

Brilliant Blue- Non Mercuric Schaudinn's Fixative Solutions Wet Mount for Identification of Intestinal Parasites in Stool Specimens

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ABSTRACT:

BACKGROUND:

Definitive diagnosis of the intestinal parasites requires the demonstration of the organisms or eggs in feces or tissues. Stool specimens should be preserved, stained and microscopically examined.

METHODS:

Copper sulfate schaudinn's fixative (CuSO₄SF) solution and zinc sulfate schaudinn's fixative (ZnSO₄SF) solution were prepared, 2 sets of 5 concentrations (serially diluted) were prepared first set Brilliant blue food color powder (B) was dissolved in CuSO₄SF solution (B-CuSO₄SF) solution second set Brilliant blue food color powder was dissolved in ZnSO₄SF solution (B-ZnSO₄SF) solution

Merthiolate-iodine-formalin (MIF) preservative and staining solution (control solution) was prepared.

- During one year 85 positive stool samples were collected from patients complaining gastro-intestinal tract disorder.
- Stool-CuSO₄SF suspension, stool-ZnSO₄SF suspension and stool-MIF suspension were prepared from each stool sample.
- Eleven slides were prepared from each stool sample and examined microscopically by wet mount technique, 5 slides were prepared from each concentration, on a slide one drop from B-CuSO₄SF and one drop from stool-CuSO₄SF suspension were mixed.

Another 5 slides were prepared from each concentration, on a slide one drop of B-ZnSO₄SF and one drop from stool-ZnSO₄SF suspension were mixed.

One wet mount smear was prepared from one drop of stool-MIF suspension (control test).

RESULTS:

- Both solutions B-CuSO₄SF and B-ZnSO₄SF were showed similar results, these two solutions were very efficient in preserving and staining trophozoites and cysts of intestinal protozoa, helminth eggs and non parasitic findings in the wet smear of stool specimen during 24h.
- The most appropriate concentration for two solutions were 0.2% wt/vol B-CuSO₄SF and 0.4% wt/vol B-ZnSO₄SF
- During one year B-CuSO₄SF and B-ZnSO₄SF were very efficient in staining of the intestinal protozoa and helminth eggs.

CONCLUSION:

Both 0.2% wt/vol B-CuSO₄SF and 0.4% wt/vol B-ZnSO₄SF solutions may be considered as an efficient one step rapid, safe and economical stain of wet mount stool smear for using in the identification of the intestinal parasites.

KEY WORDS: brilliant blue, copper sulfate schaudinn's fixative, zinc sulfate schaudinn's fixative.

INTRODUCTION:

The detection of the intestinal parasites particularly *Entamoeba histolytica* (amebiasis) is presently one

of three most common causes of death from the parasitic diseases the vast majority of these infections

are acquired in the developing world^(1, 2,13) and other pathogenic intestinal protozoa, is an important

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goal of the clinical microbiology laboratory (3, 4). Today the diagnosis of the pathogenic intestinal protozoa is most commonly attempted by combination of microscopic examination of stool and serological testing^(5,6,7), numerous studies have demonstrated the inadequacies of microscopic examination of *E. histolytica* and other pathogenic intestinal protozoa^(8,9,10). Better approach than microscopic examination of stool either antigen detection or PCR to detect intestinal protozoa in stool, current antigen detection tests suffer from the need to examine fresh or frozen (unpreserved) stool specimens they are suffer from the fact the antigen detected are denature by fixation of stool, while PCR technique today remain impractical in many developing countries^(11,12,14,15). So that the diagnosis of intestinal parasites still rest primarily on microscopic demonstration of intestinal parasites. There are several factors that adversely affect the results of fresh sample these include delayed delivery to the laboratory (motility of amebae species can cease and trophozoites can lay within 20-30 minutes difficulty in differentiation between non motile trophozoites and polymorphonuclear leucocytes and epithelial cells)^(2,16,17,18). For these reasons a long list of staining procedures for wet mounts smears are available to overcome this problems such as iodine wet mount, a preparation using for neutral red, or methylene blue in buffered saline the dye will not affect motility of the amebae by staining the background make the amebae easier to seen, protozoan cyst can also stained by malachite green stain or some workers prefer to stains the background leaving the cyst unstained, Nairs buffered methylene blue, this stain is effective in showing nuclear detail and use at low pH the mount should be examined within 30 minutes and merthiolate-iodine – formaldehyde (MIF)^(19,20). Schaulten and yang⁽²¹⁾ demonstrated a much higher recovery of intestinal protozoan in preserved specimens. Several types of preservative solutions are available like Schaudinns fixative (SF), sodium acetate acetic acid formalin (SAF) solution to prevent disintegration of some trophozoites^(22,23,24,25,26,27). In Iraq many attempt were done in preparation of many staining procedures to stain intestinal protozoa and helminth eggs^(28,29,30). Both Schaudinns and polyvinyl alcohol (PVA) fixative with mercuric chloride (HgCl₂) base have been used to preserved stool specimen for recovery and identification of parasites primarily the intestinal

