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# The Comparison of the Efficacy of Various Fixatives on Diverse Staiming Methods of *Giardia lamblia* Cyst

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**Abstract:** The definite and exact diagnosis of protozoa is possible using high magnification objective lenses, provided that suitable stained smears are prepared. Therefore, the appropriateness of both fixative and staining methods to the species of parasite, which is the main objective of this study, is important. In this study, five various fixatives including (Merthiolate iodine formalin) MIF, (Sodium acetate-acetic acid formalin) SAF, (Polyvinyl alcohol) PVA, formalin and schaudinn and four types of stains including Hematoxylin I, Hematoxylin II, Trichrome and Carbol-fuchsin were prepared using standard procedures. After the smears of stool samples containing *Giadria lamblia* cyst were prepared and kept for 24 h in various fixatives, the study was carried out using the four above-mentioned stains by changing the ingredients and time as well as by repeating the experiments. After fixing and staining all the smears in identical conditions along with the implementing interferences in the staining process, the following results were eventually obtained considering the morphologic indexes and negative and positive scores (from 1 to 20): formalin with 17 scores in hematoxylin I staining, formalin and SAF with 15 and 14 scores, respectively in Hematoxylin II staining, MIF with 13 scores in Trichrome staining and SAF, PVA, MIF with 11.5, 11.5 and 11 scores, respectively in carbol-fuchsin staining were found to be the best fixatives. Hematoxylin I staining using formalin fixative with 17 scores showed the best result while the maximum score for Carbol-fuchsin staining was 11.5 showing a necessity for more expenditure, time and expert cooperation to reach ideal results.

**Key words:** Fixatives, staining, *Giardia lamblia* cyst

## INTRODUCTION

The definite and exact diagnosis of protozoa (one-cell microscopic organisms) using photic microscopes needs sufficient skills, experience and knowledge in addition to preparing stained smears appropriate to stains and fixatives compatible with the species of protozoa. One of the samples that can be considered as the symbol of the protozoa is the *Giardia lamblia* parasite that has been spread worldwide and is easily available (Horen, 1981). The habitat of this flagellate protozoan that causes contamination is the upper part of the small intestine of human beings and some other vertebrates (Jensen *et al*., 2000; Rodney, 2001). In a study carried out in 2005 on 45128 stool samples in Iran, the prevalence of contamination by *Giardia lamblia* was found to be 10.9% (Nace *et al*., 1999).

In order to diagnose this pathogenic protozoan, the morphological diagnostic factors including the structure of the nucleus and karyosome, the number of the nuclei, median bodies, the remainder of flagella, the distance between the two membranes, as well as the difference between the parasite color with the background color are needed to be taken into consideration. Moreover, appropriate staining and fixatives which exert the least changes in the morphological structures of the parasite are essential to observe all these details. In a study carried out in California in the USA in 1992, the effect of the PVA fixative containing zinc and mercuric compounds was investigated on the intestinal protozoa in 106 stool samples stained using Trichrome staining. The results showed the fixative could substitute for other fixatives in Trichrome staining method (Garcia *et al*., 1993; Eugene *et al*., 1977). In another study performed in the US

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in 1998, Trichrome staining was found to be an appropriate way to identify the digestive parasites in concentrated stool specimens (Kellogg and Elder, 1999). In addition, a study carried out in Brazil in 1999 revealed that PVA and formalin functioned as the secondary fixatives in fixing parasitological protozoa (Piettzak-Johnston *et al*., 2000). In this study, the effects of five well-known fixatives including schaudinn, PVA, formalin, MIF and SAF on *Giadria lambia* cyst were investigated using Hematoxylin I, Hematoxylin II, Trichrome and Carbol-fuchsin staining types for 8 months.

## MATERIALS AND METHODS

This bi-agent analytical assay was conducted from June 2007 to January 2008 in Laboratory of Parasitology of Medicines Faculty of Lorestan University of Medical Sciences.

