# The Carmoisine-Sodium Acetate Acetic acid Formalin Stain-Preservative Technique in Identification of Intestinal Parasites

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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 and stained and microscopically examined. **METHODS:** 

- Stool samples were collected from patients complaining gastro-intestinal tract.
- Carmoisine red food color powder was dissolved in 2 solutions, sodium acetate acetic acid formalin (C-SAformalin) solution, and 10% formal saline (C-10% formal saline).
- Merthiolate-iodine-formalin (MIformalin) solution was prepared, (control solution).
- Eleven stool suspensions were prepared from one stool sample directly after the passage, 5 for each serially diluted solution which mentioned above, and one for MIformalin solution.
- The suspensions of 64 positive stool samples were selected to this study and subjected to periodic examination which programmed according to the schedule during one year.

# **RESULTS:**

- C-10% formal saline was inadequate in preservation of protozoan trophozoites.
- The most appropriate concentration of C-SA formalin solution was 2% wet/vol, this solution has the ability to preserve the amoeba organisms( trophozoites and cysts ), *Chilomastix mesnili* (trophozoite and cyst), *Giardia lamblia* (trophozoite and cyst), helminth eggs and the human elements for one year when suspended in this solution, at the same time it has the ability to stain the parasitic and non parasitic findings which mentioned above very efficiently.
- The protozoan trophozoites, protozoan cysts and leucocytes these cells were showed various levels of stain uptake. All C-SAformalin solutions which were stored for periods between one day up to 18 months showed the same preservation and staining capability

#### **CONCLUSION:**

The C-SA formalin solution with 2% wt/vol. concentration has proved to be highly efficient in preservation for one year and staining of the intestinal protozoa (trophozoites and cysts), helminth eggs and humane elements, which may be found in stool specimens.

KEY WORDS: carmoisine, saformalin, 10% formal saline.

#### **INTRODUCTION:**

The detection of intestinal parasites particularly *E. histolytica* and other pathogenic intestinal protozoa, is an important goal of the clinical microbiology laboratory. It is necessary to utilize accurate diagnostic tools. Molecular biology based diagnosis may become the technique of choice in the future because establishment of most protozoa in culture is still not routine laboratory process. In all cases combination of serological tests with the detection of the parasite by antigen detection or PCR, offer the best approach to diagnosis, these current tests suffer from the fact that the antigen detected are denatured by fixation of stool specimen limiting testing to fresh or frozen samples <sup>(1)</sup>, so that the diagnosis of intestinal

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parasites still rests primarily on microscopic demonstration of the intestinal parasites. The direct examination of stool samples is employed primarily for the detection of motile protozoan trophozoites, and there for requires fresh samples. Although the simplest and least costly procedure, the direct examination of stool samples is less accurate. Problems with microscopy include the misidentification of some intestinal parasites

(Entamoeba species) is due to the accompaniment of high concentration of human elements such as epithelial cells, polymorph-nuclear leucocytes, macrophages and red blood cells with low concentration of protozoan parasites. <sup>(2, 3, 4, 5)</sup>. For these reasons a long list of staining procedures for wet mounts smears are available to overcome these problems, such as iodine wet mount, merthiolate-

iodine-formaldehyde (MIformalin), Sargeant stain, Nairs buffer methylene blue, in addition to many preservative solution for stool samples like Schudinns fixative and sodium acetate acetic acid formalin(SAformalin) solution to prevent the

disintegration of some trophozoites (6, 7, 8, 9, 10, 11). In our country many attempts were done in preparation of many staining procedures to stain different protozoa and helminthes ova (12, 13, 14). SAformalin solution is a fixative and preservative solution which has many advantages, that is simple to make, relatively nontoxic (compared to other fixative), it has along shelf life, and contain buffering capabilities which tends to produce fewer osmotic changes in the organisms leads to decrease the distortion of protozoa and preserve helminth eggs and larvae. Carmoisine is a synthetic food color, has a color index number 14720, the number of color index identify the dye and its chemical constitution, while the name of the dye may vary with different suppliers <sup>(18, 19,20,21)</sup>. This food color is inexpensive 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 objective of this work is to study the characteristic of the modified SA formalin solution (carmoisine powder dissolved in SA formalin solution) in preservation and identification of the intestinal parasites.