protozoa^(31, 32, 33, 34, 35, 36, 37, 38, 39). PVA is a plastic powder that is dissolved in Schaudinns fixative this plastic powder serve as an adhesive to help glue the stool onto slide when fecal smears are prepared, while the actual fixation occurs with Schaudinns solution as a result of disposal problems related to the use of mercury compounds, many laboratories have been considered switching from mercuric chloride-base to non-mercury-base preservative using copper sulfate or zinc sulfate as a substitute of mercuric chloride^(40,41).

Brilliant blue food color powder (B) has a number of color index (C.I. NO. 4090) this number identify

the dye and its chemical constitution while the name of the dye may vary with different suppliers. It is

a synthetic dye derived from coal tar^(42,43,44,45), this food color is in expensive and available in both our local markets and all foreign markets.

The standardization of the stain is also important step which should be taken in consideration after the testing procedure of a given stain.

The aim of this work is to study the characteristics of two solutions as staining solutions of wet mount smears of stool specimen and their efficiency in the identification of intestinal parasites.

First solution

Brilliant blue food color powder dissolved in copper sulfate schaudinns fixative solution (B-CuSO₄SF). Second solution

Brilliant blue food color powder dissolved in Zinc sulfate schaudinns fixative solution (B-ZnSO₄F).

MATERIALS AND METHODS:

*ZnSO₄SF was prepared⁽¹⁹⁾

*CuSO₄SF was prepared⁽¹⁹⁾

*MIF solution was prepared⁽¹⁹⁾ control

*Brilliant blue food color powder (B) was obtained from local market

• Brilliant blue food color powder was dissolved in 2 solutions CuSO₄SF (B-CuSO₄SF) and ZnSO₄SF (B-ZnSO₄SF) and 5 serial concentrations from each solution were prepared started with 1% up to 5% wt/vol.

*During the period between 1-9-2006 to 31-8-2007, 180 stool specimens were collected from patients complaining gastrointestinal tract disorder which they were attending the private laboratory in Al-Sader city, and only 85 stool specimens were positive (intestinal parasites were present their wet smears, and many stool specimens they have

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more than one type of intestinal parasite) which they were selected for this work (table -1)

*As soon as possible 11 wet mount smears were prepared from stool-CuSO₄SF suspension, stool-ZnSO₄SF suspension and stool- MIF suspension (control test)

*Preparation of 5 wet mount smears of stool-CuSO₄SF: from 5 concentrations of B-CuSO₄SF 5 drops were distributed on 5 slides on each slides only one drop was added and then each drop was mixed with similar size drop from stool-CuSO₄SF then covered with cover glass, by similar method 5 smears were prepared from stool-ZnSO₄SF and 5 concentrations of B-ZnSO₄SF, and one wet mount smear was prepared from stool MIF suspension (control test), the edges of its cover glass were sealed by finger nail polish.

*Microscopic examination were done for the 11 wet smears directly after their preparation 100X

for protozoa and 40X for helminth eggs and larvae.

