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New and ModifiedStaining TechniquesforRapid Diagnosis of Hemoparasites in Blood Smears of Cows

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Abstract:
Blood smear stained with Giemsais considered as a gold standard for early diagnosis of hemoparasites, however, this technique is time consuming.It has low sensitivity in detecting mixed infections in endemic areas.The aim of this study is to overcome these problems through the use of new rapid modified stains (Rapid kit Giemsa, Cytochrome ™ stain, and Acridine orange)and improveproperties of traditional Giemsa by using a mixture of Leishman-Giemsa, Giemsa-Acridine and Giemsa toluidine blue stainsto enhance the visibility of parasites in blood.Thick/thin blood smears were prepared from 60 suspected parasitemic cows.Microscopic examination was performed on each set of blood smears stained with different stains.The quality indexand Cohen's kappa index (κ)) were calculated to assess the efficiencyand compatibility of each modified stain compared with traditional Giemsa.Modified stains reduced the turnaround time to 2-10 minutes in comparison to theconventionalGiemsa (45-60 minutes).Ahigher quality index (1.0) was obtained with cocktail stains, theywere better than the simple stainsin diagnosing Babesia, Theleria,Ehrlichia and Trypanosoma.From the results of the kappa values, the Giemsa rapid stain showed a complete agreement (κ = 1.000) with the traditional Giemsa in the diagnosis of blood parasites.In addition, the Giemsa acridineorange cocktail showed complete and true agreement with theGiemsa.Each of the dyes used showed distinctive coloring properties for each type of blood parasite that enable the microscopist to quickly distinguish and diagnose them accurately.This increased the reliability and ease of microscopic detection of hemoparasites in blood smear.

Key words: *Microscopy, Giemsa stain, Modified stains, Hemoparasites, Cows*

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I. Introduction

Rapid and accurate diagnosis is an essential for institution of the effective treatment of economically important hemoparasites infection of cattle, management of disease control, especially that play a role in the zoonosis and in the epidemiological studies.Haemoprotozan and rickettsia infections are a major economic constraint to the production of cattle worldwide (1). Identification of these hemoparasites has become more important with the increase in hemoparasitesinfections imported in the world in general and in our regions of Iraq particularly (2and3). The initial diagnosis of hemoparasites depends mainly on microscopic examination of stained blood smears as the gold standard technique for diagnosis and is widely used in all international laboratories according to the recommendations of the World Health Organization (4). Early diagnosis not only reduces the suffering of infection, but also reduces the transmission of the parasite between animals and herds and thus the possibility of its spread and transmission to humans.Therefore, rapid and accurate laboratory diagnosis and identification of its species is extremely necessary, despite the use of increasingly rapid diagnostic tests such as serology and immunofluorescence,microscopic examination of stained blood smears has remained a gold standard as it offers a quantitative evaluation of peripheral blood parasitism and the identification of hemoparasites stages along with information on the presence of mixed infection in the same sample(5and 6).

Blood smear staining is animportant step in the improvement of the contrast of a microscopic image in the setting of microscopy, particularly to highlight different structures in blood cells and cytoplasmic details (7). Generally,the conventional blood stainssuch as Giemsa stain belongs to the group of Romansky'sstains are useful in routine staining peripheral blood smearsduring the study of blood cellsmorphology, differentialleucocytes, and aids in the diagnosis of different hemparasitic infections (8). Conventional Giemsa stained blood smear examination by light, microscopy remains the gold standard for the diagnosis of hemoparasites in endemic region, has the advantages of being informative,sensitive,reasonablylow-cost, offers permanent record and can behelpful with other disease control programs. However, this technique suffers from being time consuming, labor intensive, requires good training and may offer poor results in low

parasitemiccases (9).Minimal turnaround time required for staining and assessment of peripheral blood smearshas inspiredmodifications in staining techniques that require minimum staining time with unequivocal parasitemorphology. To overcome these problems and improve quality of the traditional Giemsa stain for the rapid and accurate detection of parasite in blood smear, new stains have been modified and compared with conventional Giemsa staining. Modifications have been developed by many researchers (6, 7, 8, 10,11, 12 and 13) to traditional Giemsa stain in order to improve the coloring quality and/or to reduce staining time.

A few researchers in Cytology have described the cocktail staining techniques gives an excellent appearance and staining properties to the cytoplasm and nuclei of cells comparable to and superior to other dyes known to stain cells such as hematoxylin and eosin and other cell dyes such as May-Grunewald MG stain (12,14,15). Other studiesby (10,16) were conducted on cytology of fine needle aspiration smear to evaluate and compare the staining quality of Leishman-Giemsa cocktail with May-Grunewald-Giemsa stain. It was shown that it is superior to the quality of the Leishman-Giemsa stain in staining and diagnostic ability in fine needle aspiration smears.

However, all the previous scientific research and studies have indicated the use of different stains to scan the blood to detect malaria parasites in humans. There are few studies in this field regarding hemoparasites in animals, including cattle, especially those with intraerytrocytic infections such as piroplasm (Babesia and Theleria), and rickettsia infection (Anaplasma) or extraerytrocytic such as trypanosomes. In view of the difficulty of visualization and differentiating between them during rapid scanning blood stained with routine blood stains such as Giemsa, we decided to conduct this study using different modified stainsto detect parasites in cow's blood scanning.

