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Evaluation of Alcian Blue 8GS and Pyronin B Stain for Rapid Wet Mount Preparation of SAF Preserved Specimen Containing *Entamoeba histolytica* and *Giardia intestinalis*

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ABSTRACT

The microscopic examination of saline-diluted and iodine-stained wet mount preparations has satisfactorily been adopted in many laboratories as a simple and definite diagnostic measure. But, it is known that some difficulties have sometimes taken place in detection and identification of protozoa with this examination. We examined the usefulness of SAF (sodium acetate, acetic acid, and formalin) fixation, and alcian blue 8GS and pyronin B (AP) stain for wet mount preparations of fecal materials taken from amoebiasis and giardiasis patients in this study. SAF fixation was found very effective for preserving the morphological characters of protozoa, especially the inner structure, and for giving the good stainability to AP stain. The protozoa stained with AP, either trophozoites or cysts, showed the clear contour distinguishable from outer admixtures with the nuclear membrane and the karyosome stained deep purple, and the chromatoid body colored deep red. This was confirmed by the fact that the protozoa were not stained well with AP when not fixed with SAF. This method would be usable for the routine examination of fecal materials in intestinal protozoan infections, particularly *Entamoeba*

histolytica and *Giardia intestinalis* infections. SAF fixative could also be used as a long-term preservative of protozoa in fecal materials.

INTRODUCTION

In intestinal protozoan infections, particularly amoebiasis and giardiasis, the microscopic examination of fecal materials gives us the definite diagnosis. Direct or saline-diluted wet mount preparations, and iodine-stained wet mount preparations of fecal materials have so far been used routinely for detection of causative protozoa. Although these technics are simple and rapidly prepared, and usually provide us with accurate diagnostic results, we have sometimes met some difficulties for detection and identification of protozoa.

Formalin, PVA or MIF solution has usually been used for preservation of fecal materials (Brooke and Goldman 1949; .Sapero and Lawless 1942). These preservatives can be used with satisfaction if the materials are mixed with the preservative, tightly sealed and placed in refrigerator. As PVA contains sublimate, bichloride of mercury, as one of the components, particular care on environmental pollution is requested in its use (Brooke and Goldman 1949).

In this study, the usefulness of SAF fixation and AP stain was examined for wet mount preparations of fecal materials taken from amoebiasis and giardiasis patients. SAF fixative was composed of acetic acid, sodium acetate, and formalin, but did not contain sublimate (Junod 1972, Yang and Scholten 1977). AP staining solution was composed of alcian blue 8GS, one of copper-phthalocyanine dyes, and pyronin B (Sternheimer 1975). The procedure to make wet mount preparations with them was technically easy and the specimens gave us satisfactory results either in detection of protozoa or in identification of the species.

MATERIALS AND METHODS

Fecal samples

Fecal samples used were collected from patients infected with *Entamoeba histolytica* and *Giardia intestinalis*.

SAF fixative solution

SAF fixative solution is composed of 1.5 g sodium acetate, 2.0 ml

acetic acid, 4.0 ml formalin, and 92.5 ml distilled water. The fecal samples were fixed with SAF solution according to the method of Yang and Scholten (1977). One part of the fresh fecal sample was mixed well with at least three parts of the SAF solution and fixed for at least 30 minutes at room temperature.

The samples added SAF were strained through a sheet of gauze into test tubes, then washed once with physiological saline by centrifugation for one minute at 800 G. The supernatant were decanted and the sediments were collected and stored as preserved samples.

Wet mount preparations stained with AP and iodine

AP staining solution was composed of one part of 2% alcian blue 8GS (Wako pure chemical, Osaka, Japan) in distilled water and two parts of 1.5% pyronin B (Wako pure chemical, Osaka, Japan) in distilled water (Sternheimer 1975).

Wet mounting with AP solution was made by mixing a drop of AP solution and a drop of preserved sample in a test tube or on a slide glass. A drop of the mixture was placed on a slide glass and coverslipped.

