PREPARATION OF WET AND PERMANENT MOUNT STAIN FOR STAINING INTESTINAL PARASITES WITH ONE STEP PROCEDURE [+]

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Abstract:

Detection of intestinal parasites via routine microscopy considered technique demanding processes. Modified Schaudinns fixative copper base ($CuSo_4SF$) and two food color powders Amaranth (A) and Brilliant blue (B) were utilized to create a new wet and permanent stain for identification of intestinal parasites.

Eight positive stool specimens for intestinal parasites were suspended in $CuSo_4SF$. Different concentrations of food color powder (A) and (B) were prepared by dissolving each in $CuSo_4SF$ to form A-$CuSo_4SF$ and B-$CuSo_4SF$ respectively. Each solution of A-$CuSo_4SF$ was mixed at equal ratio with each of B-$CuSo_4SF$ with the addition of several concentrations of Glycerol. Wet and permanent mounts were prepared by mixing each stool suspension with each staining solution at ration 1:1 and then examined microscopically. Follow up study for two years was conducted to detect the most optimum working concentration and to determine the stability of the permanent smears and the staining solution.

Food color powders showed high dissolving capacity in $CuSo_4SF$ solution at room temperature. The solution composed of 1.6 % (A-$CuSo_4SF$), 0.4% (B-$CuSo_4SF$) and 9% glycerol showed 100% recovery for intestinal parasites, the highest staining proficiency and used effectively as both wet and permanent mount stain. This new staining solution remained stable for two years, while the permanent smears were stable for eight months.

***Keywords:*Schaudinns fixative, food color, wet, permanent stain, intestinal parasites.**

المستخلص:

يعتبر الكشف عن الطفيليات المعويه بواسطه الفحص الروتيني باستخداد المجهر الضوئي عمليه تتطلب الكثير من التقنيات. تم استخدام المحلول المعدل من شاودنز $CuSo_4SF$ مع مسحوق الباودر الغذائي الاحمر A الاماراث والبرليانت الازرق B لتحضير صبغه رطبه و صبغه دائميه لتشخيص الطفيليات المعويه.

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تم تعليق ثمان عينات خروج حاويه على الطفيليات المعويه في محلول التثبيت شاودنز المعدل. تم اعداد تراكيز مختلفه من مسحوق الملون الغذائي A و B عن طريق اذابة كل مسحوق في CuSo4SF لتكوين CuSo4SF A و CuSo4SF B على التتابع .

تم مزج وبنسبة 1:1 محلول Aمع محلول Bبدون او مع اضافة تراكيز مختلفه من الكليسيرول. تم تحضير الصبغه الدائمة والرطبه عن طريق خلط عالق البراز بنسبة 1:1مع كل محلول صبغي من المحاليل المعدة مسبقا وتم فحصه تحت المجهر. تم اعداد دراسة لاحقه لتحديد التركيز المثالي الذي يمكن العمل به وتحديد مدى ادامة المسحات الدائمه ومحلول التصبيغ

أظهرت المساحيق الملونه للطعام قدرة عالية على الذوبان في محلول CuSo4SF في درجة حرارة الغرفة. باستخدام المحلول المكون من 1.6٪ (CuSo4SF-A)، 0.4٪ (CuSo4SF-B)، و 9٪ الجلسرين تم الكشف عن 100٪ من الطفيليات في عينات الخروج حيث انه اظهرت فعاليه عاليه حيث اثبتت هذه الصبغه فعاليتها لمدة عامين، في حين اظهرت المسحات الدائمة استقرارا و ثباتا لمدة ثمانية أشهر.

من الممكن اقتراح هذة الصبغه للتشخيص الروتيني للطفيليات المعوية و كذلك للأغراض التعليمية ومن الضروري إجراء المزيد من الدراسات لتقييم هذا الصبغه و معايرتها

Introduction:

Serological tests and molecular based methods including polymerase chain reaction (PCR) offers the best approach for the detection of intestinal protozoa, however these techniques remain expensive and impractical in many developing countries [1,2,3]. Consequently stool microscopy is considered the method of choice in the diagnosis of intestinal protozoa [4].

