Use of Terpene-Based Products, with and without Anhydrous Alcohol, as Clearing Agents in Trichrome Staining Technique for Intestinal Protozoa

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BDH xylene substitute, a terpene-based product, and its mixture with anhydrous alcohol were found to be excellent replacements for xylene and carbol-xylene, respectively, in the trichrome staining technique applied to sodium acetate-acetic acid-Formalin-fixed fecal smears for detection of intestinal protozoa.

Recently, Neimeister et al. (3) successfully used Hemo-De (PMP Medical Laboratories, Inc., Irving, Tex.), a terpene-based product, as a xylene substitute in the standard trichrome staining technique (2) for polyvinyl alcohol-fixed fecal smears. However, while eliminating one toxic agent, xylene, their technique still retains another toxic agent, carbol (1). Furthermore, some laboratories, including Public Health Laboratories of Alberta, use sodium acetate-acetic acid-Formalin (SAF) as a preservative for fecal samples (5). In this study, we tried to eliminate both toxic agents and also to evaluate the suitability of Hemo-De and other similar commercially available terpene-based products as xylene substitutes in the staining technique for SAF-fixed fecal smears. The following terpene-based products were evaluated: Hemo-De, Histoclear (National Diagnostics, Inc., Manville, N.J.), BDH xylene substitute (BDH Chemicals, Ltd., Toronto, Ontario, Canada), and Shandon xylene substitute (Shandon Inc., Sewickley, Pa.).

Fresh fecal samples, mixed with SAF fixative as supplied in plastic containers (PML Microbiologicals, Richmond, British Columbia, Canada), were transported to the laboratory. Before testing, the ages of the fixed fecal samples ranged from 1 to 8 days. One patient provided three samples, and five other patients provided two samples each on different occasions. A total of 39 samples, showing the presence of intestinal protozoa by the standard trichrome technique (2), were chosen for further evaluation. In addition to the smear that was stained by the standard trichrome technique, four supplementary smears were prepared from each sample on separate slides and stained by using the following clearing agents as replacements for carbol-xylene and xylene. A mixture of one part of anhydrous alcohol and two parts of one of the commercially available terpene-based products was used in the first step of the clearing procedure, and a terpene-based product without alcohol was used in the next step. The trichrome staining technique applied to SAF-fixed fecal smears for all clearing agents tested in this study was as follows: (i) 70% alcohol and iodine (10 min); (ii) 70% alcohol (3 min); (iii) 70% alcohol (3 min); (iv) trichrome stain (8 min); (v) 90% alcohol, acidified (10 s); (vi) 95% alcohol rinse; (vii) 95% alcohol (5 min); (viii) carbol-xylene or anhydrous alcohol-terpene-based product (5 min); (ix) xylene or terpene-based product (5 min); (x) mounting with cover slip, using a mounting medium.

After being mounted, slides were examined under a microscope with a 100× oil immersion objective and evaluated on the basis of clarity, color properties, differentiation, and the physical appearance of protozoa, compared with a slide showing well-stained organisms by the standard trichrome technique. To minimize subjective influences and sample-to-sample variations, two groups of results were established in the final analysis of data: (i) unsatisfactory and (ii) satisfactory. Generally, unsatisfactory slides showed considerable distortions in morphology or deviations from the expected color of cysts or trophozoites, fuzzy cellular details, inadequate intensity of color, haziness, a lack of clearing, and poor contrast with the background, which interfered with the identification of the protozoa. However, blurring and washing out posed the major problems. Satisfactory slides showed some slight variances in staining qualities but always exhibited reasonable clarity, definition, intensity, and representation of color (red to purplish red nucleic material; blue-green cytoplasm tinged with purple), as well as distinct cellular morphology and good contrast with the background. Many times, the quality of staining for two different protozoal species was different on the same slide, and also the same species stained differently on slides originating from different specimens although the same clearing agent was used. Because of this, we could not single out any tested species which cleared or stained better than other species by using any modification of the tested staining techniques. Statistical analysis was done by the χ² method with Yates correction (4).

Cysts and/or trophozoites of more than one species were found in 10 of the 39 tested positive samples. Consequently, cysts of different species were noticed 46 times and trophozoites were seen 18 times, giving a total frequency of 64 observations for both developmental phases. Protozoal species found in the study were Giardia lamblia (cysts in 6 samples), Entamoeba histolytica (cysts in 2 samples, trophozoites in 2 samples), Entamoeba hartmanni (cysts in 10 samples, trophozoites in 4 samples), Entamoeba coli (cysts in 14 samples, trophozoites in 4 samples), Endolimax nana (cysts in 14 samples, trophozoites in 6 samples), and Dientamoeba fragilis (trophozoites in 2 samples).

An evaluation of results obtained by the five techniques is given in Table 1. We did not notice any deterioration of
staining qualities of SAF-fixed samples up to 8 days old by any staining modification tested in this study. Cysts and/or trophozoites of the same species found in repeat fecal samples provided by several patients on different occasions varied widely in staining qualities. Therefore, they were considered as separate samples. In general, there was no difference in staining properties of cysts compared with those of trophozoites when the same clearing agent was used. However, disparity was found between different clearing agents. When total numbers of satisfactory staining results were compared with unsatisfactory staining results for both developmental phases of different protozoa, BDH xylene substitute performed significantly better ($P < 0.001$) than any other clearing agent used in the study. There was no statistical difference between results obtained by Shandon xylene substitute and Hemo-De ($P > 0.5$) or between results obtained by xylene and Histoclear ($P > 0.5$). Histoclear and xylene performed better than Hemo-De or Shandon xylene substitute ($P < 0.01$ for Histoclear in both cases, and $P < 0.02$ and $P < 0.05$, respectively, for xylene). Additionally, only BDH xylene substitute gave significantly more satisfactory than unsatisfactory results ($P < 0.001$). Thus, in our study, use of BDH xylene substitute gave excellent results, far superior to any other tested clearing agent, including xylene. Furthermore, our modification successfully eliminated yet another toxic agent from the trichrome staining technique. The only potential disadvantage is that the strong citrus fragrance of BDH xylene substitute might be offensive to some technologists. Application of this technique to polyvinyl alcohol-fixed fecal smears, which were not available to us, remains to be assessed.

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LITERATURE CITED


TABLE 1. Outcome of staining of human intestinal protozoa in SAF-fixed fecal smears, using various clearing agents in trichrome staining technique

<table>
<thead>
<tr>
<th>Clearing agent</th>
<th>Cysts (no. of observations)</th>
<th>Trophozoites (no. of observations)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbol-xylene</td>
<td>28, 18</td>
<td>13, 5</td>
</tr>
<tr>
<td>Anhydrous alcohol plus:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemo-De</td>
<td>19, 27</td>
<td>8, 10</td>
</tr>
<tr>
<td>Histoclear</td>
<td>30, 16</td>
<td>13, 5</td>
</tr>
<tr>
<td>BDH xylene substitute</td>
<td>43, 3</td>
<td>18, 0</td>
</tr>
<tr>
<td>Shandon xylene substitute</td>
<td>19, 27</td>
<td>9, 9</td>
</tr>
</tbody>
</table>