Wet mounting with iodine solution was made similarly as above. Iodine solution consisted of 1.0 g iodine (Wako pure chemical, Osaka, Japan), 2.0 g potassium iodide (Wako pure chemical, Osaka, Japan), and 100 ml distilled water. Saline-diluted wet mount preparations of preserved samples used as control specimens were also prepared by mixing each a drop of saline and preserved sample.

Permanent stain smears with iron-hematoxylin

Permanent stain smears with iron-hematoxylin stain were made as follows. A slide glass was coated with 0.6% neoprene (Sigma, Tokyo, Japan) solution in toluene and dried. The smear was made with a drop of SAF preserved samples or AP stained wet mount preparations of SAF preserved samples (SAF-AP) on the coated slide glass and dried at room temperature. The smear was dipped in tap water for two to three minutes and placed in 2.5% ferric ammonium sulfate for four hours. The smear was rinsed with tap water, then placed in 0.5% hematoxylin solution for six to 12 hours. The smear was rinsed with tap water, placed in 2.5% ferric ammonium sulfate for disstain, then placed in water for two hours,

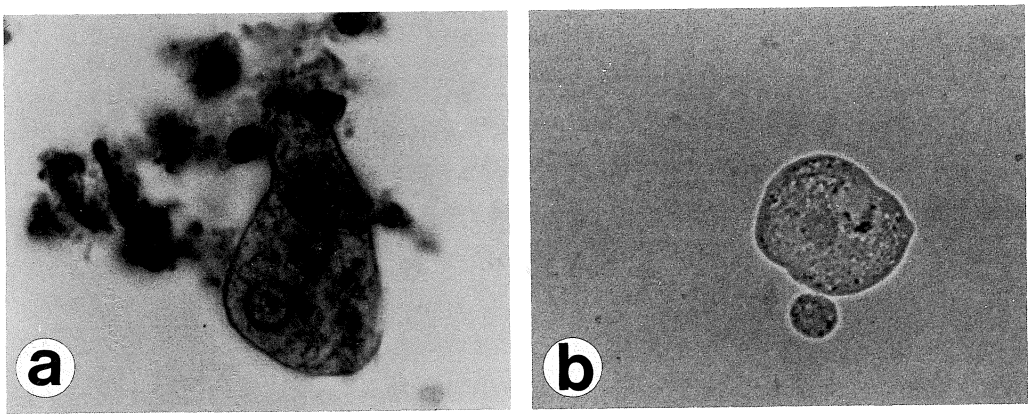

Figure 1 Light microscopic micrographs of *Entamoeba histolytica* trophozoite in wet mount preparations stained with alcian blue 8GS and pyronin B (AP stain). **a**, AP stained wet mount of SAF preserved sample. **b**, AP stained wet mount of unpreserved sample. (original magnification x 400)

dehydrated in ascending concentration of ethanol, placed in xylene, and mounted with a coverslip.

Microscopic observation of specimens

Trophozoites and cysts found microscopically were observed of their size, form, color, distinction, inner structure, and others, then figured out their species and stage.

The detection rate of protozoan cysts in wet mount preparations

Three kinds of wet mount preparations, AP-stained, iodine-stained, and saline-diluted, were compared on the presence and number of protozoa on each 20 specimens. The detection rate was expressed by percentage of the number of specimens in which protozoa were found, to 20 specimens.

RESULTS

E. histolytica trophozoites stained with AP

The trophozoite stained with AP was shown in figure 1a. The cytoplasm, both endoplasm and ectoplasm, was stained bright red. The cell membrane was stained deep purple. The nucleus was also stained bright red but clearly distinguished from cytoplasm, as the nuclear membrane and karyosome appeared equally deep purple.