II. Materials And Methods

Ethical approval

The study has been approved by the Department of Internal and Preventive Medicine, College of Veterinary Medicine, University of Mosul, Mosul, Iraq.

Sample collection

A total of 60 clinical cases of cows(1 to 4 years)with history of blood parasite infections were receivedin the Veterinary Teaching Hospital of the College of Veterinary Medicine/ University of Mosul, Iraq.Blood samples(2ml) were collectedfrom each case,and thoroughly mixed with EDTA in a special container.Processing and analysis of the samples were conducted in the clinical pathology laboratory.

Peripheral blood smears preparation and staining

Thin/ Thick smears were prepared on the same slide and stained with 7 different stains for each case. The first set of slides was stained byconventional Giemsa stain (G) (Bio lab Diagnostics Ltd, India). The other sets were stained by the following modified stains: Rapid kit Giemsa stain (RGS)(Bio lab Diagnostics (I) Pvt. Ltd., Product No. J – 245, batch No. 401501), CYTOCHROM^{TM} – Modified Leishman, working solution (Bio lab Diagnostics (I) Pvt. Ltd., Product No. J – 245, batch No. 401501) (MLS), Acridine orange (AO)(Sigma-Aldrich: No. 318337). A mixture of Leishman Giemsa stain Acridine and Giemsa (GAO), Giemsa -Toluidine blue mixture (GTB).

Giemsa stain (G): A thick blood smear was fixed by dipping for 1-2 minutes three times in acetone in a Coplin jar and air dried. They were stained with Giemsa dye (standard) at 5% concentration for 45 minutes. As for the thin smear, it was covered with absolute methyl alcohol for 5 minutes (fixation), air dried and then placed in Geimsa Standard (10%) dye for 30 minutes. Slides were gently washed in buffer solution (pH 7.2) and then air dried (7).

Rapid kit Giemsa stain (RGS): For air dried unfixed thick smear, to dehaeoglobinization process, few drops of distilled water were added to cover the smears for 1-2 minutes. Then water was removed,air dried and fixedwithEasy-Fix spray. The fixed smear wasthendipped in Rapid- Giemsa stain-II for 5-6 seconds and washed in adistilled water. The next step is dipping the smear in Rapid – Giemsa Stain-I for 35-45 seconds,washing in water and air dried.For thin smear, it was fixed with Easy-Fix spray and air dried. Then dipped in Rapid-Giemsa stain –II for 3-5 seconds andwashed in distilled water.The next step is dipping the smear in Rapid – Giemsa stain –I for 35-45 seconds, washing in distilled water and finally, the smear was air dried.

Cytochrome ^{TM} stain (Modified – Leishman's (MLS):Thick smear, one volume of Cytochrome ^{TM} stainwas diluted with two volume of buffer (pH 7.3), mixed welland covered with diluted stain. After waiting for 6 minutes, the stain was carefully removed by briskly flushing with plenty of water and air dried. For unfixed thin smear,it was coveredwith 0.5 ml (8-10 drops) Cytochrome ^{TM} stain, waited for 60- 80 seconds than double the quantity (1 ml)of working buffer was added. The next step was washing carefully after 6 minutes and finally, the smear was air dried and was examined under 100 X objective.

Acridine Orange stain: A stock solution was prepared of Acridine orange (AO) at 1 mg/ml in distilled water. A working solution of AO was prepared by diluting the stock solution 1:10 in phosphate buffered saline

(PBS) pH 7.4. The final AO concentration in the working solution will be 0.1 mg/ml. A thick blood smear was prepared on a microscopy slide. Thin smears were fixed with Easy-fix spray.The smears were allowed to dry for 20-30 seconds.The slides were dipped with freshly prepared AO working solution, and let to stain for 2 minutes. Finally, the slides were rinsed carefully with PBS and allowed to dry in air.

Leishman Giemsa cocktail (LG), was prepared by the using the protocol of (12)with modification,the unit volume of Giemsa was thoroughly mixed with a samevolume ofdistilled water to arrange Giemsa working solution; A sameamount of Leishman's stain was filtered and thoroughly mixed witha same amount of the prepared Giemsa working solution;A dry blood smear was immersed in an LG mixture and left for 1 minute. An equal volume of both dyes (1:1), diluted with buffer solution instead of distilled water and left for 6 minutes. Then, they were washed in distilled water and dried in air.

Giemsa –Acridine Orange stain mixture (GAO)was prepared in a ratio 2:3 in which 40 ml of concentrated Acridine Orange was mixed with 60 ml of concentratedGiemsa. The mixture of both stains wasflooded on each slide and left for 10 minuteson the bench at room temperature beforerinsing with buffered distilled water with PH6.8(6).

Giemsa Toluidine blue stain mixture (GTB): TheToluidine stain was prepared according to ref. (13). Then the Toluidine stain was mixed with an equal amount of Giemsa stain. The air-dried blood smear was immersed in the Giemsa-toluidine blue mixture for one minute. Then smearwas washed with deionized water and air-dried.

