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Evaluation of Intestinal Protozoan Morphology in Polyvinyl Alcohol Preservative: Comparison of Zinc Sulfate- and Mercuric Chloride-Based Compounds for Use in Schaudinn's Fixative

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As a result of disposal problems related to the use of mercury compounds, many laboratories have considered switching from mercuric chloride-based Schaudinn's and polyvinyl alcohol (PVA) stool preservatives to other non-mercury-based preservatives. The primary use for PVA-preserved specimens is the permanent stained smear, the most important technique in the routine ova and parasite examination for the identification and confirmation of intestinal protozoa. A comparison of organism recovery and morphology of the intestinal protozoa was undertaken with PVA containing either a zinc sulfate base or the "gold standard" mercuric chloride base. Paired positive fecal specimens (106 from 64 patients) were collected and examined microscopically by the trichrome stain technique. There were 161 instances in which organism trophozoite and/or cyst stages were identified and 3 in which human cells were identified. Morphology, clarity of nuclear and cytoplasmic detail, overall color differences, and the ease or difficulty in detecting intestinal protozoa in fecal debris, as well as the number of patients with a missed diagnosis, were assessed from the permanent stained smear. Overall organism morphology of the intestinal protozoa preserved in zinc sulfate-PVA was not always equal in nuclear and cytoplasmic detail or range of color after permanent staining to that seen with mercuric chloride-PVA. However, the same organisms were usually identified in both specimens, with the exception of situations in which organism numbers were characterized as rare (no organisms per 10 oil immersion fields at ×1,000 magnification but at least one organism in the smear) [9 of 161 (5.6%)] or the organism was missed because of poor morphologic detail [12 of 161 (7.5%)]. In only six of these cases [6 of 161 (3.7%)] did the results involve pathogens. The patient diagnosis was missed in four cases of amebiasis and two cases of giardiasis; in both situations the organism numbers were rare. There were no discrepant results with *Dientamoeba fragilis*. Overall agreement between the two PVA-based results was 87.0% (140 of 161); when the instances of rare organisms were disregarded, the overall agreement was 92.5% (149 of 161). On the basis of these findings, zinc-PVA is a viable substitute for mercuric chloride-PVA used for trichrome permanent stained smears.

Both Schaudinn's and polyvinyl alcohol (PVA) fixatives with a mercuric chloride ($HgCl_2$) base have been used to preserve stool specimens for the recovery and identification of parasites, primarily the intestinal protozoa (1–5, 7, 9–11). PVA is a plastic powder that is dissolved in the Schaudinn's fixative; this plastic powder serves as an adhesive to help glue the stool onto the glass slide when the fecal smears are prepared, while the actual fixation occurs with the Schaudinn's solution. The permanent stained smear prepared from Schaudinn's or PVA fixatives is used primarily for the identification of intestinal protozoa and is considered to be the most important technique for this purpose (3–5, 7, 11). For several years, the issue of mercury disposal has been raised by clinical laboratories, since many facilities have neither funds nor access to limited commercial options for the disposal of small quantities of materials contaminated with mercury. It is becoming almost impossible to find companies that will accept mercury-containing waste, and even if an appropriate company can be located, the cost is prohibitive. The use of Schaudinn's fixative prepared with a compound other than $HgCl_2$ would be advantageous. Several studies using the substitute copper sulfate ($CuSO_4$) indicated that this compound did not provide consistent fixation for adequate protozoan morphology, although some laboratories use this option rather than institute costly and inconvenient disposal mechanisms for materials contaminated with $HgCl_2$ (6, 8). Some laboratories have switched to the use of sodium acetate-acetic acid-formalin (SAF) fixative coupled with the iron hematoxylin stain for the permanent stained smears. Although this approach is a good alternative, other laboratories want to maintain use of the trichrome stain (12, 13). The combination of SAF fixative and trichrome stain may not always provide the same quality results seen with the SAF-iron hematoxylin combination. For these reasons, a clinical study was undertaken to compare organism recovery and morphology with both types of PVA fixative, one with $HgCl_2$ and one with zinc sulfate ($ZnSO_4$). The main objective of the study was to determine whether the same organisms, regardless of morphologic differences or numbers other than rare (see Materials and Methods), could be identified with either type of PVA fixative.

MATERIALS AND METHODS

Fecal specimens were collected in ~300 paired vials, one containing PVA with $HgCl_2$ and the other containing PVA prepared with $ZnSO_4$, of which 106 pairs from 61 patients were positive for intestinal protozoa and/or human cells.