#### **MATERIALS AND METHODS:**

• Stool samples were collected from patients complaining the gastro-intestinal tract disorder, attending the privet laboratory in Al-Sader city, for the period between 1-6-2004 to 31-12-2005, 6 months for stool samples collection.

• Carmoisine powder was obtained from local market.

- SAformalin solution was prepared (16).
- 10% Formal saline was prepared (16)
- MIformalin solution was prepared (7)

• Carmoisine red food color powder was dissolved in 2 solutions, sodium acetate acetic acid formalin (C-SAformalin) solution, and 10% formal saline

(C-10% formal saline) solution, and 5 serial concentrations from each solution were prepared started with 1% up to 5% wt/vol, (test solutions).

• All the solutions which were used in this work were prepared at the same day, then they were stored and used , the results of preservation, and staining capabilities were followed up for 18 monthes.

• For each collected stool sample 11 screw capped tubes (10ml capacity) were prepared, and preparation of 11 stool suspensions from one stool sample which were done directly after the passage (table1).

• Microscopic examination for each collected stool sample by saline wet mount smear, iodine wet mount smear in addition to the wet mounts smears of the stool suspensions in 11 tubes were done simultaneously and directly after preparation of stool suspensions.

• From 230 collected stool samples, the suspensions of 64 positive stool samples were selected to this study they were as follow:

**1-** Ten stool samples included trophozoites of amoeba organisms.

**2-** Ten stool samples included cysts of amoeba organisms.

**3-** Ten stool samples included cysts of *Giardia lamblia*.

**4-** Ten stool samples included trophozoites of *Giardia lamblia*.

**5-** Ten stool samples included cysts of *Chilomastix mesnili*.

6- Ten stool samples included trophozoites of *Chilomastix mesnili*.

7- Two stool samples included eggs of *Entrobius vermicularis*.

**8**-one stool samples included eggs of *Ascaris lumbricoides*.

**9-**one stool samples included eggs of *Taenia* saginata.

Table (1): Explanation for the method of preparation of 11 stool suspensions from one stool sample by using two sets of five tubes, the tubes of each set containing 5 serial dilutions of one solution under test, and a control tube for MIformalin formalin solution.

	The conc	Control solution				
Test	1 <sup>st</sup> tube	2 <sup>nd</sup> tube	3 <sup>rd</sup> tube	4 <sup>th</sup> tube	5 <sup>th</sup> tube	
solutions	1%	2%	3%	4%	5%	
	3gm if formed	11 <sup>th</sup> tube				
	stool or	stool or 1ml if	stool or	stool or	stool or	containing
C-	1ml if liquid	liquid suspended	1ml if liquid	1ml if liquid	1ml if liquid	stool-
SAformali	suspended in	in 7ml C -	suspended in	suspended in	suspended in	MIformalin
n	7ml C -	SAformalin	7ml C -	7ml C -SAF	7ml C -SAF	suspension
	SAformalin	solution	SAformalin	solution	solution	
	solution		solution			
	3gm if formed	(3gm if formed				
C-10%	stool or					
Formal	1ml if liquid					
saline	suspended in					
	7ml C -10%	7ml MIformalin				
	formal saline	solution)				
	solution	solution	solution	solution	solution	

• The 64 positive stool suspensions, they were also subjected to the following processes:

♦ 1<sup>st</sup> Periodic microscopic examinations of their wet mounts smears and examining the colors and shapes of the organisms and other structures during one year.

The schedule of the microscopic examination was as follow: 1. Daily during the first week (once a day). 2. Once a month during one year.

◆ 2<sup>nd</sup> Checking up the amount of the solution of the suspended stool in each tube it was kept approximately with constant volume (7ml) during whole year.