Microscopic examination of stained blood smears

All slides were screened blindly by two microscopistunder oil immersion of the light microscope. In case of disagreementbetween the microscopist indiagnoses of hemoparasites, the blood smears were reexamined by both of them to reach a consensus. In addition to screening for detection of hemoparasites in the blood smears, the slides were also evaluated in terms of speed, ease of reading and the turnaround time of the results. The quality of modified staining was assessed by considering scores which were given for five parameters;turnaround time of staining, overall staining, RBC morphology, clarity ofparasite and ease of speciationaccording to the morphological characteristics. The maximum possible score for a single casetaking into consideration all of the five parameters is 15. Quality Index (QI) of the stain was obtained by dividing the actual score obtained by the maximum possible (10, 17). The calculated quality index for each modified stain was compared with the corresponding quality index ofconventional Giemsa stain. Agreement between each modified stain and conventionalGiemsa for hemoparasites detection using these modified staining techniques was compared using Cohen's kappa index(κ) and its 95% CIas follows: poor, $\kappa < 0$; light, $\kappa = 0$-0.2;fair, $\kappa =$ 0.21-0.4; moderate, $\kappa = 0.41$-0.6; substantial or true,$\kappa = 0.61$-0.8; and almost perfect, $\kappa = 0.81$-1.

Statistical analysis: Descriptive statistical analysis was done using SPSS software version 21.0. The results for continuous measurements were mean ± standard deviation (SD), P value < 0.05was considered as significant.

III. Results

Table 1 and 2 show the scores and quality indexes (IQ) for simple rapid and mixture stains.In conventional Giemsa stain the minimum obtained score was 0.53 (for Theleria) and the maximumobtained score was0.74(for Anaplasma).Compared to the conventional Giemsa stain the QI of RG stain is better. The minimum obtained quality index was 0.71(for Trypanosoma) and the maximum was 0.97 (for Anaplasma) in a comparison to the standard Giemsa which was 0.74. In ML stain the minimum QI was 0.85 (for Trypanosoma) and the maximum index was 0.98 (for Theleria), whereas in conventional Giemsa was 0.53. The minimum index of AO obtained was 0.74 (for Theleria)and maximum index was 0.90 (for Ehrlichia) with 0.53 (for Theleria) and 0.72 (for Ehrlichia) in the Giemsa stain. The cocktail stains are the best in the assessment of parasite morphology. The QI of LG was 1.00 for Ehrlichia in comparison to the Giemsa which was 0.72. The maximum quality index of GAO was 1.00 for Babesia. In GTB stain the maximum index was 1.00 for Babesia,Theleria and Trypanosoma.

Table 1: Quality index (mean ± SD) scores of simple Modified stains and conventional Giemsa stain

Hemoparasites	G		RG		ML		AO	
	Score	QI	Score	OI	Score	QI	Score	QI
Babesia	10.05/15	0.67 ± 0.02	12.3/15	0.82* ± 0.03	13.8/15	0.92* ± 0.01	12.9/15	0.86* ± 0.05
Theleria	7.95/15	0.53 ± 0.06	13.8/15	0.92* ± 0.01	14.7/15	0.98* ± 0.02	11.1/15	0.74* ± 0.06
Anaplasma	11.1/15	0.74 ±	14.5/15	0.97* ±	13.8/15	0. 92* ± 0.01	12.3/15	0.82* ±

		0.06		0. 02				0.04
Ehrlichia	10.8/15	0.72 ± 0.01	12.9/15	0.86* ± 0.02	13.5/15	0.90* ± 0.08	13.5/15	0.90* ± 0.08
Trypanosoma	10.5/15	0.69 ± 0.02	10.65/15	0.71* ± 0.04	12.75/15	0.85* ± 0.06	12.45/15	0.83* ± 0.03

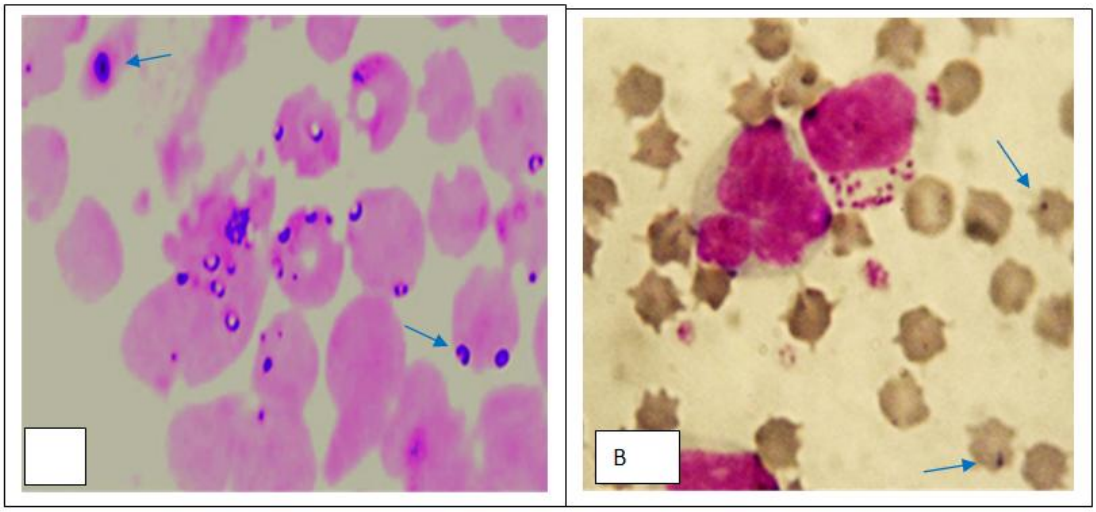
G=Giemsa, RG= Rapid kit Giemsa, ML=Cytochrome ™ stain, AO=Acridine Orange, Q.I. =Quality index. *P value < 0.05 was considered as significant.