*According to microscopic examination 2 wet mount smears out of 10 wet mount smears of the same stool sample which they were showed the most suitable results in the identification of the intestinal parasites one from stool-CuSO₄SF and the other from stool-ZnSO₄SF were selected, the edges of the cover glass of the two slides were sealed by finger nail polish then subjected to the schedule of periodic microscopic examination

• 4 hours after 1st examination., 14 hours after 1st examination.,24 hours after 1st examination.

• Staining capabilities of the 2 solutions B-CuSO₄SF and B-ZnSO₄SF were followed up, we were keeping all concentrations of these solutions at room temperature, and we were using them in staining of all collected stool samples during one year.

Table 1: The types, stages and numbers of intestinal parasites which they were identified in 85 wet smears of stool-MIF suspension

Intestinal parasites	Stage	Number
Amebae species	Trophozoite	14
Amebae species	Cyst	19
Giardia lamblia	Trophozoite	18
Giardia lamblia	Cyst	27
Chilomastix mesinili	Trophozoite	13
Chilomastix mesinili	Cyst	17

RESULTS:

*Brilliant blue food color powder was dissolved completely in CuSO₄SF solution and ZnSO₄SF solution

• Staining capability of all concentrations of B-CuSO₄SF solution and B-ZnSO₄SF solution, were very efficient and stable at room temperature during one year.

*The most suitable result in staining of the intestinal parasites, and clear differentiation between leucocytes and protozoan trophozoites and protozoan cysts, particularly in the presence amebae

species trophozoites and cysts were 0.2% wt/vol B-CuSO₄SF with stool-CuSO₄SF and 0.4% wt/vol B-ZnSO₄SF with stool-ZnSO₄SF, their smears of the same stool specimen were showed a similarity in staining capability, morphological characteristics and in preservation of the parasitic cells in the wet smear during 24 hours, and the detailed appearance of the main structures inside the stained and unstained protozoan trophozoites, protozoan cysts, leucocytes and semi-digested food

materials were appeared very clear during the whole period of 24 hours (figures 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16).

*The microscopic examination of wet mount smears both 0.2% wt/vol B-CuSO₄SF with stool-CuSO₄SF and 0.4% wt/vol B-ZnSO₄SF with stool-ZnSO₄SF compared with wet mount smears of stool-MIF were revealed that the morphological characteristic and the number of the intestinal parasites during 24 hours are approximately similar, which they were done from the same stool specimen. And both wet smears were appeared with colorless back ground and the cells were appeared with different levels of stain up take (description of cells coloration bellow). While the wet mount smears of the solutions which have more concentrations than these 2 solutions were appeared with colored back ground and most stained cells were predominantly appeared with one dark color. On the other hand the wet mount

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smears of the solutions which have less concentrations than these 2 solutions, were appeared with colorless background and all the cells were predominantly appeared with one pale colors.

Description of different levels of stain up take of stained intestinal parasites :

◆**Amebae species trophozoites.** Were appeared stained with stable moderate green color in microscopic examination which was done directly after preparation of stool suspension and in all microscopic examination during 24 hours (table 2),(figures 1).

◆**Amebae species cysts.** Were appeared as a bright colorless organisms in microscopic examination which was done directly after preparation of stool suspension (table 2) (figure 2)., 4 hours after preparation of stool suspension were appeared with light green color (table 2) (figure 3)., while the period between 14-24 hours after preparation of stool suspension these organisms were appeared with dark green color, (table 2) (figure 4).

◆**G.lambli**a trophozoites These organisms were appeared stained with stable dark green color, in

microscopic examination which was done directly after preparation of stool suspension and in all microscopic examination during the whole 24 hours (table 2) (figure,7).

◆**G.lambli**a cysts. Were appeared as a bright colorless organisms in the direct microscopic examination (table 2) (figure,8), 4 hours after preparation of stool suspensions the organisms were appeared with light green color (table 2) (figure 9), in the period between 14-24 hours after preparation of stool suspension these organisms were appeared with dark green color, (table 2) (figure 10).