In the routine work of the clinical laboratories, stool examination by saline wet mount has the advantage to detect the trophozoites, however their nuclei cannot be easily seen, particularly when artifacts (epithelial cells, RBC and charcot – leyden) are present in stool smear [5].

Several staining solutions have been used for preparation of wet mount of faecal specimens including Nairs buffered methylene blue stain which is very efficient in staining the internal organelles of microorganisms, facilitating their detection and identification in the stool [6, 7].

Detection and precise identification of intestinal protozoa frequently depend on the examination of the permanent stained smear which provides the physicians a permanent record of the protozoan organisms identified and also can be used for medical consultations and educational purposes [8, 9].Trichrome stains are considered the most common permanent staining techniques for identification of intestinal protozoa, while Iron hematoxylin stain is considered to be long time consuming method [10].

Preserved stool specimen showed high recovery of intestinal protozoan than the non-preserved [11,12]. Immediate preservation of protozoan provides reliable diagnosis [13,14]. Schaudinns fixative is considered the mainstandard for preservation of intestinal parasites [15]. Many laboratories have switched from mercuric base to non-mercuric base because due to disposal problem [16].

Artificial food colors have been synthesized from organic compounds. Food colorants are sodium salts of sulfonic acid [12, 17].Each type of food color has a number of color index (C.I. NO.) which identify the dye and its chemical constitution, however the name of the dye vary with different suppliers [18,19,20].Food color powders are non-expensive and commercially available. Previous studies reported a novel one step wet mounts staining solutions for theroutine diagnoses [21, 22].The aim of this study is to prepare a staining solution to diagnose intestinal parasites.

Materials and methods:

Twenty five human fecal specimens were collected in this study from private clinical laboratory in Al- Saddar city at Baghdad during 2013. Eight stool specimens were suspected positive for the presence of Amoebae species (*Entamoebahistolytica, Entamoeba coli Entamoebadispar), Giardia lamblia*, *Chilomastixmesnili* and *Enterobiusvermicularis* parasites after direct examination of wet mount stool smearsasindicated in table (1). In this study a modified non mercuric Schaudinn's fixative copper base ($CuSo_4SF$) was prepared [23]. Mercuric chloride was substituted with copper sulfate. Each stool specimen was suspended in ($CuSo_4SF$) solution in the ratio of 3x ($CuSo_4SF$) solution: 1x of stool specimen [24]then kept at room temperature.

Table (1): Parasites detected in 8 positive stool specimens.

Parasitic organisms detected in the positive stool specimens	
Entamoebahistolytica	troph. & cyst
Giardia lamblia	troph.& cyst
Chilomastixmesnili	troph. & cyst
Enterobiusvermicularis	Ova

Two food color powders, Amaranth (A) C.I. NO. 16185 and Brilliant blue (B) C.I. O.42090 (product of international food craft company) were purchasedfrom local markets, then dissolved in$CuSo_4SF$ solution at the following concentrations wt./vol. (0.2%, 0.4%, 0.8%, 1.6%, 3.2%) to form two sets of solutions, A($CuSo_4SF$) andB($CuSo_4SF$) as shown in figure (1). Solution in the first set A($CuSo_4SF$) was mixed with each solutionof the second set B($CuSo_4SF$) at ratio of 1:1 to form a matrix of 25 staining solutions (Table 2).Five glycerol concentrations (0-12%) was added to each solution in set 1 to form five sets as indicated in Figure (2).