Table 2: Quality index (mean ± SD) scores of cocktail stains and conventional Giemsa stain

Hemoparasites	G		L-G		G-AO		G-TB	
	Score	QI	Score	OI	Score	QI	Score	QI
Babesia	10.05/15	0.67 ± 0.02	12.9/15	0.86*± 0.05	15/15	1.00*± 0.001	15/15	1.00*± 0.001
Theleria	7.95/15	0.53 ± 0.06	11.1/15	0.74* ± 0.06	13.8/15	0.92*± 0.01	15/15	1.00* ± 0.001
Anaplasma	11.1/15	0.74 ± 0.06	12.75	0.85*± 0.01	13.5	0.90*± 0.08	13.8/15	0.92* ± 0.01
Ehrlichia	10.8/15	0.72 ± 0.01	15/15	1.00*± 0.001	14.5/15	0.97*± 0.02	13.5/15	0.90* ± 0.08
Trypanosoma	10.5/15	0.69 ± 0.02	11.85/15	0.79* ± 0.03	14.25/15	0.95* ± 0.03	15/15	1.00*± 0.001

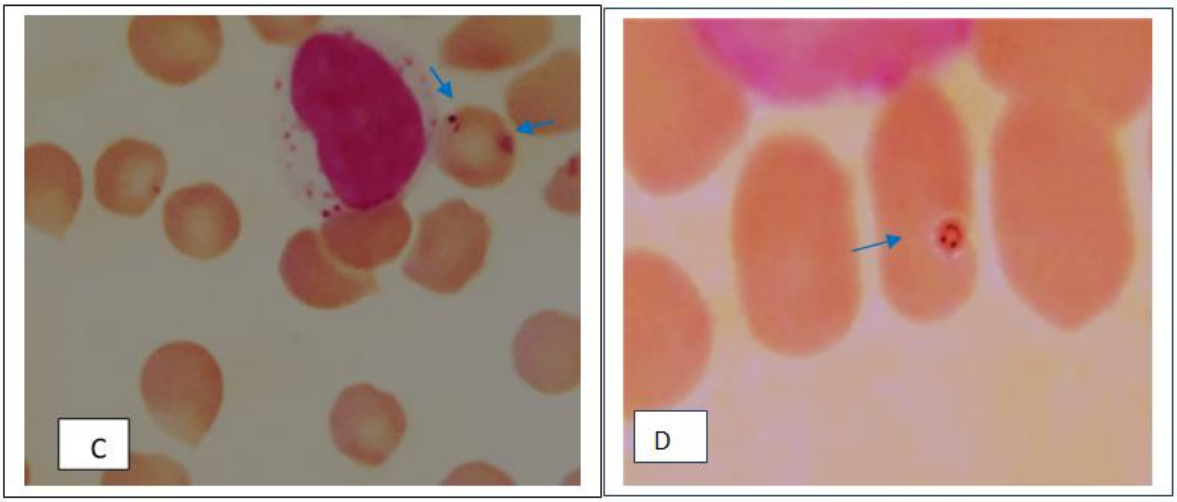
G= Giemsa, LG= Leishman- Giemsa, GAO=Giemsa- Acridine Orange, GTB= Giemsa -Toluidine blue, Q.I. =Quality index. *P value < 0.05 was considered as significant.

Rapid kit Geimsa stain with a dilution of 1:10 was better than the traditional Giemsa (G) with a dilution of 1:5 by its applicability to thick and thin blood smears, and the time required to perform staining (2 minutes) is shorter compared to the GS stain (30-45 minutes). The piroplasm (Theleria and Babesia) are stained in blue-violet color and Anaplasma where seen as a drake purple dot in the dry thin blood smears, where stained with Rapid Geimsa stain compared to the coloration of the red blood cells, which were stained with a pale pinkish color against a gray background, which distinguished the parasites from the rest slide contents (Figure1: A, B).



Figurer 1: (A) microscopic graphs of thin blood smears of cows, Theleria annulata stained with blue-violet color with Rapid Giemsa stain, (B) Theleria piroplasm appered dark brown inside RBC in blood smear staind with traitional Giemsa. Light microscope, 1000X.

