td>
<td>28 (24.1)</td>
<td>116 (20.4)</td>
</tr>
<tr>
<td><em>Hymenolepis</em> sp.</td>
<td>13 (25.0)</td>
<td>30 (57.7)</td>
<td>9 (17.3)</td>
<td>52 (9.1)</td>
</tr>
<tr>
<td><em>C. sinensis</em></td>
<td>6 (19.4)</td>
<td>17 (54.8)</td>
<td>8 (25.8)</td>
<td>31 (5.4)</td>
</tr>
<tr>
<td><em>Taenia</em> sp.</td>
<td>2 (11.1)</td>
<td>11 (61.1)</td>
<td>5 (27.8)</td>
<td>18 (3.2)</td>
</tr>
<tr>
<td><em>Enterobius</em> vermicularis</td>
<td>1 (33.3)</td>
<td>0 (0)</td>
<td>2 (66.7)</td>
<td>3 (0.5)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>221 (38.8)</td>
<td>266 (46.7)</td>
<td>83 (14.6)</td>
<td>570 (100)</td>
</tr>
</tbody>
</table>

* The total number seen in the concentrate was 487 (85.4%); this number represents the sum of the species seen in the concentrate only plus those seen in both the concentrate and the stained smear. The total number seen in the stained smear was 349 (61.2%); this number represents the sum of the species seen in both the concentrate and the stained smear plus the stained smear only.

For concentrate preparation, 2 ml of emulsified Formalin-preserved stool was filtered through a double layer of gauze into a 15-ml conical centrifuge tube. Formalin (10%) and 2 ml of ethyl acetate were added to the filtrates to make a total volume of 12 ml. The tubes were capped, agitated vigorously for 30 s, centrifuged (1,150 x g) for 2 min at room temperature, and carefully decanted. One drop of each concentrate sediment was combined on a slide with one drop of diluted D’Antoni iodine. The entire surface area of a cover slip (22 by 40 mm) was examined microscopically under low-power magnification (100 x), and subsequently, a random examination was made with 400 x magnification.

For the trichrome-stained smears, 2 ml of stool emulsified in PVA was centrifuged (1,150 x g) for 2 min at room temperature. This step served to pellet the larger fecal debris to the bottom of the tube, as well as to remove excess liquid, thereby concentrating the specimen. The supernatant material was decanted, and smears were made from the top layer of fecal sediment (8). A wooden applicator stick was used to procure a small amount of fecal material and gently rotate it onto the surface of a glass slide. The material was spread or rolled across the slide in one direction and with minimal stroking, until the desired thickness of the smear was achieved, as diagrammed by Markell and Voge (7). When thoroughly dry, these smears were stained by the Wheatley modification of the trichrome method (11), mounted on cover slips immediately after removal from the xylene clearing agent, allowed to dry, and examined microscopically (2, 8). The entire surface of each cover slip (22 by 40 mm) was examined under low-power magnification (10 x ocular and 10 x objective). Suspicious forms were studied in more detail with 400 x magnification and oil immersion (1,000 x) magnification. The search for protozoans was always conducted with an oil immersion objective.

Examinations of the trichrome-stained smears and the concentrate sediments were performed daily by technologists working in the parasitology laboratory. The two preparations of each specimen were usually examined independently by different technologists and read at random, the concentrates not necessarily being read before the stained smears.

The data generated in this retrospective study were treated in two ways. First, the individual stool specimens were treated as separate, distinct laboratory examinations, disregarding the fact that many were multiple specimens from the same patients. Second, the data were analyzed with respect to detection of intestinal helminths in individual patients. The treatment of the data for the individual patients was complex since various numbers of specimens were submitted and patients were infected with varying numbers of helminths which may have been detected by one or both of the methods being compared here. For these reasons, the helminths identified in these patients were tabulated as to the method by which they were first detected, although in some patients from whom more than one specimen was obtained, the helminth(s) may have been detected in subsequent specimens by the other method.

**RESULTS**

Data analysis. A total of 3,997 stool specimens were examined between 11 February 1980 and 6 May 1981. Of these, 31% (1,239 of 3,997) were positive for one or more parasites, either helminths or protozoans or both. Since protozoan detection is not pertinent to this discussion, no further mention of them will be made. Table 1 shows the data obtained when each specimen was treated independently and the data for the detection of each helminth genus recovered by the two methods. A total of 570 separate helminth identifications were made among the positive specimens. It can be seen that in 61.2% (349 of 570) of the identifications, helminth ova/larvae were detected in the stained smear, although the concentrate was positive in 85.4%
(487 of 570). It is interesting to note that in 14.6% (83 of 570) of the identifications, the helminth ova/larvae were missed in the concentrate and yet were found and identified in the stained smear. Of the individual genera, hookworm ova, *Trichuris* ova, and *Ascaris* ova were the most commonly encountered, and of these, *Trichuris* ova were most often missed in the concentrate and seen in the stained smear. *Clonorchis* and *Taenia* ova were also more frequently seen in the stained smear than in the concentrate.