◆**C.mesnili** trophozoites. These organisms were appeared stained with stable light green color, in microscopic examination which was done directly after preparation of stool suspension and during the whole 24 hours, (table 2) (figure 11).

◆**C.mesnili** cysts. These organisms were appeared as a bright colorless organisms, in microscopic examination which was done directly after preparation of stool suspension and during the whole 24 hours, (table 2) (figure)

Table 2: Descriptive table represent the various levels of stain up-take and the colors of some intestinal protozoa human elements which were recognized in the wet mounts smears of 0.2% wt/vol B-CuSO4SF with stool-CuSO4SF, which they were microscopically examined according to the schedule of the periodic microscopic examination .

Organism The schedule of microscopic examination according to the period of preparation of the wet mount smear of 0.2%wt/vol B-CuSSF with Stool-CuSSF	Colors of some intestinal protozoa							
	Trophozoite	Cyst	Trophozoite	Cyst	Trophozoite	Cyst	Leukocyte	Semi digested food particle
Direct	Moderate green	Unstained bright colorless	Light green	Unstained bright colorless	Moderate green	Unstained bright colorless	Dark green	Dark green
After 4h	Moderate green	Light green	Light green	Unstained bright colorless	Moderate green	Light green	Dark green	Dark green
After 14h	Moderate green	Moderate green	Light green	Unstained bright colorless	Moderate green	Moderate green	Dark green	Dark green
After 24h	Moderate green	Moderate green	Light green	Unstained bright colorless	Moderate green	Moderate green	Dark green	Dark green



Figure 1: Amebae species trophozoite was recognized in the wet mounts smear of 0.2% wt/vol B-CuSO₄SF with stool-CuSO₄SF which was examined directly after preparation of the smear and of all periodic examination during 24 hours. The organism is appeared stained with moderate green color. The arrows indicate the internal details of the organism. Objective 100 X



Figure 2: Amebae species cyst (quadrinucleated) was recognized in the wet mounts smear of 0.2% wt/vol B-CuSO₄SF with stool-CuSO₄SF, which was examined directly after preparation of the smear, The organism is appeared unstained. The arrows indicate the internal details of the organism. Objective 100 X.



Figure 3: Amebae species cyst (uninucleated) was recognized in the wet mount smear of 0.2% wt/vol B-CuSO₄SF with stool-CuSO₄SF, which was examined after 4h of preparation of the smear and of all periodic examination. The organism is appeared stained with light green color. The arrows indicate the internal details of the organism. Objective 100 X.

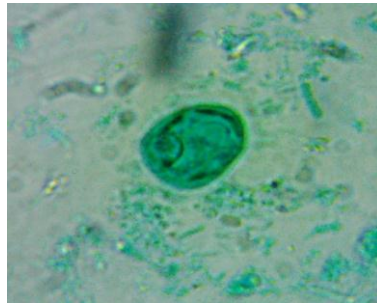


Figure 4: Amebae species cyst (uninucleated) was recognized in the wet mount smear of 0.2% wt/vol B-CuSO₄SF with stool-CuSO₄SF, which was examined after 14 and 24h of preparation of the smear. The organism is appeared stained with moderate green color. The arrows indicate the internal details of the organism. Objective 100 X.

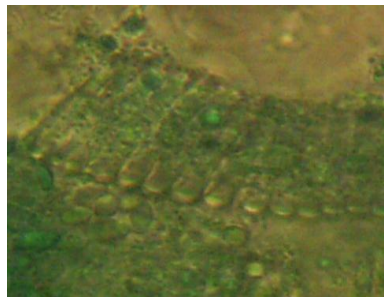


Figure 5: A particle of semi digested food material, which was recognized in the wet mounts smear of 0.2% wt/vol B-CuS O₄SF with stool-CuSO₄SF the smear was examined directly after preparation of smear and of all periodic examination during 24h the particle was stained with light green color. Objective. 100X.