The Cytochrome ™ stainwas more homogenous stain in comparison to the conventional Giemsa.Piroplasmwaseasily demonstrated which stained with orange color and their nucleus with dark red color against brown coloration of erythrocyte and gray background. This helps to distinguishhemoparasites from thebackground over staining of traditional Giemsa stain (GS) which may be confused for backgroundmaterial (Figure 2C, D).



Figurer 2: (C) Consistent staining and clear background of the blood smears stained with Cytochrome ™ stain, Theleria piroplasm easily detected. (D) Babesia piroplasm stained with orange color and their nucleus with dark red color against brown coloration of erythrocyte and gray background, Light microscope, 1000 X.

Due to consistent staining and clear background of the blood smears stained with Cytochrome ™ stain (modified Leishman stain), Theleria could be detected in 14 (82.35%) of the cases on microscopy of the thick films stained with the modified method in comparison to only 10 (58.8%) with the conventional Giemsa stain. Furthermore, the turnaround time for reporting the microscopy test was reduced to 1–10 min for the modified staining method in comparison with90 min for the standard staining methods.

Acridine orange (AO) staining method was easily applied and required less time (2-5 minutes) versus 60 minutes of traditional Giemsa stain. Also, AO stain was able to determine 13 (72.22%) cases of Babesia and 10 (100%) cases of Ehrlichia in fixed thin blood smears of cattle under oil immersion (100 X) of the light microscopy compared to only 11(61.11) and 7 (70.00%) cases of Babesia and Ehrlichia respectively with standard Giemsa. The AO staining method provides an alternative to Giemsa for the rapid screening of hemoparasites in cattle under light microscopy. Acridine orange dye showed that it is a simple and fast technique for scanning large numbers of dry blood smears in a short time (2-5 minutes)versus 60 minutes of Giemsa stain.The bright yellow luster of the blood parasites was observed with Babesia piroplasminside red blood cells and trypanosomes were freebetween cells in a brilliant yellow color in dark room orminimizing the light intensity of microscope(Figure 3:E,F).

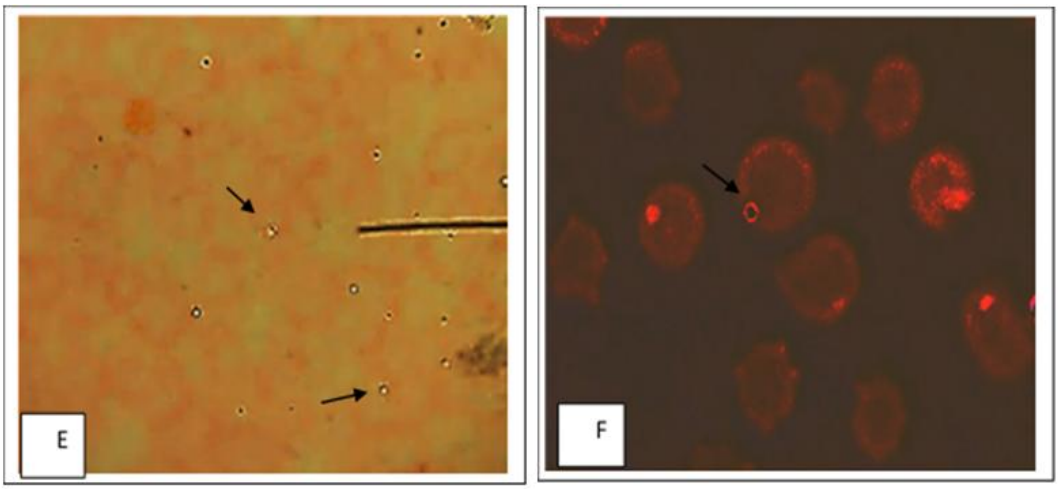


Figure 3 :(E) Brilliant Babesia piroplasm inside RBC in thick blood smear. (F) Orange color with luster of piroplasm inside RBCs in thin smear, when decrease the intensity of light.Acridine Orange stain, light microscope, 1000X.

The morulae of Ehrlichia clearly appeared as a largeround vacuole with blue to lavender in color, with a clumped or spotted appearancein the cytoplasm of monocytes or neutrophilswith LG staining.The color of the morulae is different from the color of the leucocytes nucleus and this canhelp todifferentiate an organism from a small nuclear lobe,whereas this structure is not clearly appeared in blood smears when stained with Giemsa or Leishman alone, the identification of such cells is quite subjective and clinical pathologist-dependent(Figure 4:G, H)