## **RESULTS:**

- Carmoisine powder was completely dissolved in SAformalin solution, and10% formal saline. Precipitates were never appeared in the wet mounts smears during the whole year.
- No obvious differences in the results of preservation and staining between all stool samples of one intestinal parasite during the whole year.
- All concentrations of C-SAformalin solution have proved to be efficient to preserve protozoan trophozoites and cysts like amoeba organisms, and *Chilomastix mesnili*, *Giardia lamblia*, helminths eggs and the human elements like leucocytes, and semi-digested food materials of stool specimens when suspended in these solutions for one year.
- All concentrations of C-10% formal saline, were appeared inadequate for preservation of protozoan trophozoite,
- Most appropriate concentration of C-SAformalin solution, in staining of the intestinal parasites, and clear differentiation between leucocytes and protozoan trophozoites and protozoan cysts, particularly in the presence amoeba trophozoites and amoeba cysts was 2% wt/vol. In this concentration the stain uptake of these cells were appeared as follow.

◆ Amoebic trophozoites. Were appeared stained with stable moderate red color in microscopic examination which was done directly after preparation of stool suspension and in all microscopic

◆ Amoebic cysts. Were seen as bright colorless organisms during the period between direct preparation of stool suspensions up to the end of 48h (table 2) ( figures 3, 4 ), after that and during the one year these organisms were stained with stable moderate red color (table 2), (figures 5, 6).

•*G.lamblia* cyst. Was appeared as a bright colorless organisms during the period between direct preparation of stool suspensions up to the end of 24h (table 2) (figure7), after that and during the whole year this organism was stained with stable pink color (table 2), (figure 8).

◆ *G.lamblia* trophozoite This organism was stained with stable pink color, in microscopic examination which was done directly after preparation of stool suspension and in all microscopic examination during one year (table 2) (figure,9).

♦ C. mesnili cysts.

This organism was unstained and appeared as a bright colorless, in the microscopic examination was done directly after preparation of stool-C-SAformalinformalin suspensionb and in

all microscopic examination during the whole year (table 2), (figures, 10).

 $\bullet$ *C. mesnili* trophozoite. This organism was stained with stable pink color, in microscopic examination which was done directly after preparation of stool suspension and in all microscopic examination during one year (table 2) (figure 11).

•Leucocytes were stained with stable dark red color in microscopic examination which is done directly after preparation of stool suspension and in all microscopic examination during the whole year,(table 3),(figure 12).

♦ Semi-digested food materials. Were stained with stable pink color in microscopic examination which was done directly after preparation of stool suspension and in all microscopic examination during the whole year (table 3), (figures 13, 14).

◆Helminth eggs: *Ascaris lumricoides*, and *Taenia* saginata eggs were stained with moderate red color, (table 3), (figures 15, 16), *Entrobius* vermicularis egg appeared with pink color (table 3), (figure 17).

No obvious differences between preservative and staining capabilities of C-SAformalin solutions when stored for periods between one day up 18 months.

- The detailed appearance of the main structures inside the stained and unstained protozoan trphozoites, protozoan cysts, helminthes eggs, leucocytes and semi-digested food materials were appeared very clear during the whole year (figures 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17).
- In stool- MIformalin suspensions all parasitic and non parasitic findings were stained and appeared clear in the smears which were done directly after preparation of stool suspension (figures 18, 19, 20, 21, 22, 23, 24, 25, 26). Through the periodic examination and after one month of preparation of stool-MIformalin suspension precipitates were appeared, made the appearance of parasitic and non parasitic finding not clear.