The data were then analyzed as they applied to individual patients. Among the 1,570 patients from whom one or more specimens were obtained, 14.1% (221 of 1,570) were positive for one or more helminth species. In 156 patients, ova/larvae of only one helminth species were found, 48 had two helminth species, 15 had three helminth species, and 2 were infected with four helminth species. No patient was found to have more than four helminth species. A total of 305 helminth identifications were made with the combination of the Formalin concentration technique and the trichrome-stained smear technique. Table 2 summarizes the 305 helminth ova/larvae identified in the 221 patients, separated into species and the method by which they were first detected in each patient. From these data, it can be seen that nearly half (137 or 44.9%) of the helminths were first identified in the concentrate only but that 51 or 16.7% of the helminths were identified in the stained smear only and not seen in the corresponding concentrate. This percentage is close to the 14.6% obtained for the same category when the specimens were treated independently. The remaining 38.4% (117/305) of the helminths were first seen in both the concentrate and the stained smear. Of the 51 ova/larvae first identified in the stained smear only, 35 were seen in the concentrate in subsequently submitted specimens. However, 16 ova/larvae were seen only in the stained smear in 14 patients (2 patients had two helminth species and 12 patients had only one helminth species). In an additional 13 of 65 patients who were infected with more than one helminth species, at least one helminth species was identified in the stained smear only, although other species were seen in the concentrate. Therefore, in 12.2% (27/221) of the patients found to be positive for helminth ova/larvae, at least one of the helminths was identified in the trichrome-stained smear only, and finally, in 6.3% (14/221) of the patients harboring only one helminth species, that helminth was identified in the trichrome-stained smear only.

**Morphological characteristics.** Following are descriptions of the appearance of the helminth ova/larvae as they were found in trichrome-stained stool smears. One of the hallmarks of the trichrome stain is the variety of colors displayed. We found that with the exception of *Ascaris* ova, the colors of the helminth ova/larvae described below were consistent from one preparation to the next. *Ascaris* ova displayed a variety of colors but were consistent morphologically, and color variation did not prevent their recognition. Other helminth ova with thicker shells, *Trichuris* and *Taenia*, maintained their shape and size after the staining procedure as did *Ascaris* ova. *Clonorchis* ova were more delicate and sometimes folded slightly, but this distortion was minimal. The more thin-shelled helminths and the larvae tended to shrink, distort, or collapse but consistently stained pink or red. This red-staining characteristic, plus a clear zone or halo which was present around some and represents the space left by shrinkage or the collapsed shell, allowed them to stand out and be detectable under low-power magnification. Sometimes the cysts of *Entamoeba coli*, having a wall similar in thickness to a thin-walled helminth egg, did not preserve well in the PVA-fixative and stained red, with a clear zone around them. The nuclei could be seen and counted upon careful examination at a higher magnification, making identification possible. In the same way, certain features of the helminth ova/larvae, such as hooklets in *Hymenolepis* ova and radial striations in *Taenia* ova, could be seen with the oil immersion objective. These morphological characteristics differentiate helminths from artifacts and pseudoparasites and allow a definite identification of helminths to be made.

**Ascaris lumbricoides** ova. The albuminoid cortex of both fertilized and unfertilized *Ascaris* ova (Fig. 1A) stained very dark brown, blue-black, or sometimes green. The coarse mammillations of the shell were visible and, due to their large size, focusing on several places was necessary at a higher magnification to reveal the embryo inside the fertilized ovum. There was no distortion of the oval shape and no shrinkage.