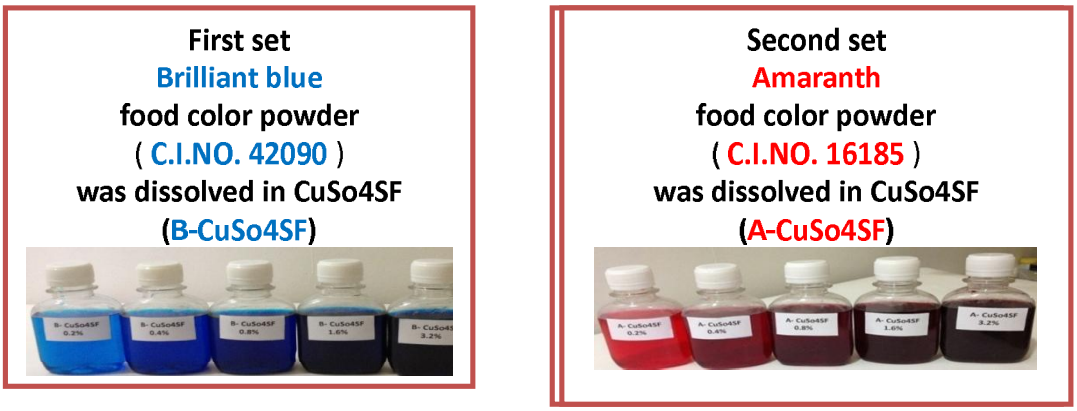


Figure (1): Sets B($CuSo_4SF$) and A($CuSo_4SF$), the following concentrations was prepared for each solution (0.2%0,4%0.8%1.6%,3.2%) wt./vol.

Table (2): A matrix of 25 staining solutions (Set 1) prepared by mixing each solution in the first set A($CuSo_4SF$) with each solution of the second set B($CuSo_4SF$) at equal ratio. Solution numbers presented in the table refer to the final concentration of A($CuSo_4SF$) and B($CuSo_4SF$).

% A-$CuSo_4SF$ &% B-$CuSo_4SF$					
	solution 1 0.2 &0.2	solution 2 0.4 &0.2	solution 3 0.8 &0.2	solution 4 1.6 &0.2	solution 5 3.2 &0.2
	solution 6 0.2 &0.4	solution 7 0.4 &0.4	solution 8 0.8 &0.4	solution 9 1.6 &0.4	solution 10 3.2 &0.4
	solution 11 0.2 &0.8	solution 12 0.4 &0.8	solution 13 0.8 &0.8	solution 14 1.6 &0.8	solution 15 3.2 &0.8
	solution 16 0.2 &1.6	solution 17 0.4 &1.6	solution 18 0.8 &1.6	solution 19 1.6 &1.6	solution 20 3.2 &1.6
	solution 21 0.2 &3.2	solution 22 0.4 &3.2	solution 23 0.8 &3.2	solution 24 1.6 &3.2	Solution 25 3.2 &3.2

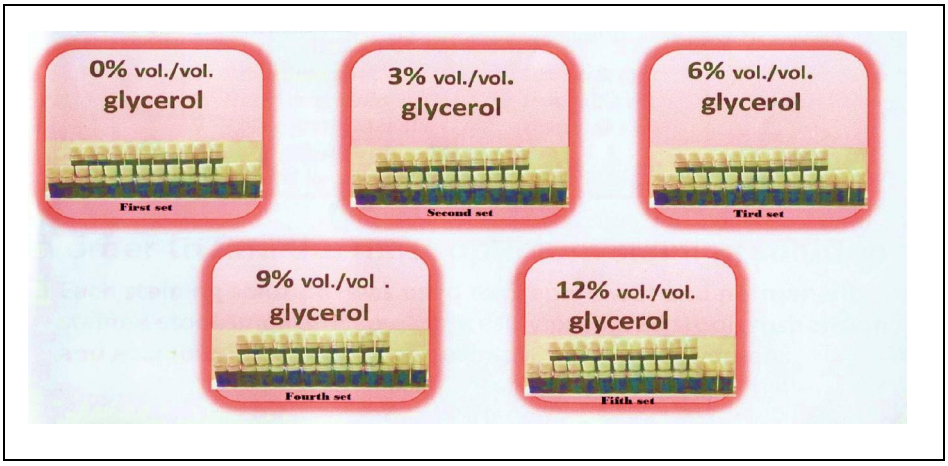


Figure (2): Sets of 25 staining solutions

Eight freshly collected, positive stool samples were suspended in CuSo4SF. In order to find the most optimum staining solution,each staining solution was used to prepare wet and permanent stained stool smears from each freshly prepared stool suspension and examined microscopically using 10X and 40X magnification.

