Evaluation of Commercially Available Preservatives for Laboratory Detection of Helminths and Protozoa in Human Fecal Specimens

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Formalin and mercuric chloride-based low-viscosity polyvinyl alcohol (LV-PVA) are widely used by most diagnostic parasitology laboratories for preservation of helminth eggs and protozoan cysts and trophozoites in fecal specimens. Concerns about the toxicity of formalin and the difficulty of disposal of LV-PVA are powerful incentives to use alternate preservatives. Such alternatives have been marketed by several companies and are often presented as one-vial, non-mercuric chloride fixatives that aim at performing the same role as formalin and PVA combined. We compared five, one-vial commercial preservatives, two from Meridian Diagnostics, Inc. (Ecofix and sodium acetate-acetic acid-formalin), and one each from Scientific Device Laboratories, Inc. (Parasafe), Alpha Tec Systems, Inc. (Proto-fix), and Streek Laboratories, Inc. (STF), with 10% formalin and LV-PVA. Fecal specimens obtained from patients in a Brazilian hospital were aliquoted within 12 h of collection into the seven preservatives mentioned above and were processed after 1 month at the Centers for Disease Control and Prevention. Direct and concentrated permanent smears as well as concentrates for 20 positive specimens (a total of 259 processed samples) were prepared, stained according to the manufacturers’ instructions, examined, and graded. Positive specimens contained one or more parasites with stages consisting of eggs, larvae, cysts, and a few trophozoites of Giardia intestinalis. Criteria for assessment of the preservatives included the quality of the diagnostic characteristics of helminth eggs, protozoan cysts, and trophozoites, ease of use, and cost. Acceptable alternatives to formalin for wet preparations were found. Ecofix was found to be comparable to the traditional “gold standard” LV-PVA for the visualization of protozoa in permanent stained smears. This study suggests that more acceptable alternatives to the traditional formalin and LV-PVA exist.

Formalin and low-viscosity polyvinyl alcohol (LV-PVA), two traditional stool fixatives, have been widely used in most public, private, and commercial laboratories for many years. These two fixatives have been and are still considered the “gold standard” in parasitology because they allow excellent long-term preservation of intestinal parasites (2). Formalin is considered an all-purpose fixative used to preserve helminth eggs, larvae, and protozoan cysts (2, 7). PVA is a plastic resin that contains Schaudinn’s fixative, which is used to preserve protozoan cysts and trophozoites for the preservation of permanent stained smears (2, 7).

Formalin contains formaldehyde (5 or 10%), which is a toxic carcinogen (1, Southwest Environmental Health Sciences Center, University of Arizona College of Pharmacy, [http://www.pharmacy.arizona.edu/centers/tox_center/exp_path/formaldehyde.html]), and LV-PVA contains mercuric chloride, which must be disposed of according to biosafety regulations to limit mercury contamination of the environment (5; Southwest Environmental Health Sciences Center, University of Arizona College of Pharmacy). The high cost of disposal of LV-PVA has become a problem in most laboratories within the United States (3, 4). Thus, many laboratories are assessing or adopting non-mercuric chloride- and/or nonformalin-based fixatives. Biomedical supply companies that currently produce one-vial, non-mercuric chloride fixatives state that these new preservatives play the same role as formalin and LV-PVA combined. These one-vial fixatives aim at allowing the laboratory to perform all tests from one vial, with the results presumably being equivalent to or better than those obtained with formalin and LV-PVA. Some of these new fixatives allow concentration procedures that require less centrifugation time or that use staining procedures that are faster and require fewer reagents. We compared five one-vial preservatives (Ecofix, sodium acetate-acetic acid-formalin [SAF], STF, Parasafe, and Proto-fix) with 10% formalin and LV-PVA in terms of their suitability in preparation of concentrated wet preparations and permanent stained smears for microscopic examination, ease of use, diagnostic accuracy, and cost. The objective of these findings was to find a fixative that could replace the traditional combination of formalin and mercuric chloride-based PVA (LV-PVA).