Figure (1): Amebic trophozoite was recognized in the wet mounts smear of stool-C-SAformalin suspension, which was examined directly after preparation of stool suspension and of all periodic examination during one year. The organism is appeared stained. The arrows indicate the internal



Figure (2): Amoebic trophozoite was recognized in the wet mounts smear of stool-C-SAformalin suspension, which was examined directly after preparation of stool suspension and of all periodic examination during one year. The organism is appeared stained. The arrows indicate the internal details of the organism. Objective 100 X



Figure (3): Amebic cyst (uninucleated ) was recognized in the wet mounts smear of stool-C-SAformalin suspension, which was examined directly and within 48 h after preparation of stool suspension, The organism is appeared unstained. The arrows indicate the internal details of the organism. Objective 100 X.



Figure (4): Amoebic cyst (uninucleated) was recognized in the wet mounts smear of stool-C-SAformalin suspension, which was examined after 48 h of preparation of stool suspension and of all periodic examination during one year. The organism is appeared stained. The arrows indicate the internal details of the organism. Objective 100 X.



Figure (5): Amebic cyst (qudranucleated) was recognized in the wet mounts smear of stool-C-SAformalin suspension, which was examined directly and within 48 h after preparation of stool suspension. The organism is appeared unstained. The arrows indicate the internal details of the organism. Objective 100 X.

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Figure (6): Amoebic cyst (quadranucleated) was recognized in the wet mounts smear of stool-C-SAFformalin suspension, which was examined after 48 h of preparation of stool suspension and of all periodic examination during one year. The organism is appeared stained. The arrows indicate the internal details of the organism. Objective 100 X.



Figure (7) :*Giardia lamblia* cyst was recognized in the wet mounts smear of stool-C-SAformalin suspension, which was examined directly and within 24 h after preparation of stool suspension. The organism is appeared unstained stained. 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 stool-C-SAformalin suspension, which was examined after 24 h of preparation of stool suspension and of all periodic examination during one year. The organism is appeared stained. The arrows indicate the internal details of the organism. Objective 100 X.



Figure (9): Giardia lamblia trophozoite was recognized in the wet mounts smear of stool-C-SAformalin suspension, which was examined directly after preparation of stool suspension and of all periodic examination during one year. The organism is appeared stained. The arrows indicate the internal details of the organism. Objective 100 X.



Figure (10): *Chilomastix mesnili* cyst was recognized in the wet mounts smear of stool-C-SAformalin suspension, which was examined directly after preparation of stool suspension and of all periodic examination during one year. The organism is appeared unstained. The arrows indicate the internal details of the organism. Objective 100 X.



Figure (11): *Chilomastix mesnili* trophozoite was recognized in the wet mounts smear of stool-C-SAformalin suspension, which was examined directly after preparation of stool suspension and of all periodic examination during one year. The organism is appeared stained. The arrows indicate the internal details of the organism. Objective 100 X.



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Figure (12): Leucocyte was recognized in the wet mounts smear of stool-C-SAformalin suspension, which was examined directly after preparation of stool suspension and of all periodic examination during one year. The cell is appeared stained. The arrows indicate the internal details of the cell. Objective 100 X.



Figure(13) : A particle of semi digested food material, which was recognized in the wet mounts smear of stool-C-SA formalin suspension, the smear was examined directly after preparation of stool suspension and of all periodic examination during one year Objective 100 X.



Figure (14): A particle of semi digested food material, which was recognized in the wet mounts smear of stool-C-SA formalin suspension, the smear was examined directly after preparation of stool suspension and of all periodic examination during one year. Objective 100 X.



Figure(15) : Ascaris lumbricoides egg, which was recognized in the wet mounts smear of stool-C-SAformalin suspension the smear was examined directly after preparation of stool suspension and of all periodic examination during one year. Arrows indicate the details of the egg. Objective 40X.



Figure (16) : *Taenia saginata* egg, which was recognized in the wet mounts smear of stool-C-SAformalin suspension, the smear was examined directly after preparation of stool suspension and of all periodic examination during one year. Arrows indicate the details of the egg. Objective 40 X.



Figure(17) : *Entrobius vermicularis* egg, which was recognized in the wet mounts smear of stool-C-SAformalin suspension, the smear was examined directly after preparation of stool suspension and of all periodic examination during one year. Arrows indicate the details of the egg. Objective 40 X.