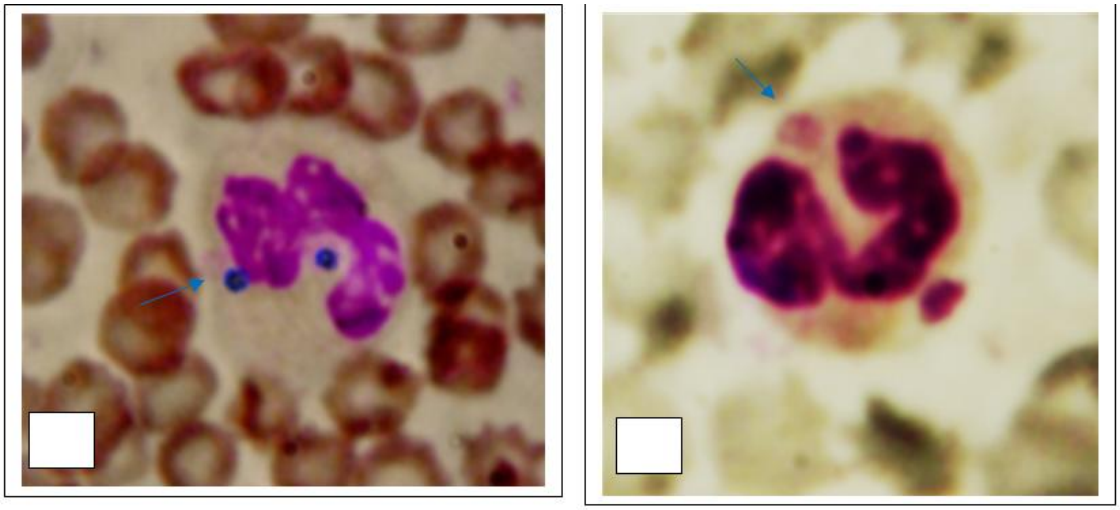


Figure 4 :(G) Ehrlichia morulae clearly appeared as a large round vacuoles with blue color, in the cytoplasm of neutrophils with Leishman Giemsa cocktail stain (H) Large morulae intracytoplas of neutrophil appeared in purple color like nucleous of WBC, Conventional Giemsa stain, light microscope, 1000X.

The combination of Giemsa and Acridine orangestains enhanced the performance of the microscopy technique for detecting blood parasites, as the combination of both dyes proved its superior ability in accurate diagnosis of hemoparasites.Babesia piroplasmclearly appeared inside RBC. They werestained in a bright orange color in the center of the reddish-gray color red blood cells, while the Babesiamerozoites stained with dark orange and the chromatin in the color of red and gave a clear luster when examined under the light microscope (Figure 5:I, J).

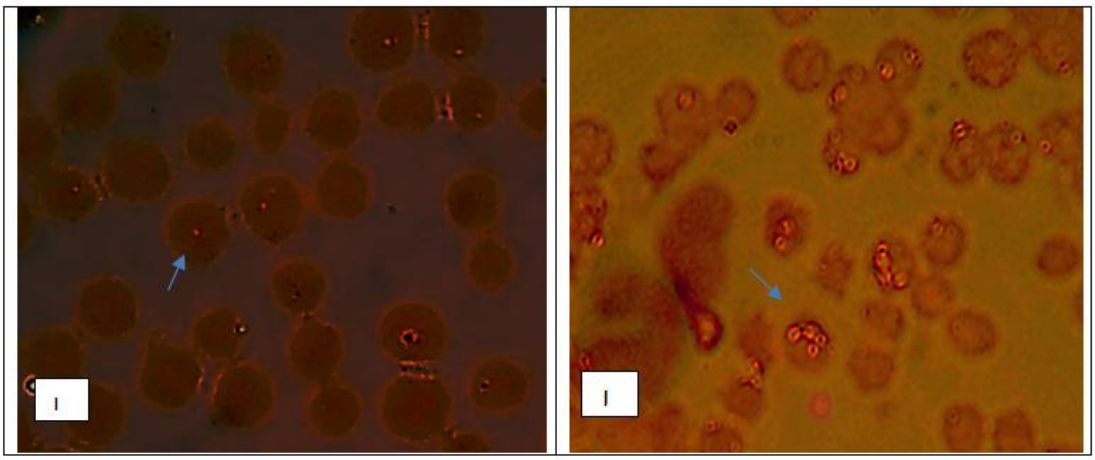


Figure 5 : Thin blood smears of cows stained with Giemsa - Acridine Orange (GAO). (I)Babesia inside and out sideRBC in a bright orange color in the center of the reddish-gray color RBC. (J) Bright orange piroplasm of Babesia multiplication gave a clear luster, light microscope, 1000X.

Giemsa toluidine blue mixture (GTB) was the best stain in terms of its purity, clear of stain artifacts, and stained hemoparasites with a clear blue color, the nuclear features of piroplasm (Babesia and Theleria)and Trypanosoma were better appreciated in the GTB stain in comparison to other stains which helped in the speed of their diagnosis and the possibility of identifying its stage or stages and even its species were well distinguished in comparison to the Giemsa alone.The trypanosomes were also clearly stained in blue, red blood

cells appeared in purple, and the background of the slide was greenish-gray, which showed the parasite more clearly, as well as the clarity of some of its basic structures such as the elongated nucleus, kinetoplast, and undulating membrane. Whereasit was stained with a dark purple color with traditional Giemsa stain (GS)(Figure 6:K,L).