The fixatives used in the present study were as follows: 5% formalin, SAF (E.Merck, KGaA Darmstadt, Germany), a mono-solution compound which, as the name suggests, contains sodium acetate, glacial acetic acid and formalin in distilled water (Vandenberg *et al*., 2006); MIF(Merck KGaA 64271, Germany), a bi-solution compound, with the first one being Lugol's solution and the second one containing formalin, thimerosal (Merthiolate 0.001) and glycerin, which is combined with an 0.6 to 9.4 mL ratio immediately before being used and can be prepared if it is needed; Schaudinn, a combination of the saturated mercuric chloride solution ($HgCl_2$) and ethylic alcohol, of which 100 mL is added for 5 mL of acetic acid (E.Merck KGaA Darmstadt, Germany); and the fifth and the last fixative, polyvinyl alcohol (PVA), a milky solution made from combining schaudinn, polyvinyl alcohol powder and glycerin(E.Merck KGaA Darmstadt, Germany) (Garcia, 2007).

The applied stains were as follows:

- **Hematoxylin I:** It contains two stored solutions including iron Hematoxylin powder (crystal) (E. Merck KGaA 64271, Germany) in absolute ethylic acid-kept for two months to be ripped, as well as the combination of ferrous and ferric ammonium sulfate and condensed chloride acid
- **Hematoxylin II or Improved Hematoxylin:** It is a powerful decolorizer and contains carbol-fuchsin stain and picric acid (E. Merck KGaA Darmstadt, Germany) in addition to Hematoxylin I (Garcia, 2007)
- **Trichrome:** It is produced by combining chromotrope powders, light green SF, phosphotungstic acid and glacial acetic acid solution (E.Merck KGaA Darmstadt, Germany) and then dissolving the combination in distilled water (Kellogg and Elder, 1999)
- **Carbol-fuchsin or Kinyon:** (carbolic acid = phenol acid) In this staining method, in addition to carbol-fuchsin, there are similar more condensed combinations to Carbol-fuchsin in Hematoxylin II as well as alkaline methylene blue (E. Merck KGaA 64271, Germany) (Garcia, 2007)

After the positiveness of the stool samples sent from clinical diagnosis laboratories were reconfirmed, the samples were rinsed with physiologic serums. After being passed through two-layered wet gauze dressings, the samples were relatively condensed and then were added, in equal amounts, to glass containers containing the fixatives and finally the combinations were mixed. The following day, after the fixed samples were centrifuged, four appropriate smears of stool for each sample were prepared through combing equal amounts of the sample and the Mayer's glue-a combination of glycerin and the whites of eggs in equal amounts. Finally, the samples were dried and prepared for being stained with the four stains. To this point, all the procedures were nearly the same; however, each staining method has its own specific characteristics that will be briefly discussed below:

- **Hematoxylin I:** First, the smears were stained in iron Hematoxylin and then dehydrated with ethylic acid having densities of 70-100 degrees
- **Hematoxylin II:** The smears were first stained with carbol-fuchsin stain and, after being decolorized using alcohol-acid, were stained again with Hematoxylin. Then, they were dehydrated as in Hematoxylin I after picric acid was added. It needs to be explained that in the intervals between the stages, the slides were rinsed by being dipped in the containers of tap water
- **Trichrome:** The smears were decolorized using alcohol-acid after being stained with Trichrome and, like the above-mentioned stages, they were used after being dehydrated in xylene
- **Carbol-fuchsin:** The smears were stained with carbol-fuchsin and after they were decolorized using 1% sulfuric acid, methylene blue was finally added and the smears were rinsed using water