Figure (18): Amoebic trophozoite which was recognized in the wet mounts smear of stool-MIformalin suspension, the smear was examined directly after preparation of stool suspension and of all periodic examination during one year. Arrows indicate the internal details of the cell. Objective 100 X.



Figure(19) :Amoebic cyst which was recognized in the wet mounts smear of stool-MIformalin suspension, the smear was examined directly after preparation of the suspension and of all periodic examination during one year. Arrows indicate the internal details of the cell. Objective 100 X.



Figure (20):*G.lamblia* trophozoite which was recognized in the wet mounts smear of stool-MIformalin suspension, the smear was examined directly after preparation of stool suspension and of all periodic examination during one year. Arrows indicate the internal details of the cell. Objective 100 X.

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Figure(21) :G.lamblia cyst which was recognized in the wet mounts smear of stool-MIformalin suspension, the smear was examined directly after preparation of stool suspension and of all periodic examination during one year. Arrows indicate the internal details of the cell. Objective 100 X.



Figure(22) :*C. mesnili* cyst which was recognized in the wet mounts smear of stool-MIformalin suspension, the smear was examined directly after preparation of stool suspension and of all periodic examination during one year. Arrows indicate the internal details of the cell. Objective 100 X.



Figure(23) :*C. mesnili* trophozoite which was recognized in the wet mounts smear of stool-MIformalin suspension, the smear was examined directly after preparation of stool suspension and of all periodic examination during one year. Arrows indicate the internal details of the cell. Objective 100 X.



Figure(24) : *Taenia saginata* egg, which was recognized in the wet mounts smear of stool-MIformalin suspension, the smear was examined directly after preparation of stool suspension and of all periodic examination during one year. Arrows indicate the details of the egg. Objective 40 X.



Figure(25) : Ascaris lumbricoides egg, which was recognized in the wet mounts smear of stool-MIformalin suspension the smear was examined directly after preparation of stool suspension and of all periodic examination during one year. Arrows indicate the details of the egg. Objective 40X.



Figure(26) : *Entrobius vermicularis* egg, which was recognized in the wet mounts smear of stool-MIformalin suspension, the smear was examined directly after preparation of stool suspension and of all periodic examination during one year. Arrows indicate the details of the egg. Objective 40 X.

Lists of abbreviations:								
1	en	Endoplasm	9	ec	Ectoplasm	17	cw	Cell wall
2	v	Vacuole	10	ch	Chromatoidal body	18	su	Sucking disk
3	cy	Cytostome	11	1	Larva	19	a	Axonemal component
4	al	Albuminus layer	12	eg	Egg cell	20	me	Membrane layer
5	ml	Middle layer	13	s	Shell			
6	ho	Hook let	14	e	Envelope			
7	f	Flagellum	15	le	Leucocytes			
8	р	Parabasal body	16	n	Nuclease			

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Table (2): Descriptive table represent the various levels of stain up-take and the colors of some intestinal protozoa which were recognized in the wet mounts smears of 2% wt/vol. stool - C-SAformalin suspensions, which were microscopically examined according to the schedule .

$\mathbf{N}$ .	Colors of some intestinal protozoa						
Organism	Amoeba organisms		Chilomastix mesni	ili	Giardia lamblia		
The							
schedule	Trophozoite	Cyst	Trophozoite	Cyst	Trophozoite	Cyst	
of	Tophozotic	Cyst	TTOPHOZOIC	Cyst	Tophozotte	Cyst	
microscopic							
examination							
according to the							
period of preparation							
of stool C-SAformalin N							
suspension.							
	Stable moderate	Unstained	Stable pink	Unstained	Stable pink	Unstained	
Direct	red	bright	color	bright	color	bright	
		colorless		colorless		colorless	
	Stable moderate	Unstaied	Stable pink	Unstained	Stable pink	Unstained	
Within	red	bright	color	bright	color	bright	
24 h		colorless		colorless		colorless	
	Stable moderate	Unstained	Stable pink	Unstained	Stable pink	Stable pink	
Within	red	bright	color	bright	color	color	
48 h		colorless		colorless			
Daily at the3 <sup>rd</sup> day up	Stable moderate	Stable	Stable pink	Unstained	Stable pink	Stable pink	
to7 <sup>th</sup> day and once a	red	moderate red	color	bright	color	color	
month				colorless			
during one year							