**Table 2. Method of initial detection of helminth ova/larvae in individual patients**

<table>
<thead>
<tr>
<th>Helminth detected</th>
<th>No.* detected in:</th>
<th>Concentrate only (44.9)</th>
<th>Concentrate and stained smear (38.4)</th>
<th>Stained smear only (16.7)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ascaris</em> sp.</td>
<td>21</td>
<td>28</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><em>Strongyloides</em> sp.</td>
<td>17</td>
<td>12</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Hookworm</td>
<td>55</td>
<td>33</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td><em>Trichuris</em> sp.</td>
<td>31</td>
<td>18</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td><em>Hymenolepis</em> sp.</td>
<td>6</td>
<td>13</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><em>Clonorchis</em> sp.</td>
<td>5</td>
<td>7</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><em>Taenia</em> sp.</td>
<td>1</td>
<td>6</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><em>Enterobius</em> sp.</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in parentheses represent percentages.

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FIG. 1. (A) _A. lumbricoides_ ovum. Trichrome stain. ×1,000. The coarse mammillations of the shell are apparent. Focusing on several planes revealed the embryo inside the fertilized ovum. (B) _T. trichiura_ ovum. Trichrome stain. ×1,000. This ovum retained its characteristic size and barrel shape. The diagnostic polar plugs are readily visible. (C) _C. sinensis_ ovum. Trichrome stain. ×1,000. Careful focusing revealed the operculum with definite shoulders, and focusing on a different plane revealed a prominent knob at the opposite end (not visible here). These characteristics identify this as a _Clonorchis_ ovum. (Color slides of Fig. 1 are available upon request from J.C.W.)
Trichuris trichiura ova. The thick-shelled Trichuris ova (Fig. 1B) were the most easily identified of all the helminths. These ova appeared much as they would in a wet mount, stained dark red-brown, and retained their size and characteristic barrel shape. The diagnostic polar plugs and the embryo inside the shell are easily seen.

Clonorchis, Heterophyes, or Metagonimus ova. The ova of these three fluke genera can be difficult to differentiate in a well-preserved wet mount. In the stained smear, these ova retained their size and oval shape or sometimes collapsed slightly longitudinally. They had thick shells that stained red-brown, contrasting with the green background. At a high magnification, some of the features that differentiate Clonorchis sinensis ova (Fig. 1C) from the ova of other genera could be seen, if present. Careful focusing revealed these features, the operculum with definite shoulders and a prominent knob at the opposite end.

Taenia ova. The Taenia ova (Fig. 2A) retained their round shape and stained dark brown or blue-black due to their thick shells. The onchosphere was visible but stained especially dark, so the hooklets could not be seen. However, identification was made when the diagnostic radial striations in the outer wall were revealed with careful focusing.

Strongyloides stercoralis rhabditiform larvae. These larvae (Fig. 2B and 2C) stained red and often shrunk slightly, leaving a clear zone around them. The larvae were rounded at the head and pointed at the tail. They must be differentiated from plant fibers, which they can resemble in size and color. Whereas the larvae were granular and often curved or coiled, plant fibers are straight and have nongranular clear interiors with definite central canals running the length of the fibers. A high magnification revealed more detail of the granular appearance of the intestinal structure of the larvae. Careful focusing sometimes revealed the length of the buccal cavity, well defined by the presence of fecal material staining in a contrasting manner. This permitted differentiation of Strongyloides larvae from hookworm larvae.

Hookworm ova. Hookworm ova (Fig. 3A) had delicate, thin shells that ruptured during the staining process and were rarely visible. Only the embryo, which stained red and retained its size and oval shape, could be seen. Due to the original presence on the shell, there was often a clear halo around the embryo, which had a granular appearance. Sometimes the round individual cells of the embryo were visible. Some plant material resembled these ova but was not as uniformly shaped and had an irregular, thick-walled cellular structure, unlike the granular appearance of hookworm embryos.

Hymenolepis ova. The ova of Hymenolepis nana and Hymenolepis diminuta (Fig. 3B) had thin shells that stained pink and collapsed and wrinkled but remained visible, unlike hookworm ova. The darker pink onchosphere was readily visible inside the shell, and careful focusing revealed the hooklet pairs, which distinguish these ova from any similar plant material. Due to the collapse of the shell, the polar fibrils could not be seen, and size was not reliable. Therefore, differentiation of the two Hymenolepis species had to be made from a wet mount.

DISCUSSION

The data presented here show that, by a routine search of a trichrome-stained smear under low-power magnification (100×), helminths that would have been missed had the Formalin concentrate been depended upon entirely were detected in 14 patients (6.3%). Additional species of helminths were found by examinations of stained smears for 13 more patients (5.9%) from whom other helminths were found by examinations of concentrates. If stool specimens were considered as independent examinations regardless of the patient from whom they were obtained, 14.6% of the total helminth ova/larvae identified would have been missed if the stained smear had not been examined for helminths. In our laboratory, under routine working conditions, infection with helminths can be diagnosed for more patients with this simple additional examination of the trichrome-stained smear: this procedure is already used in most laboratories for the recovery of protozoan trophozoites.