It should be explained that if the fixatives containing mercuric chloride are used, the smears have to be passed through 70%-alcoholic solutions and iodine-alcohol. Since, the purpose of the present study was to determine

the factors affecting the diagnosis, the morphological indexes had to be chosen as the criteria of the study. There had to be assessment criteria because the best one was to be chosen. Therefore, the indexes were divided into two groups. The first group of indexes included the nucleus (the nucleolus and the membrane), the flagella and their interior effects, axonemes, median bodies and the contrast between the parasite colors with the background color. These indexes were considered as the positive indexes to which from 1 to 4 scores and in total in the ideal conditions up to 20 scores, were allocated in terms of their separation power and the characteristic clarity. Additionally, the negative indexes including the distance between the two membranes and cytoplasm compression, which seemed to be of less importance, were each allocated up to 2 negative scores. After the experiments were repeatedly performed 15 times during an 7-month period and after the means of the negative and positive scores were calculated, the obtained results were shown in a Table 1. It deserves explaining that the densities of the materials included in the study and the frequency of the phases of the study were repeatedly modified and attempts were made to perform the study in an ideal condition. To analyze the data from this experiment, the SPSS 15.0 software was utilized. In design of this study the two factorial design of experiments procedure was conducted thus analysis of data was done by two way ANOVA assay (Douglas, 2004).

## RESULTS

After the five various fixatives including MIF, SAF, PVA, formalin and schaudinn and the four types of stains including Hematoxylin I, Hematoxylin II, Trichrome and carbol-fuchsin were prepared and used and after the negative and positive scores were calculated in terms of the morphological indexes and after the experiments were performed 15 times, the following results shown in Table 1, were obtained. According to the results, formalin with 17 scores in Hematoxylin I staining, formalin and SAF with 15 and 14 scores, respectively in Hematoxylin II, MIF and PIV each with 13 scores in Trichrome staining and finally SAF and PVA each with 11.5 scores in Carbol-fuchsin staining were found to be the most suitable fixatives (Table 1).

Table 1: Means of the obtained scores for each staining type in various fixatives for *Giardia lamblia* cyst

| | Fixative type | | | | |
|---|---|---|---|---|---|
| Staining type | MIF | SAF | Formalin | Schaudinn | PVA |
| Hematoxylin I | 13.0 | 10.0 | 17 | 13 | 13.0 |
| Hematoxylin II | 13.5 | 14.0 | 15 | 9 | 10.0 |
| Trichrome | 13.0 | 9.0 | 8 | 10 | 13.0 |
| Carbol-fuchsin | 11.0 | 11.5 | 10 | 9 | 11.5 |

The results also revealed that in Hematoxylin I staining with Formalin as a fixative, most of the parts of the cyst was recognizable, while carbol-fuchsin was incompatible with all the fixatives in staining *Giardia lamblia* cyst. Meanwhile, some of the fixed smears with PVA separated during the experiments.

## DISCUSSION

Considering the difficulty of the exact diagnosis of digestive protozoa using direct parasitological methods due to the smallness of the parasite and the inability to apply higher microscopic magnification (Vandenberg *et al*., 2006), the present study aimed to obtain simpler and more reliable diagnostic methods for protozoan parasites staining needing appropriate procedures to separate the morphological indexes. The abundance of the stains, on the one hand and the unavailability of these stains to diagnostic laboratories, on the other hand, led to finding the most suitable supplements compatible with staining types, namely fixatives prepared easily in every laboratory, to send the samples to more equipped diagnostic centers with the least morphological disorders. After group working for hours and hours and repeating the experiments and considering the morphological indexes, the researchers found the results shown in Table 1. According to the results, 5% Formalin as a fixative with 17 scores out of 20 in Hematoxylin I staining was found to be better than the other applied fixatives in the present study (Fig. 1, 2). Our results are similar to the results of Garcia *et al*. (1993) study on the staining of intestinal parasites, but we have made some changes in the times and ingredients of the stains to reach better results. Moreover, the results of El-Taweel and Abou Holw (2008) study revealed that trichrome staining of methanol fixed smears was the most