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TABLE 1. Pathogenic protozoa identified on trichrome permanent stained smears with mercuric chloride or zinc sulfate in PVA fixative

Organism (*n*)	No. of protozoa recovered:		Comments
	With $HgCl_2$	With $ZnSO_4$	
Entamoeba histolytica[a]			
Trophozoites (7)	6	6	One *E. histolytica* trophozoite (rare) missed in each fixative[b]
Cysts (7)	7	4	Diagnosis missed in three patients (rare cysts)
Giardia lamblia			
Trophozoites (0)	0	0	Diagnosis missed in two patients (rare cysts)
Cysts (7)	7	5	
Dientamoeba fragilis trophozoites (14)	14	14	Organism morphology very similar in both preparations

[a] In one instance where *E. histolytica* was identified but either the trophozoite or cyst form was missed from either the $HgCl_2$ or $ZnSO_4$ PVA, this would affect therapy since different drugs are often used for the two stages. The diagnosis of amebiasis would have been made, regardless of whether the trophozoite or cyst form was present.

[b] One *E. histolytica* trophozoite was missed in a $HgCl_2$-preserved specimen and one *E. histolytica* trophozoite was missed in a $ZnSO_4$-preserved specimen. In both instances, the trophozoites were found in the opposite, matched specimen.

Permanent stained smears were prepared from each vial by the trichrome-staining procedure (5). The $HgCl_2$ vial was considered the "gold standard" against which the results of the $ZnSO_4$ were compared. Both smears were examined by reviewing approximately 300 oil immersion fields (magnification, ×1,000), and the results were recorded by technologists other than those preparing the smears. This approach is generally more consistent than reading each smear for a set period of time, since individuals scan smears at different speeds. The selection of a $ZnSO_4$- or $HgCl_2$-based smear for examination was blinded with no specific identification as to fixative. Numbers of organisms and cells, clarity of nuclear and cytoplasmic detail, overall staining differences, and the ability to detect organisms in fecal debris were assessed from the permanent stained smear for both trophozoite and cyst stages and human cells by two experienced technologists. The permanent stained smears were read with the 100× oil immersion objective. Although there were organism morphology and color differences, specimens were considered comparable if the same organisms were identified in both specimens. Quantitation was based on the number of organisms per 10 oil immersion fields at ×1,000 magnification (many = ≥10; moderate = 3 to 9; few = ≤2; rare = 0, but at least one identifiable organism is seen on the smear).

RESULTS

A wide range of intestinal protozoa were identified from the 61 positive patients and, in most cases, the comparative morphology of the intestinal protozoa was equal, although the best overall nuclear and cytoplasmic detail and clarity were seen with $HgCl_2$. Results with the pathogenic intestinal protozoa and information on the patient diagnosis status are shown in Table 1, while results with the nonpathogenic intestinal protozoa and human cells are shown in Table 2. Discrepancies in numbers of organisms detected on the permanent stained smears were noted in 12 of 161 (7.5%) smears in which the organism numbers were few or more, but the morphology was poor. There were 9 of 161 (5.6%) discrepancies where the number of organisms was rare. In only 6 of 161 (3.7%) discrepant situations did the results involve pathogens. The patient diagnosis was missed in four cases of amebiasis and two cases of giardiasis; in both situations the organism numbers were rare. There were no discrepancies in 14 cases of infection with *Dientamoeba fragilis*. The majority of the smears prepared from both fixatives showed minimal differences in color which would influence the ability to recognize the organism. When there were differences, color obtained from $HgCl_2$ and subsequent trichrome staining was better than that obtained with $ZnSO_4$. *Endolimax nana* cysts were the most difficult to identify in the Zn preparation, with the problem being one of morphology, not numbers. Overall agreement between the two PVA-based systems was 87.0% (140 of 161); if the discrepancies related to rare organism numbers were disregarded, the overall agreement was 92.5% (149 of 161).

DISCUSSION

Although $HgCl_2$ has been used in the preparation of Schaudinn's and PVA fixatives, several other compounds have been tried as substitutes in order to eliminate the numerous problems and high cost associated with mercury disposal. This study was designed to test one of the possible substitutes, $ZnSO_4$, and to determine its effectiveness as a fixative compared with that of $HgCl_2$. When permanent stained smears were examined, the overall differences in recovery and morphology were minimal in terms of organism identification. Although the range of colors was present (pink, red, purple, blue, green), stained smears prepared from $ZnSO_4$-fixed material were more green and $HgCl_2$ smears were more uniformly blue with better differential colors (purple, red, pink); however, organisms were detectable on both types of smears.