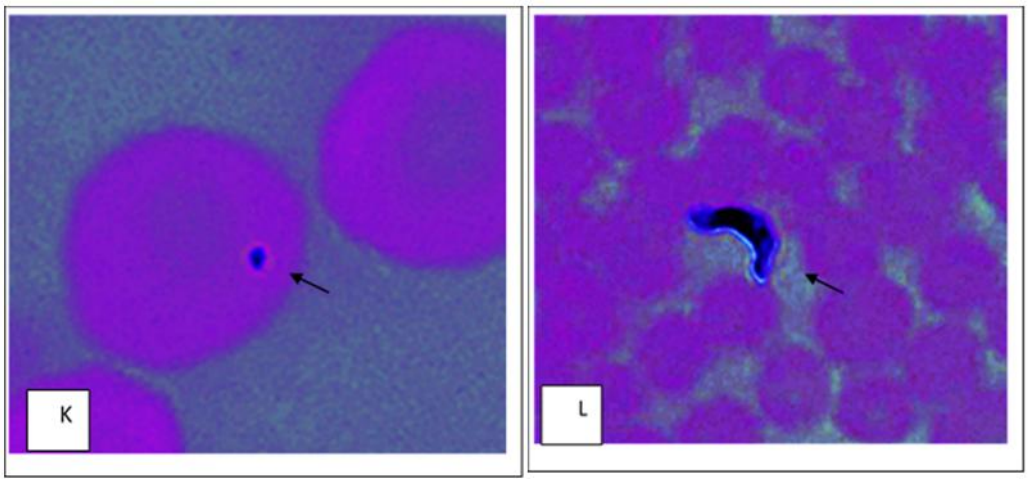


Figure 6 : Microscopic images of blood smears (K) Babesia piroplasm is blue in color. (L) Trypanosoma clearly stained in blue, red blood cells appeared in purple, and the background of the slide was greenish-gray, Giemsa-toluidine Blue stain (GTB), 1000X.

The confirmed cases of hemoparasitesinfections in 60 cowsafter pooling the results of microscopic examination wereBabesia (18), Theleria (17), Anaplasma (11), Ehrlichia (10), and Trypanosoma (17). From the results of comparing the compatibility between the simple modified techniques and Giemsa stain based on the Kappa value(κ),Giemsa rapid kit had complete agreement (κ=1.000) in its ability to diagnose allparasites for all positive cases in comparison with standard Giemsa and a true agreement(κ =0.653) in the diagnosis of trypanosomes cases in comparison to the Giemsa stain. As for the modified Leishman stain, it gave true agreement in its ability to diagnose Trypanosoma (κ = .761) and Ehrlichia (κ=.737). The concordance was complete (κ = 1.000) between Acridine orange stain and Giemsa in its ability to diagnose Anaplasma (κ = .814) andTrypanosoma (κ = (1.000)(Table 3).

Table 3: Comparison Kappa value (κ) of Simple modified stains with conventional Giemsa for the detection of hemoparasites in cattle

Blood parasites	Total No. cases	Giemsa (G)	Rapid GiemsaKit (RGS)		Cytochrome ™ stain (MLS)		Acridine Orange stain (AO)	
		+(%)	+(%)	kappa index(κ)	+(%)	kappa index(κ)	+(%)	kappa index(κ)
Babesia	18	11(61.11)	11(61.11)	1.000	11(61.11)	.532	13 (72.22)	.443
Theileria	17	10 (58.8)	10 ((58.8)	1.000	14(82.35)	.443	6 (35.29)	.459
Anaplasma	11	6 (54.54)	6 (54.54)	1.000	6 (54.54)	.441	5 (45.45)	.814
Ehrlichia	10	7 (70.00)	7 (70.00)	1.000	7 (70.00)	.737	10 (100)	.600
Trypanosoma	17	11(64.70)	7 (41.17)	.653	9 (52.94)	.761	11 (64.70)	1.000

The results of the kappa values also showed a complete agreement between the Leishman – Giemsa cocktail stain in its ability to detect Anaplasma (κ = .814) with Giemsa gold standard and a true agreement in its ability to detect Theleria (K = .761) and Ehrlichia (K = .737). As for the Giemsa-Acridine orange cocktail, it had a kappa value of (κ = 1,000), which means perfectagreement in the diagnosis of trypanosomes, and true agreement with Giemsa criterion in the diagnosis of Babesia (κ = .800), Anaplasma (κ = .633) and Ehrlichia (κ = .761). Giemsa toluidine stain had perfect agreement (κ = 1,000) with standard Giemsa in the diagnosis of Trypanosoma. The agreement was true between Giemsa-toluidine blue stain and the standard Giemsa alone in the detection of Babesia and Anaplasma. From the results of Kappa values of all modified techniques used had showed true and complete agreement in diagnosing of all parasitic infections in blood smear with Giemsa. Giemsa-Acridine orange stain technique for thin and thick smears is a good substitute to Giemsa stain for diagnosing hemoparasites (Table 4).

Table 4: Comparison Kappa value (κ) of cocktail stains with standard Giemsa for the detection of hemoparasites in cows.