 Table (3): Descriptive table represent the colors of the helmiths eggsand some human elements which were recognized in the wet mounts smears of 2% wt/vol. stool – C-SAformalin suspensions, which were microscopically examined according to the schedule .

· Organism	Colors of some helminths ova and some human elements						
Tha		Helmin	Human elements				
schedule of microscopic examination according to the period of preparation of stool C-SAformalin suspension.	Entrobius vermicularis	Taenia saginata	Ascaris lumbricoides	Leucocytes	Semi-digested Food materials		
	Stable pink	Stable moderate	Stable moderate	Stable dark	Stable pink		
Direct	color	red	red	red	color		
Within 24 h	Stable pink color	Stable moderate red	Stable moderate red	Stable dark red	Stable pink color		
Within 48 h	Stable pink color	Stable moderate red	Stable moderate red	Stable dark red	Stable pink color		
Daily at the3 <sup>rd</sup> day up to7 <sup>th</sup> day and once a month during one year	Stable pink color	Stable moderate red	Stable moderate red	Stable dark red	Stable pink color		

## **DISCUSSION:**

All concentration of C-SAformalin solution have been showed high efficiency in preservation of parasitic and non-parasitc findings that found in stool-C-SAformalin suspension, and these organisms were appeared very clear during the period of one year, this may suggest that the Carmoisine powder when dissolved in SAformalin solution has no contraindication effects on the preservation capability of SAF solution which tends to produce fewer osmotic effects changes in the organisms leads to decrease the distortion of protozoa and helminthes ova and larvae (22, 23, 24, 25). Another interesting point regarding the efficiency of 2% wt/vol .concentration of C-SAformalin solution is the variability of stain uptake which provide specificity in coloration for each type of cell or organism when suspended in this staining solution, e g . G. lamblia cyst was stained after 24h of preparation of stool C-SAformalin suspension, and C. mesnili when suspended in this solution was never stained and appeared as a bright colorless organism during one year preservation. 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  $^{(26)}$ . 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 <sup>(19)</sup>. The variability in stain up take make each of the following cells appeared with specific color, the protozoan trophozoites were stained with light red color, protozoan cysts either unstained and appeared as bright colorless organism or stained with moderate red color, and leucocytes were stained with dark red color. This unique characteristic in this staining solution in compared to other common stains of wet mounts stool smears, which makes the differentiation between these three cells very easily particularly in the presence of amoeba organism, and may play an important role in reducing the problems of confusion between amoebic trophozoites, amoebic cysts with the leucocytes. 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) difficulty in differentiation between non motile trophozoites and polymorph-nuclear leucocytes, macrophages and tissue cells <sup>(23, 27)</sup>. On the other point, this work may need further investigations is the

standardization of stain in some details as well as photo-colorimetric assessment since it has been several authors that suggested by these investigations are of great importance in selecting dyes <sup>(28, 29)</sup>. The results presented in this work has demonstrated the C-SAformalin solution with 2% wt/vol. concentration has the ability to provide dual function with high efficiency, first preservation of all parasitic and non parasitc findings (protozoan trophozoites and cysts, helminthes ova, leucocytes, semi-digested food materials ) which may be present in stool specimen for one year, second a precise identification of intestinal protozoa, and helminthes ova and has a high efficiency in differentiation between parasitic findings and the artifacts. Our final conclusion is that C-SAformalin solution of 2% wt/vol. concentration can be used as an efficient stain one step rapid stain for wet mounts stool smear and preservative solution for all parasitic and non parasitic findings which may be found in stool specimens.

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