Preparation method of wet mount stained stool smear was as follow; two drops of stool suspension and staining solution were mixed on a glass slide. The mixture of drops was covered with cover slip. For the preparation of permanent stained stool smear, two drops of stool suspension and staining solution were mixed on a glass slide. The air dried smear of the mixture was mounted with DPX (Distyrene, Plasticizer, and xylene) and covered with cover slip.

Microscopic examination was done directly after each smear preparation.Periodic microscopic examinations (once per two weeks for the duration of two years) were done for the first group of eight permanent stained smears those were stained with the most optimum staining solution. Periodic preparation of wet and permanent stained stool smears were done for all stool suspensions using the most optimum staining solution and then examined microscopically (once per two weeks for two years).

Results:

Food color powders were rapidly and completely dissolved in CuSo4SF at room temperature. Microscopic examinations of all wet mounts stained stool smears showedthe that food color powders played a role in the coloration of stool smears. Based on microscopic examinations, the colors of stained smears were classified in to three categories pale gray, purple, pale pink as shown in table (3).

In the present study solution No 9 (0.4% B-CuSo4SF, 1.6% A- CuSo4SF & 9% glycerol)showed the most optimum staining results, since it had a unique staining characteristic and showed color contrast in staining the freshly collected stool samples. Validity period of solution No 9 was six months while the validity period of the permanent stained stool smears (prepared with solution No 9) was up to six months at room temperature.

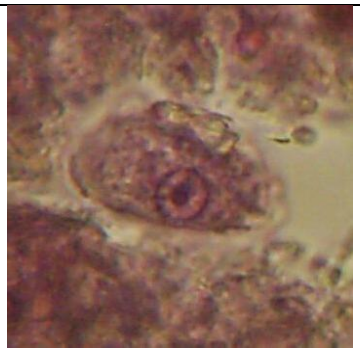
Microscopic findings using the 1stset, 2ndset, and 3rdset with0%, 3%, and 6% glycerol respectively were unclear. Whereas microscopic examinations of parasitic and non-parasitic findings in smears prepared by applying staining solutions of the 4thset, and 5th set, with 9% and 12% glycerol, were observed with different clarity. The best staining results observed by applying staining solutions number 9 (0.4% B-CuSo4SF, 1.6% A-CuSo4SF) with 9% glycerol in fourth set. Parasitic findings looked with purple color.

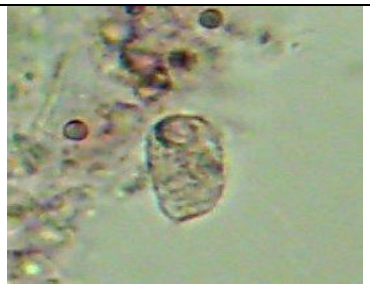
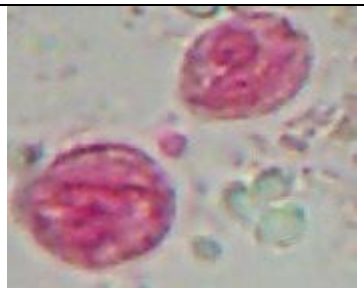
Microscopic findings other than parasites were stained with violate colorwith colorless back ground. Thiscontrast in color allowed easy identification (Table 3).Microscopic findings of wet and permanent stool smears stained using staining solution No 9 (0.4% B CuSo4SF, 1.6% A- CuSo4SF& 9% glycerol) were shown in Tables (4) and (5) respectively.