Figure 6: A clump of leucocytes was recognized in the wet mounts smear of 0.2% wt/vol B-CuO₄SF with stool-CuSO₄SF, which was examined directly after preparation of smear and of all periodic examination during 24h. The cell is appeared stained with dark green color. Objective 100 X.

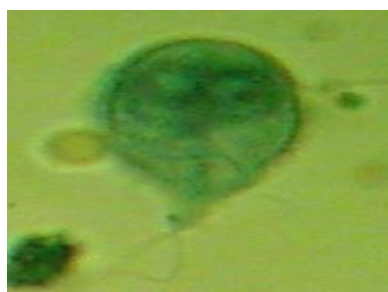


Figure 7: *Giardia lamblia* trophozoite was recognized in the wet mounts smear of 0.2% wt/vol B-CuSO₄SF with stool-CuSO₄SF directly afte preparation of the smear and of all periodic examination during 24h. The organism is appeared stained with moderate green color . The arrows indicate the internal details of the organism. Objective 100 X.



Figure 8: *Giardia lamblia* cyst was recognized in the wet mounts smear of 0.2% wt/vol B-CuSO₄SF with stool-CuSO₄SF, which was examined directly after preparation of the smear and of all periodic examination during 24h. The organism is appeared unstained stained. The arrows indicate the internal details of the organism. Objective 100 X.

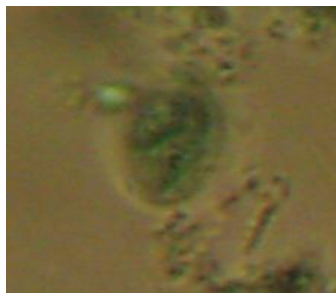


Figure 9 :*Giardia lamblia* cyst was recognized in the wet mounts smear of 0.2% wt/vol B-CuSO₄SF with stool-CuSO₄SF, which was examined 4h after preparation of the smear. The organism is appeared stained with light green color. The arrows indicate the internal details of the organism. Objective 100 X.



Figure 10 :*Giardia lamblia* cyst was recognized in the wet mounts smear of 0.2% wt/vol B-CuSO₄SF with stool-CuSO₄SF which was examined after 14h and 24 h after preparation of the smear. The organism is appeared stained with moderate green color. The arrows indicate the internal details of the



Figure 11: *Chilomastix mesnili* trophozoite was recognized in the wet mounts smear of 0.2% wt/vol B-CuSO₄SF with stool-CuSO₄SF, which was examined directly after preparation of the smear and of all periodic examination during 24h . The organism is appeared stained with light green color. The arrows indicate the internal details of the organism. Objective 100 X.



Figure 12: Chilomastix mesnili cyst was recognized in the wet mounts smear of 0.2% wt/vol B-CuSO₄SF with stool-CuSO₄SF, wt/vol.



Figure 13: Amebae species trophozoite which was recognized in the wet mounts smear of stool-MIF suspension, the smear was examined directly after preparation of stool suspension of all periodic examination during 24h. The organism is appeared stained. Arrows indicate the internal details of the cell. Objective 100X.



Figure 14 : Amebae species cyst which was recognized in the wet mounts smear of stool-MIF suspension, the smear was examined directly after preparation of the suspension and during 24h , The organism is appeared stained . Arrows indicate the internal details of the cell. Objective 100 X.



Figure 15: *G.lamblia* trophozoite which was recognized in the wet mounts smear of stool-MIF suspension, the smear was examined directly after preparation of stool suspension of all periodic examination during 24h. The organism is appeared stained. Arrows indicate the internal details of the cell. Objective 100 X.



Figure 16 : *G.lamblia* cyst which was recognized in the wet mounts smear of stool-MIF suspension, the smear was examined directly after preparation of stool suspension and of all periodic examination during 24h. The organism is appeared stained. Arrows indicate the internal details of the cell. Objective 100 X.