MATERIALS AND METHODS

Human fecal specimens were obtained from patients at the Hospital Universitário Pedro Ernesto in Rio de Janeiro, Brazil. An initial examination of the specimens was completed in Brazil by the Baermann technique, by the zinc sulfate flotation procedure, and with a modified Kato thick smear. On the basis of these examinations, 20 positive specimens were selected. These specimens were aliquoted within 12 h of passage into seven different fixative vials, coded as a set with identification numbers, and placed in a Ziploc bag. The set of seven fixatives included 10% formalin and LV-PVA (Meridian Diagnostics, Inc.), Ecofix (Meridian Diagnostics, Inc.), SAF (Meridian Diagnostics, Inc.), STF (Streek Laboratories, Inc.), Parasafe (Scientific Device Laboratories, Inc.), and Proto-fix (Alpha Tec Systems, Inc.). When the stool quantity was insufficient, one or more of the seven preservative vials were left out on a rotating basis. A total of seven fixative vials were randomly left out of the study due to leakage during transport back to the United States.

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Fixed stool specimens arrived in the Centers for Disease Control and Prevention laboratory within 1 month after initial collection. Accompanying the 20 positive specimens was a data sheet from Brazil which listed the organisms identified in each of the samples. Individual vials of the 20 sample sets were recorded with new identification numbers so that the microscopists at the Centers for Disease Control and Prevention would identify organisms on the basis of morphology and not on the basis of previous knowledge of the initial identification. Since 10% formalin and LP-VPA work together as a set, we considered these two fixatives as “one system,” with formalin being labeled “A” and LP-VPA being labeled “B.” The traditional formalin-ethyl acetate concentration procedure was compared for samples in 10% formalin and STF fixatives (Table 1). The SAF and Parasafe manufacturers recommend a modified formalin-ethyl acetate concentration procedure in which saline is used instead of formalin. The manufacturer’s instructions state that the saline in maternal was red (as shown in Fig. 3). Staining of LV-PVA-fixed samples with Wheatley’s trichrome did not have the same detail as samples stained with Trichrome-Plus. Samples preserved in STF and Parasafe stained with Ecostain displayed similar morphologic details compared with those for samples preserved in LV-PVA and stained with Wheatley’s trichrome. Material fixed in Parasafe and stained with iron hematoxylin was brownish-grayish, samples fixed in STF and stained with Wheatley’s trichrome were pink or purple, material fixed in Parasafe and stained with trichrome was bluish green, and samples preserved in Proto-fix and stained with Trichrome-Plus were red (as shown in Fig. 3).

### RESULTS

Twenty positive specimens had one or more of the following stages of parasites: eggs of Ascaris lumbricoides and Trichuris trichiura, larvae of Strongyloides stercoralis, and cysts of Blastocystis hominis, Endolimax nana, Enterobius vermicularis, and Giardia intestina laris (see Table 2). Two trophozoites of this organism were also found. These samples did not contain Cryptosporidium parvum or Isospora belli. Therefore, no data could be collected on these organisms in the seven preservatives.

Concentration procedures for samples from six fixatives (with LP-VPA being the exception) were easy to perform and took approximately 10 to 20 min to complete. Wet mounts of samples from four fixatives were clean and easy to visualize. The concentrated material from Parasafe preparations was viscous (Fig. 1), and wet mounts prepared by the Consed procedure were found to be dense red in color, which sometimes obscured elements such as eggs of Ascaris lumbricoides because of blending with background material. The morphology of eggs of Ascaris lumbricoides from concentrated samples in these six preservatives can be found in Fig. 1. Five of these images clearly show morphologic characteristics necessary for identification of Ascaris lumbricoides eggs.