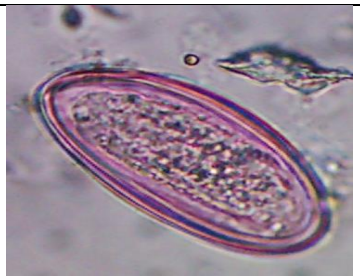
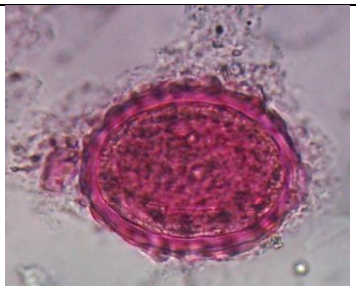
Table 3: Color categories of staining solutions according to microscopic examination of wet and permanent stained stool smears

Glycerol concentration of each set	Color categories of staining solutions according tomicroscopic examinationof wet and permanent stained stool smears		
	Pale gray	Pale pink	Red – Violate
1st set 0% glycerol	1, 6, 7, 11, 12, 13, 16, 17, 19, 21, 22, 23, 24, 25	2, 8, 14, 20	3, 4, 5, 9, 10, 15
2nd set 3% glycerol	1, 6, 7, 11, 12, 13, 16, 17, 19, 21, 22, 23, 24, 25	2, 8, 14, 20	3, 4, 5, 9, 10, 15
3rd set 6% glycerol	1, 6, 7, 11, 12, 13, 16, 17, 19, 21, 22, 23, 24, 25	2, 8, 14, 20	3, 4, 5, 9, 10, 15
4thset 9% glycerol	1, 6, 7, 11, 12, 13, 16, 17, 19, 21, 22, 23, 24, 25	2, 8, 14, 20	3, 4, 5, 9,10, 15
5thset 12% glycerol	1, 6, 7, 11, 12, 13, 16, 17,19, 21, 22, 23, 24, 25	2, 8, 14, 20	3, 4, 5, 9, 10, 15

Table 4: Microscopic examinations of wet stool smear stained by staining solution No 9 (0.4% B CuSo4SF, 1.6% A- CuSo4SF& 9% glycerol)(Magnification: 1000x).

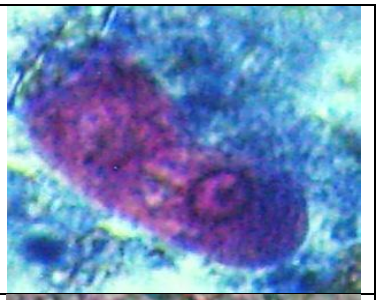
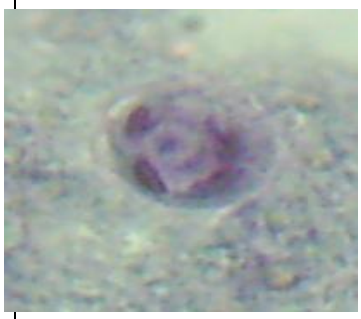
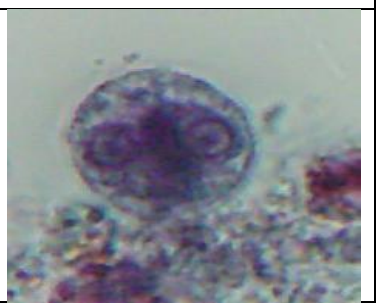
Entamoebahistolytica or *Entamoebadispar**	Trophozoite stage	
Entamoebahistolytica or *Entamoebadispar**	Cyst	

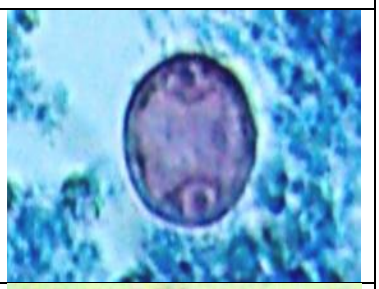
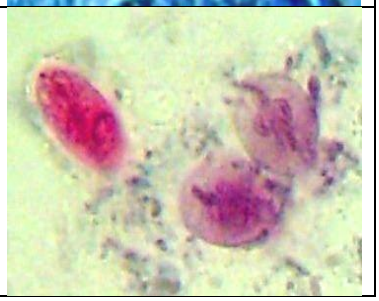
Giardia lamblia	trophozoite stage	
Giardia lamblia	Cyst	
Chilomastixmesnili	trophozoite stage	
Chilomastixmesnili	Cyst	

Enterobiusvermicularis	*(Egg)*	
Ascarislumbricoides	*(Egg)*	

***These intestinal parasites are morphologically identical. Further diagnosis are needed for specific differentiation e.g polymerase chain reaction and / or isoenzyme electrophoresis [25].**

Table 5: Microscopic examination of permanent stool smear stained with staining solution No 9 during a period less than one month of samples collection (Magnification: 1000x).