Figure 17 : *C. mesnili* cyst which was recognized in the wet mounts smear of stool-MIF suspension, the smear was examined directly after preparation of stool suspension and of all periodic examination during 24h. The organism is appeared stained. Arrows indicate the internal details of the cell. Objective 100 X.



Figure 18 : *C. mesnili* trophozoite which was recognized in the wet mounts smear of stool-MIF suspension, the smear was examined directly after preparation of stool suspension and of all periodic examination during 24h . The organism is appeared stained. Arrows indicate the internal details of the cell. Objective 100 X.

DISCUSSION:

Both concentrations of the two wet mount smears 0.2% wt/vol B-CuSO₄SF with stool-CuSO₄SF and 0.4% wt/vol B-ZnSO₄SF with stool-ZnSO₄SF were showed high efficiency in preservation of parasitic and non-parasitic findings that found in 2 stool smears, and these organisms were appeared very clear during the period of at least 24h, this may suggest that the Brilliant blue powder when dissolved in CuSO₄SF and ZnSO₄SF has a weak contraindication effects on the preservation capability of the 2 solutions which tends to produce fewer osmotic effects changes in the organisms leads to decrease the distortion of protozoa and helminthes ova and larvae^(46,47,48,49). Another interesting point regarding the efficiency of 0.2%wt/vol B-CuSO₄SF and 0.4%wt/vol B-ZnSO₄SF concentrations is the variability of stain uptake which provide specificity in coloration for each type of cell or organism when examined in the stained wet smears, for example amebae species and *G. lamblia* cysts was stained light green color after 4h of preparation of the 2 smears, and during the period between 14h to 24h after preparation of the two smears was appeared with dark green color, while *C. mesnili* cyst in the two smears never stained and appeared as a bright colorless organism during 24h this may be due to the nature of the cystic wall of each organism, the cystic wall of *G. lamblia* is describe as a thin hyaline membrane, whereas cystic wall of *C. mesnili* is describe as a thick hyaline wall⁽⁵⁰⁾. On the other hand osmosis and capillarity are simple physical forces which are considered by some workers as being at least partly responsible for penetration of some stains into the porous tissue⁽⁴³⁾. The variability in stain up take makes the differentiation between leucocytes which stained with dark green color and both protozoan trophozoites which either appeared with light green or moderate green color, and protozoan cysts which appeared either unstained as a bright colorless organisms or stained with light green or moderate green, is very easily particularly in the presence of amebae organisms (both trophozoites and cysts), this unique characteristic may play an important role in reducing the problems of confusions between these three types of cells. Many investigators were reported that there are several factors that adversely affect the results of microscopy. These include delayed delivery of stool samples to the laboratory (motility can cease and trophozoites can lyses within 20 to 30 minutes) leads to difficulty in differentiation between non motile trophozoites from polymorph-

nuclear leucocytes, macrophages and tissue cells^(47,51).The points which needs further investigations as the detailed standardization since it has been suggested by several authors that this process is an important one for any given stain to be tested^(52,53). In addition to that the photo-colorimetric study of the stain is another point which needs farther future investigations because it reflects the ability of the stain regarding turbidity, change in color, properties, fungal and bacterial growth or formation of surface impurities layer.

The results presented in this work has demonstrated that both 0.2% wt/vol B-CuSO₄SF and 0.4%wt/vol B-ZnSO₄SF. solutions has the ability to provide dual function with high efficiency, the preservation of all parasitic and non parasitic findings (protozoan trophozoites and cysts, helminthes ova, leucocytes, semi-digested food materials) which may be present in the wet smear of stool specimen during 24h and they are also efficient staining solutions which provides precise identification and differentiation of intestinal protozoa (trophozoites and cysts), helminth ova and human elements (leucocytes and semi-digested food particles).

CONCLUSION:

Our final conclusion is that both 0.2%wt/vol B-CuSO₄SF and 0.4%wt/vol B-ZnSO₄SF solutions may be considered as an efficient one step rapid, safe, stable for at least one year, have a simple method of preparation and they are economical stains of wet mount stool smear for using in the identification of the intestinal parasites.

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