Permanent stained smears had many color ranges that either aided or hindered identification of organisms within the samples. LP-VPA-fixed samples, when stained with trichrome, were red, purple, and blue, while Ecofix-preserved material stained with Ecotain was more of a greenish blue with areas of red. For the other fixatives, material preserved in SAF and stained with iron hematoxylin was brownish-grayish, samples fixed in STF and stained with Wheatley’s trichrome were pink or purple, material fixed in Parasafe and stained with trichrome was bluish green, and samples preserved in Proto-fix and stained with Trichrome-Plus were red (as shown in Fig. 3).

### Table 1. Procedures completed with the seven preservatives

<table>
<thead>
<tr>
<th>Fixative</th>
<th>Concentration technique</th>
<th>Permanent stain</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% Formalin</td>
<td>FEA</td>
<td>Wheatley’s trichrome</td>
<td></td>
</tr>
<tr>
<td>LP-VPA</td>
<td>FEA</td>
<td>Wheatley’s trichrome</td>
<td></td>
</tr>
<tr>
<td>Ecosafe</td>
<td>FEA (saline)</td>
<td>Iron hematoxylin</td>
<td></td>
</tr>
<tr>
<td>SAF</td>
<td>FEA</td>
<td>Wheatley’s trichrome</td>
<td></td>
</tr>
<tr>
<td>STF</td>
<td>FEA</td>
<td>Wheatley’s trichrome</td>
<td></td>
</tr>
<tr>
<td>Parasafe</td>
<td>FEA (saline)</td>
<td>Wheatley’s trichrome</td>
<td>Conc. PS</td>
</tr>
<tr>
<td>Proto-fix</td>
<td>Consed</td>
<td>Trichrome-Plus</td>
<td>Conc. PS</td>
</tr>
</tbody>
</table>

Abbreviations: Conc. PS, concentrated permanent smear; FEA, formalin-ethyl acetate concentration procedure.
Parasafe and Proto-fix did not score well, as shown in Fig. 5. The morphologic characteristics of organisms preserved in Parasafe or Proto-fix and then stained were sometimes difficult to observe because the stained sample was blurry, was faint, or lacked the necessary clarity of morphologic characteristics for identification of the organisms.

Among the permanent stained smears, there were few missed positive specimens among the specimens that were either unconcentrated or concentrated with various fixatives. As mentioned earlier, organisms were missed primarily due to low parasite density, although the staining quality of the smear and the inability to differentiate between amebae such as *Entamoeba coli* and *Entamoeba histolytica/E. dispar* also contributed to some discrepancies.

Readers 1 and 2 graded 129 of the 259 processed fixed samples, identically. Discrepancies between readers 1 and 2...
DISCUSSION

Fixatives play an important role in the preservation and transport of human fecal specimens and in the accurate diagnosis of parasitic diseases. Formalin and LV-PVA have been trusted preservatives in the past, but with mounting concern over environmental issues and health concerns for laboratorians, alternative fixatives need to be explored. The two objectives of this study were to compare various fixatives (10% formalin and LV-PVA, Ecofix, SAF, STF, Parasafe, and Protofix) in terms of diagnostic quality both with wet preparations of concentrates and by techniques for the preparation of permanent stained smears.

We found that all fixatives except Parasafe performed well for the preservation of helminth eggs and protozoa when used with concentration procedures for wet preparations (Fig. 1 and 2). The concentrate of Parasafe-fixed samples was viscous and dense, which made it difficult to see all the morphologic characteristics of the various organisms necessary for identification. Studies by Yang and Scholten (8) found that the SAF fixative works well in concentration procedures but that SAF contains formalin and hence poses the same health concerns as standard 10% formalin. Nace et al. (6) found STF to be an excellent substitute for formalin in concentration techniques. Therefore, acceptable alternatives to traditional 10% formalin for wet preparations exist.