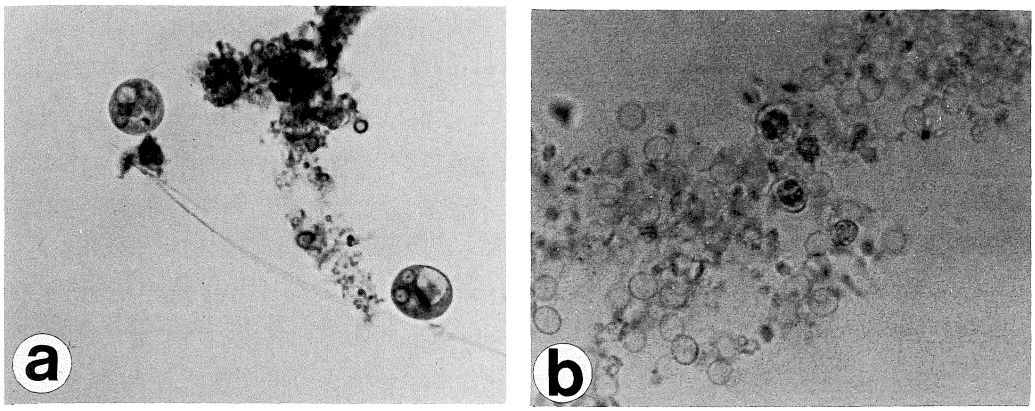

Figure 2 Light microscopic micrographs of *Entamoeba histolytica* cyst (**a**) and leukocytes (**b**) in AP stained wet mount preparations of SAF preserved samples. (original magnification x 400)

When the living trophozoite was not fixed with SAF and stained with AP, the cytoplasm appeared slightly reddish, and the nuclear membrane and the karyosome were not clearly visible (Fig. 1b). It was assumed that the living protozoa might not be stained with AP.

E. histolytica cysts stained with AP

The cyst stained with AP was shown in figure 2a. The cytoplasm was stained bright red similarly to the cytoplasm of trophozoite. Most of the chromatoid bodies were colored deep red as compared with cytoplasm, but some of them were stained bluish hue. The nuclei appeared bright red with the nuclear membranes and karyosomes stained deep red or bluish red, similarly to the nucleus of trophozoite.

It was known that leukocytes were apt to be confused with protozoan cysts. But they were easily distinguished from cysts as their appearance stained with AP was quite different from cysts. The cytoplasm was stained slightly red, most of the nuclei were not stained but their structures were finely visible, and some nuclei were stained bluish (Fig. 2b).

G. intestinalis trophozoites stained with AP

The cytoplasm was stained bright red. The cell membrane, median body, and flagellates were stained deep red or purple. The adhesive disk was stained pale pink. The nuclear membrane and karyosomes stained deep red, similarly to those of *E. histolytica* trophozoite (Fig. 3a,b).

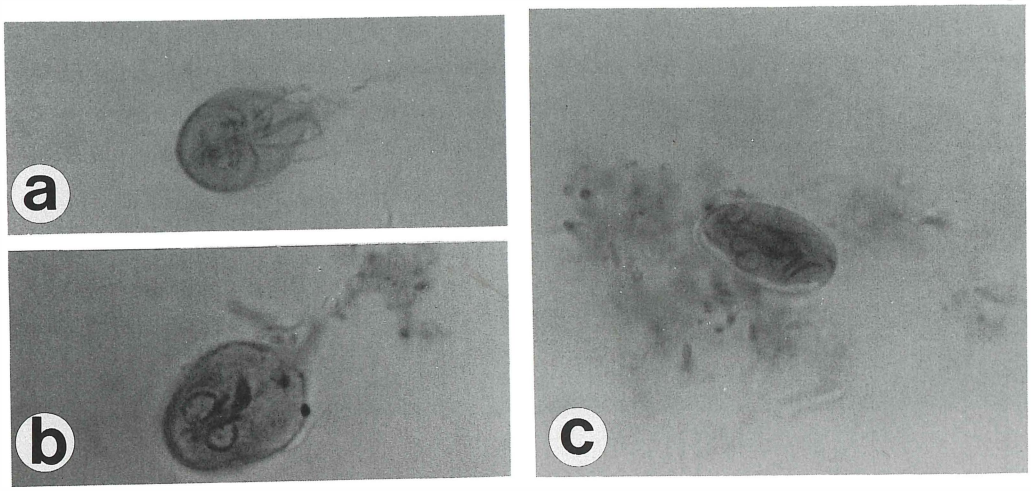

Figure 3 Light microscopic micrographs showing *Giardia intestinalis* trophozoite (**a**, **b**) and cyst (**c**) in AP stained wet mount preparations of SAF preserved samples. (original magnification x 1,000)

G. intestinalis cysts stained with AP

The cytoplasm was stained bright red. The nuclear membrane, curved bristle, and flagellates were stained deep red or purple. The cyst wall was not colored with AP but clearly visible (Fig. 3c).