*Entamoebahistolytica or Entamoebadispar**	Trophozoite stage	
*Entamoebahistolytica or Entamoebadispar**	Cyst	
*Entamoebahistolytica or Entamoebadispar**	Uninucleated cyst	
*Entamoebahistolytica or Entamoebadispar**	Binucleated cyst	

Entamoeba coli	Multinucleated cyst	
Entamoeba coli	Binucleated cyst	
Giardia lamblia	(trophozoite& cyst)	

***These intestinal parasites are morphologically identical. Further diagnosis are needed for specific differentiation e.g polymerase chain reaction and / or isoenzyme electrophoresis [25].**

Discussion:

Modified Schaudinns fixative copper base ($CuSo_4SF$) and two food color powders Amaranth (A) and Brilliant blue (B) has improved the visual identification and clarified the morphological criteria of parasites in clinical specimens which is of high value in any laboratory procedure of diagnostic parasitology. It is established that fragile trophozoites of intestinal amoebae are rapidly being deteriorated after passage [26]. Immediate preparation of permanently stained smear upon arrival of the stool at the laboratory or immediate preservation of portion of stool in Schaudinn'sfixative is therefore necessary to maintain trophozoites in the optimal condition [27].Many laboratories do not routinely perform permanent staining due to the time and cost constraints [28].

Food color powders consist of sulfonic acid which is the main active factor in staining parasitic organisms. The clear appearance of the stained smear is attributed to the physical properties of Sulfonic acidwhich provides food color powder the ability to dissolve rapidly and completely in water [29,30]. All sulphonic acids are relatively strong acids, colorants will be ionized and carry negative charge while carboxyl and amino groups of protein materials giving protein a net positive charge. Electrostatic bonding

will then initiate between positively chargedprotein andnegatively charged colorant molecule. This will probably accounts for most of the bonding of the colorant stained or parasitic organisms in stained smears [31].
Each staining solution of wet mount stains was able to stain all of the stool smears, though concentration of food color powders influenced staining efficiency and the levels of thestain up take of microbes in wet mount stained stool smears.

A previous study [32] reported that the addition of glycerol to polystyrene sulfonate leads to high conductivity. In the present study $CuSo_4SF$ was efficient to provide good protozoan morphology and serves as adhesive agent, which glues stool material to the slide. Previous studies reported that Schaudeinns fixative act as glue due to mercury and/or due to PVA polyvinyl aniline [15, 9], though the modified Schaudeinns fixative $CuSo_4SF$ is free of mercury or PVA.

In the present study, many factors played a role in staining efficiency of the permanent stained stool smears including food color powders and glycerol concentration. Staining solutions with less than 9% glycerol concentration showed low clearance and visualization which may be attributed to the evaporation of fluid in preservative and staining solution during preparation of air dried smear. Here as in a previous report [32] it is important to highlight the main role the glycerol played in increasing the level of visualization of permanent stool smears.

In all sets of the following staining solutions; 3, 4, 5, 9, and 15, improving visualization and clear morphological characteristic of parasites was recorded (Table 2).These results confirmed that the glycerol did not play any role in staining process of wet mounts stained stool smears.

Conclusions:

The staining solution can be suggested for the routine diagnosis of intestinal parasites and for educational purposes. Further studies are necessary for standardization and evaluation of this procedure.

Recommendations:

Using preservative solution $CuSo_4SF$ and the dual function staining solution for wet and permanent stool smear together for identification of intestinal parasites and for educational purposes.

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