The preparation of permanent stained smears is an important technique associated with routine examination of intesti-
nal protozoa (7). LV-PVA has always been a superior fixative for the preparation of permanent stained smears of protozoan cysts and trophozoites. In our study, LV-PVA proved to be superior. Nonetheless, Ecofix was comparable to LV-PVA in terms of preservation of diagnostic characteristics when the recommended stain was used. Garcia and Shimizu (2) also found this to be true in their study with specimens preserved in Ecofix and stained with Ecostain or Wheatley’s trichrome. They found that specimens fixed in Ecofix and stained with Ecostain had well-defined nuclear detail, with some parasites being easier to identify than the parasites in traditional specimens fixed in LV-PVA.

Direct or concentrated permanent stained smears from various samples preserved in Parasafe, Proto-fix, SAF, and STF were sometimes fuzzy or faint in color, with detailed morphologic characteristics not visible when the smears were stained with appropriate stains. In samples fixed in Parasafe, STF, and sometimes Proto-fix and SAF, amebae (such as *Entamoeba histolytica*, *E. dispar*, *Entamoeba coli*, and *Endolimax nana*) were the most difficult to identify because of poor preservation. In such samples that did not receive high scores, amebae were distorted or shrunken or the cyst wall was deteriorated; internal structure was poor or nuclei were hard to visualize (these were reported as *Entamoeba* species). It has been well recognized that good preservation and staining are critical for the identification of amebae, much more so than for the identification of *Giardia*, for instance. This was again demonstrated in the present study.

Permanent stained smears made from the various fixatives had many color ranges. We found that stains with contrasting
colors worked well for the identification of protozoan para-
sites. Garcia and Shimizu (2) found that the primary difference
between Ecostain and Wheatley’s trichrome was the color of
the organisms. Overall, better performance was obtained for
samples preserved in LV-PVA and Ecofix because of preser-
vation of morphology and ease of visualization of parasites on
smears stained with contrasting colors.

We were not able to thoroughly evaluate the issue of quan-
titation between fixatives. However, we noted only a small
difference in the number of organisms between aliquots of the
same samples in various fixatives. Since a consistent pattern
could not be found among the samples in the various fixatives,
it does not appear that one fixative performs better or worse
for the detection of organisms.

When comparing fixatives in terms of ease of use, Ecofix and
Proto-fix had the shortest staining procedures; these two pro-
cedures took approximately 15 min, whereas the procedure
with trichrome required 55 min and that with iron hematoxylin
required 2 h.

Many laboratories today are also concerned with the high
cost of preservatives. The approximate cost of each fixative is
$1 to $2 per preserved sample. One-vial fixatives cost almost as
much as the traditional formalin and LV-PVA Para-Paks. To
use new concentration techniques such as Consed or Spincon
with samples preserved in Proto-fix or Ecofix, respectively,
laboratorians will need to purchase additional concentration
kits, which usually include specific vials for use in centrifuga-
tion and filters. These kits are relatively expensive, costing
anywhere from $1 to $2 per sample, which adds an additional
charge to the cost of processing each sample. Therefore, when
considering the total cost of sample preservation and process-
ing, the difference in cost between new and traditional fixatives
is negligible.

New one-vial fixatives are entering the commercial market.
In the present study, several of the seven fixatives (Ecofix,
SAF, STF, and Proto-fix) provided the best overall fixative for
subsequent parasitological examination with wet preparations.
However, all fixatives studied except Ecofix fell short in terms
of quality of staining compared to that achieved with LV-PVA.
Continuing evaluations of these new preservatives need to
be made by both public health and clinical laboratories to find
suitable alternatives to the traditional 10% formalin and PVA.
A new one-vial preservative that has characteristics similar to
those of the traditional formalin and LV-PVA preservatives
would be advantageous in the laboratory not only because of
health and disposal issues but also in terms of ease of use, cost,
and shorter staining times. Additional work on staining quality
for permanent stained smears is needed before a one-vial pre-
servative can replace LV-PVA.

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