Blood parasites	Total No. cases	Giemsa (G)	Leishman - Giemsa LG		Giemsa-Acridine orange GAO		Giemsa-Toluidine blue GTB	
		+ (%)	+ (%)	kappa index (κ)	+ (%)	kappa index (κ)	+ (%)	kappa index (κ)
Babesia	18	11(61.11)	11 (61.11)	.532	12(66.66)	.830	12(66.66)	.766
Theleria	17	10 (58.8)	9 (52.94)	.761	10 (58.82)	.860	8(47.05)	.532
Anaplasma	11	6 (54.54)	7(63.63)	.814	5(45.45)	.633	6 (54.54)	.633
Ehrlichia	10	7 (70.00)	7 (70.00)	.737	7(70.00)	.737	8 (80.00)	.400
Trypanosoma	17	11(64.70)	11(64.70)	.523	11(64.70)	1.000	11(64.70)	1.000

IV. Discussion

In order to accelerate and facilitate the detection of hemoparasites in blood smears of cattle by dependent on their morphological properties in staining.The current study concentrate on the improvement ofthe appearance of hemoparasites in blood smears through staining different hemoparasites with different colors which distinguishes them from other elements of blood smear (erythrocyte, White blood cell, and background).

However, the rapid staining techniques reduced thetime for staining and also reduced the turnaround time formicroscopic examination due to homogeneous staining of all elements of blood smear, allowed determination of different mixed infection even in the thick blood smears.The better performance of the modifiedtechniquesismainly due to the fixation of boththick and thin blood films witheasy fix spray,before stainingwith Rapid kit Giemsa stain (RG). The significant difference between Giemsa stain and CYTOCHROM^{TM}is the composition of the stain. Giemsa stain is a combination of Azure B,eosin and methylene blue. While CYTOCHROM^{TM}is a methanolic mix that contains methylene blue (4,18).Giemsa staining is convenient in the staining of DNA regions of different chromosomes to investigate aberrations such as translocations and rearrangements, while Leishman stain is convenient in blood staining to distinguish and identify trypanosomes, leucocytes and malaria parasites (7,8 and10). The distinctive features of various blood parasites inside or outside RBCappeared with different colors in relation to the coloration of erythrocyte and clearer background. This will improve visualization of parasites were easily recognized,even a thick filmdue to the further fixation steps in the modified staining techniqueswhich made the erythrocytes membranes more permeable and thus released the hemoglobin out of the erythrocytes by distilled water thusmaking it easier to stain and visualize the hemoparasites.Thick smear is also helpful for parasites species differentiation by askilledmicroscopist.The otheradvantage of having both thick and thin blood smears onthe same slide is the ease with which the slides can beexamined without the loss or delayed examination ofeither smear.

Acridine orange (AO) is a common fluorescent stain that improves the brightnessof parasites under fluorescent microscope but it is generally considered of little value in study of morphological features of small blood protozoa with low parasitaemiain comparison with the Giemsa stain (5,19,20,21).In the current studyAOshowedhigh ability in uses for the rapid screening and determine species of *Babesia* spp. and other hemoparasites in cattle blood smearunder the light microscope.

Although hemoparasites are difficult to identify in a stained blood smear, some steps can increase the reliability of this technique, Giemsa stain is used under the light microscope, and whereas Acridine orange (AO) is a common fluorescent stain that enhances the brightnessof parasites, this result was agreed with previous studies by (19,20 and 22). In addition to AO stain could be able to enhance visualization hemoparasites during scanning blood smears of cows under the light microscope, combination of both stains could further improve the performance of microscopy technique. Mixture of Giemsa-Acridine stain was also used by (6) for the detection of malaria parasites in human under fluorescent microscopy.

Studyhas also indicated the use of mixture of Giemsa-Toluidine blue stain in the diagnosing of piroplasm (Babesia and Theleria) and Trypanosomainfections in which the level of parasitism is low. The parasites is blue in color and seems clearly as a ghost in the blood smear, which distinguishes it from the blood smearelements and considers it more efficient than Giemsa stain when used a lone for rapid diagnosis of hemoparasites in cattle.Toluidine blue being an acidophilic stain is taken up more by cytoplasm which enhanced morphology of parasites in blood smear (23). Trypanosomesare takes up more of the stain whichcontains much more deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) than other due to the large elongated nucleus and kinetoplast. Further the mixture of Giemsa toluidine blue stain visualized parasites also exhibited enhanced cellular and nuclear details under light microscopy, so use of this staining with toluidine blue is based on the fact that it selectively stains acidic constituents, which help in rapid detection of these parasites. This could be attributed to the synergistic effect of toluidine blue along with Giemsain improving the staining(24,25).However,toluidine blue stain was DNA-nonspecific, the reason why Giemsa stain was more

improvedvisualization of hemoparasites by toluidine blue application permits further evaluation as scientific literatures lack optimum related data in context to use of toluidine blue stain with Giemsa.

V. Conclusions

Techniques for modifiedstains processing, preparation and examination of blood smears by light microscope can help to, reduce artifacts and turnaround time of result, identify parasites morphology, andcorrect diagnosis of some hemoparasites of cows. Our study found out, for the first time, theutility of three new mixtures for blood parasites smear staining, the parasites have been efficiently stained to facilitate visual identification, through the use of dyes. Modified dyes can be used as an alternative to routine stains for rapid scanning blood smear under a light microscope.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this work.

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