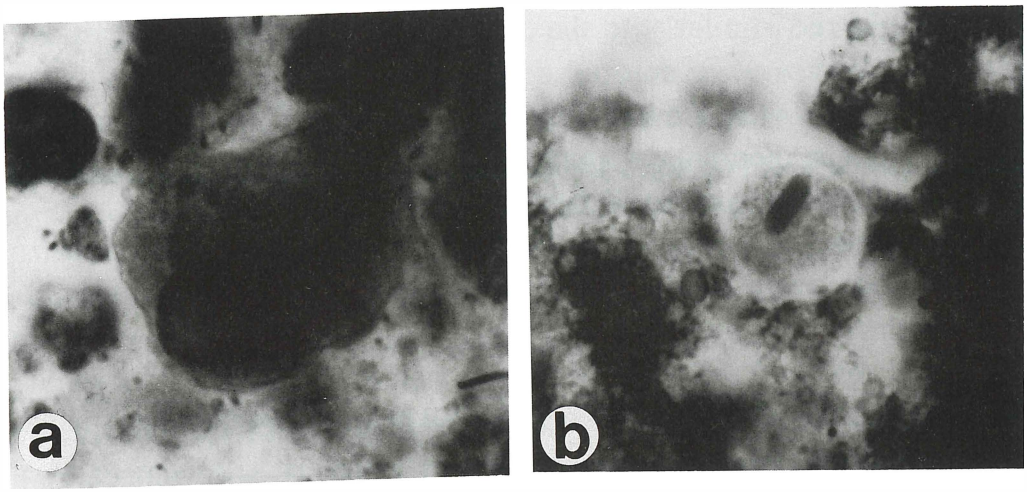

Figure 4 Light microscopic micrographs showing *Entamoeba histolytica* trophozoite (**a**) and cyst (**b**) on iron-hematoxylin permanent preparations of SAF preserved and AP stained samples. (original magnification x 1,000)

Vegetable cells, mucus, and other admixtures in specimens

Vegetable cells were commonly stained deep red and the mucus was stained bluish hue. They were easily distinguishable from intestinal protozoa.

Iron-hematoxylin permanent stain of SAF preserved and SAF-AP samples

Both the cytoplasms of *E. histolytica* and *G. intestinalis* were stained brightly bluish. The internal structures of these protozoa, nuclear membrane, karyosome, chromatoid body, curved bristle, and flagellates, were stained deep blue (Fig. 4). Since AP staining solution was rinsed out from protozoa during the process of iron-hematoxylin stain, AP stain which was preliminarily given to the samples had no affection to the result of iron-hematoxylin stain.

Detection rate of protozoan cyst in wet mount preparations stained with AP, iodine, and diluted with saline

The result was shown in Table 1. SAF-fixed and AP-stained wet mount preparations showed the highest rate of 95% (19/20) either in *E. histolytica* or in *G. intestinalis*. SAF-fixed and iodine-stained preparations showed 75% in *E. histolytica* and 65% in *G. intestinalis*. The detection rate in saline wet mount preparations was the lowest in either species of protozoa, *E. histolytica* or *G. intestinalis*.

DISCUSSION

The technology of laboratory diagnosis in amoebiasis and giardiasis has been becoming improved and more accurate. Recently, the serological diagnosis has been commonly used and the detection of specific antigens

Table 1 Detection rates of *E. histolytica* cyst and *G. intestinalis* cyst in each 20 AP stained, iodine stained wet mount and saline diluted wet mount preparations of SAF preserved samples.