Identification of helminth ova/larvae in trichrome-stained smears has not been recommended in the past. However, their presence in the smears has been recognized, although the stained smear technique has not been compared to concentration techniques in previous publications (2, 4, 8). A brief, general description of the appearance of helminths in trichrome-stained smears was given by Garcia and Voge (4). Melvin and Brooke (8) and Garcia and Ash (2) pointed out that many helminth ova stain red and stand out against the green background material of the smear. The tendency of thin-shelled hookworm ova to collapse was also mentioned by Garcia and Voge (4). In this article, we have expanded the descriptions of the appearance of the ova/larvae in stained smears and have emphasized the variability of the staining characteristics of the different ova and larvae. The helminth ova/larvae present in stained stool smears are often overlooked by untrained observers, but once an awareness of them is gained and their morphological characteristics and variations are learned, the ova/larvae can be easily recognized. The morpholog-
FIG. 2. (A) *Taenia* ovum. Trichrome stain. ×1,000. The round ova of this genus have very thick walls; the diagnostic radial striations can be seen. (B) *S. stercoralis* rhabditiform larva. Trichrome stain. ×100. Larvae of this species often curve or coil, and, as shown here, tend to shrink slightly, leaving a clear zone around them and allowing for easy detection. (C) *S. stercoralis* rhabditiform larva. Trichrome stain. ×1,000. A high magnification facilitated study of the granular appearance of the intestinal structure and the head of the larva. Careful focusing sometimes revealed the length of the buccal cavity, well defined by the presence of fecal material staining in a contrasting manner, as shown here. (Color slides of Fig. 2 are available upon request from J.C.W.)
FIG. 3. (A) Hookworm ovum. Trichrome stain. ×1,000. The hookworm ova had thin shells that collapsed during the staining process and were rarely visible. All that can be seen here is the embryo, which retained the characteristic size and oval shape. (B) Hymenolepis ovum. Trichrome stain. ×1,000. H. nana and H. diminuta ova had thin shells that collapsed and wrinkled but remained visible. The onchosphere is readily visible inside the shell, and careful focusing revealed the hooklet pairs. (Color slides of Fig. 3 are available upon request from J.C.W.)

Physical characteristics can be learned by identifying helminths in a wet mount, searching the corresponding stained smear, and studying the helminths that are found. The smears may be examined very quickly under low-power magnification as experience is gained in recognizing the outstanding features of the helminth ova/larvae. Most ova/larvae retain enough diagnostic characteristics to be definitely identified to the species level, although occasionally some may require additional examinations of wet mounts of Formalin-preserved concentrated specimens to confirm the identification of ova/larvae that were previously missed. In general, *Ascaris*, *Trichuris*, *Taenia*, and *Clonorchis* ova have consistent appearances and are readily identifiable. Certain artifacts may be confused with *Strongyloides*, *Hymenolepis*, and hookworm ova; therefore, attention to the details of their appearance is more important.

The ability to find and identify helminths in trichrome-stained smears has several advantages. Most laboratories perform both a trichrome stain and a concentrate. Identifying the same organism by both methods serves as a double check and also guards against specimen misidentification. On occasion, the helminth ova or larvae can be missed in the concentrate and
yet be seen in the stained smear. Thus, the stained smear can help as a check on the effectiveness of the concentration procedure and further assure diagnosis of some helminth infections that might otherwise be overlooked. Many laboratories use a two-vial collection kit (8), one vial containing PVA and the other vial containing Formalin. Occasionally, a specimen will be received with no Formalin vial. In this case, it is still possible to perform an examination of the specimen for helminth ova/larvae since the stained smear can be used to search for helminths. Finally, trichrome-stained smears can be conveniently kept for any length of time as a permanent record of helminths, as well as of protozoa.

In conclusion, although the identification of helminth ova/larvae in trichrome-stained smears has not been emphasized in the past, the advantages of doing so are many. The technique cannot be used alone to detect and identify all the helminth ova/larvae that may be present in a specimen. However, when used in conjunction with a wet mount of a fecal concentrate, this procedure increases the accuracy of detection of helminths. Learning to recognize the appearance of helminth ova/larvae in stained smears is not difficult, and with experience, an individual with this ability is more effective at diagnosing intestinal parasitic infections.

LITERATURE CITED