The actual nuclear and cytoplasmic detail and clarity of the intestinal protozoa were different when the two smears were compared. Smears prepared from $HgCl_2$ were of consistently better quality, with clear, well-defined morphologic details. Those smears prepared with $ZnSO_4$ did not always provide the same clarity and detail definition; in some specimens the fixation did not appear to be adequate (fuzzy detail, more shrinkage, poorly defined nuclear detail) and definitive identification was somewhat more difficult. However, in this study the ability to identify the intestinal protozoa was the diagnostic objective, and in 87.0% of the instances this goal was not compromised when the $ZnSO_4$-PVA fixative was used.

When organisms are present in rare numbers, it may be difficult to obtain consistent results from two smears, even with the identical fixative. However, in all instances but one,

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TABLE 2. Nonpathogenic protozoa identified on trichrome permanent stained smears with mercuric chloride or zinc sulfate in PVA fixative

Organism (*n*)	No. of protozoa or human cells recovered:		Comments
	With $HgCl_2$	With $ZnSO_4$	
Entamoeba hartmanni			
Trophozoites (6)	6	6	No discrepancies between $HgCl_2$ and $ZnSO_4$
Cysts (2)	2	2	
Entamoeba coli			
Trophozoites (9)	9	8	One *E. coli* trophozoite (rare) missed
Cysts (8)	8	7	One *E. coli* cyst (rare) missed
Endolimax nana			
Trophozoites (24)	24	20	One *E. nana* trophozoite (rare) missed; three *E. nana* trophozoites missed
Cysts (28)	28	23	Five *E. nana* cysts missed[a]
Iodamoeba bütschlii			
Trophozoites (5)	5	4	One *I. bütschlii* trophozoites missed
Cysts (3)	3	3	
Blastocystis hominis central body form (38)	38	35	Three *B. hominis* isolates missed
Chilomastix mesnili			
Trophozoites (2)	2	2	No discrepancies between $HgCl_2$ and $ZnSO_4$ results
Cysts (1)	1	1	
Human cells (3)	3	3	No discrepancies between $HgCl_2$ and $ZnSO_4$ results

[a] *E. nana* cysts were the most difficult to identify in the $ZnSO_4$ preparation. In the cases noted, the problem did not seem to be one of numbers, but of morphology. Again, the key question to ask is that of clinical relevance of the results obtained.

when organisms were rare in number, they were found in the $HgCl_2$ preparation and not in the $ZnSO_4$-base preparation. The implication is that minor changes in the quality of morphology and color differences in the Zn preparation, coupled with low organism numbers, may prevent identification of the organism present. However, the significance of these findings always needs to be assessed within the context of clinical relevance: was the diagnosis missed, and, if so, what was the clinical relevance of these findings?

Definitive identification of intestinal protozoa frequently depends on the permanent stained smear, and it is important that this technique be performed. The ability to identify the organisms after staining depends on obtaining the best possible fixation of the specimen as soon as possible after passage (5). Because the overall agreement in identification of the intestinal protozoa and human cells was almost as good with $ZnSO_4$-PVA as with $HgCl_2$-PVA, this fixative is an acceptable alternative that can be used with trichrome stain. Although the main difference is one of consistency of morphology, the differences related to actual organism identification appear to be minimal. However, as seen in this study, when organisms are found in rare numbers, they may be missed by using $ZnSO_4$-PVA compared with $HgCl_2$-PVA. This is certainly relevant if the organisms are pathogens, as seen with *Entamoeba histolytica* and *Giardia lamblia*. The search for quality alternatives to the mercury-based fixatives has been ongoing for many years; to date the $ZnSO_4$ substitute appears to be an acceptable alternative when used with the trichrome stain. Studies are in progress to compare the results of $ZnSO_4$-based versus $HgCl_2$-based PVA by using iron-hematoxylin stain. Sampling errors and organism shedding cycles contribute to the failure to always identify all intestinal protozoa present in the stool sample or infecting the patient; however, the laboratory community accepts these limitations as normal factors related to diagnostic test results in parasitology. Organisms fixed in $ZnSO_4$ do not have the same clarity and morphologic detail as organisms fixed in $HgCl_2$ and stained with trichrome. The laboratory will need to accept the fact that until the "perfect" replacement for $HgCl_2$ fixatives is found, we must learn to review organism morphology that is not "picture perfect" but that will allow identification of the organism. Although the percent agreement was not higher than 87% (adjusted to 92.5% when rare organism number discrepancies were disregarded), for many laboratories, the use of this or some other mercury substitute is not optional but is mandated by the current situation related to disposal limitations and high cost. It is also important to remember that any permanent stain will certainly increase the chances for protozoan recovery and identification over that with concentration sediment alone (4). With the substitute fixatives and methods available at this time, we would select the combination of $ZnSO_4$ and trichrome stain as our replacement for the current use of $HgCl_2$ and trichrome stain with a second, equally acceptable, choice being the SAF fixative coupled with iron hematoxylin stain.

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