	Detection rate		
Protozoa	AP stain	Iodine stain	Saline dilution
Entamoeba histolytica	19/20 (95%)	15/20 (75%)	11/20 (55%)
Giardia intestinalis	19/20 (95%)	13/20 (65%)	10/20 (50%)

or DNA to causative protozoa in the fecal material or serum of patients has been studied (Abd-Alla et al. 1993, Kasprzak and Majewska 1995, Vidal et al. 1991, Yamaura et al. 1990). But, the first choice of diagnostic measures is still the detection of protozoa with microscopic examination of feces (Ravdin 1995).

Fecal materials should be subjected to microscopic examination as soon as possible after collected, otherwise the protozoa in feces, especially trophozoites, would collapse or die within a few hours. The microscopic examination of saline-diluted and iodine-stained wet mount preparations has satisfactorily been adopted in many laboratories as a simple and definite diagnostic measure. But, it is known that some difficulties have sometimes taken place in detection and identification of protozoa with this examination (Krogstad et al. 1978).

SAF fixation was found very effective for preserving the morphological characters of protozoa, especially the inner structure, and for giving the suitable stainability to AP stain. The protozoa stained with AP, either trophozoites or cysts, showed the clear contour distinguishable from outer admixtures, the nuclear membrane and karyosome were stained deep purple, and the chromatoid bodies colored deep red. On the other hand, admixtures, especially leukocytes that were apt to be confused with protozoan cysts (Krogstad et al. 1978), were easily distinguished from cysts by their appearance stained with AP.

AP staining method is routinely used in clinical laboratories to prepare the wet mounts of urinary sediments for microscopic examination (Sternheimer 1975). This method has not been used previously in any wet mount preparation for demonstration of intestinal protozoa. Alcian blue 8GS is a basic copper-phthalocyanine dye with high molecular weight that stains acid mucopolysaccharides and nucleic acids, but has low staining affinity to cytoplasmic ribonucleic structures (Mowry 1963). On the other hand, pyronin B is a basic xanthene dye with low molecular weight that interacts strongly and rapidly with polynucleotides, particularly ribonucleic acids (Scott 1967). Both of alcian blue 8GS and pyronin B do not or slightly stain the living cell (Chapman-Anderson 1962, Sternheimer 1975). This was confirmed by the fact that protozoa were not stained well with AP when not fixed with SAF. Concerning the

fixation of protozoa in fecal materials, it was recognized later that formalin could be substituted to some extent for SAF fixative.

The detection rate of cysts in SAF-AP wet mount preparations was 95%, although iodine and saline-diluted wet mount methods showed a lower detection rate. Yang and Scholten (1977) reported that intestinal protozoa in SAF preserved specimen showed a higher detection rate compared with unpreserved specimen. It was noticed that the permanent preparations stained with iron-hematoxylin of SAF preserved samples showed a higher detection rate than the wet mount preparations of SAF preserved samples. The SAF-AP wet method also had the advantage that to find protozoa and to identify the species were easier in this method than in others, although the motility of trophozoites was lost in this wet mount preparations. It was pointed out that the SAF preserved samples and even the SAF-AP samples were equally stained well with iron-hematoxylin. In view of the facts mentioned above, the SAF-AP wet mount method would be better to use together with saline-diluted method, or with both of saline-diluted and iodine wet mount method, than use solely.

It is a routine procedure so far used for fecal examination that the fecal materials collected from patients are mixed with one of preservatives such as formalin, MIF and PVA, sealed tightly and stored in refrigerator until microscopic examination. SAF fixative proved itself effective as a long-term preservative, as the fecal materials added SAF fixative and preserved for several years displayed the clear image of protozoa in wet mount preparations stained with AP and in permanent preparations stained with iron-hematoxylin.

These results strongly suggested that SAF-AP wet mount method be rapid and satisfactory enough for routine microscopic examination in intestinal protozoan infections, especially *E. histolytica* and *G. intestinalis* infection.

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