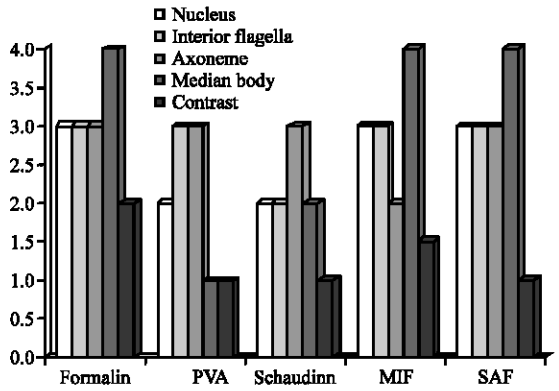


Fig. 1: The effects of various fixatives on the morphological indexes of *Giardia lamblia* cyst in Hematoxylin I staining

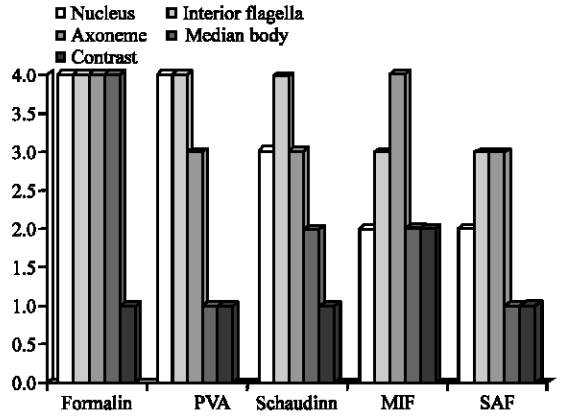


Fig. 2: The effects of various fixatives on the morphological indexes of *Giardia lamblia* cyst in Hematoxylin II staining

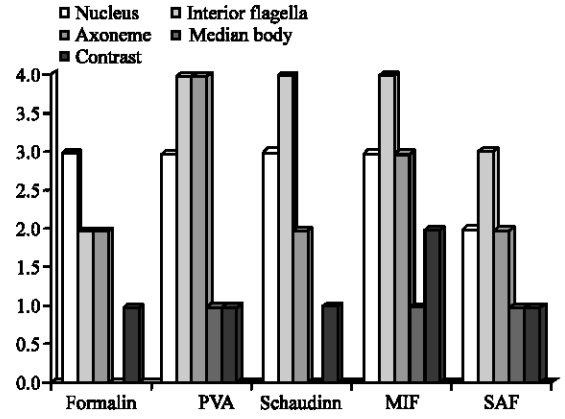


Fig. 3: The effects of various fixatives on the morphological indexes of *Giardia lamblia* cyst in Trichrome staining

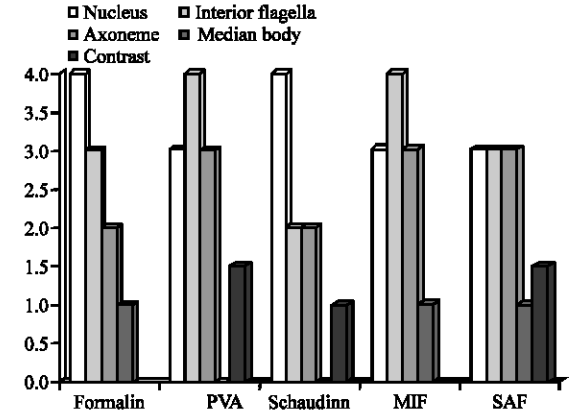


Fig. 4: The effects of various fixatives on the morphological indexes of *Giardia lamblia* cyst in Carbol-fuchsin staining

sensitive technique for *Giardia* trophozoite detection followed by MIF direct smear method. For *Giardia* cysts, trichrome staining and MIFC (direct smear method and MIF concentration) had nearly equal sensitivity and were more sensitive than MIF direct smear method. Easily reachable, more cheapness and had no toxicogenic and teratogenic effects in its component are unique specialties that make it greater than other fixatives. Although, there was a difference between this score and the ideal score of 20 showing an urgent need for more attempts and expenditure to reach the ideal point, formalin showed better results in comparison with other fixatives. Particularly, in carbol-fuchsin staining it resulted in a great amount of ambiguity and its priority to other methods might have been its excessive expenditure (Fig. 3, 4).

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