Shortened Staining Procedures of Thin Blood Films with Leishman Stain: A Suitable Alternative Under Emergency.

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ABSTRACT

Background: The relevance of Leishman stained thin blood film for assessment of blood cells morphology, White Blood Cells (WBC) differentials and inclusion bodies had long been established. The time it takes to generate results on laboratory bench is still long when compared to what is obtainable in automation.
Methods: To investigate effects of reduced Leishman staining time on cell morphology, 3 thin blood films were made for 60 subjects. First group were flooded with Leishman stain for 3 minutes and further stained with diluted stain for 12 minutes while second group were stained for 6 minutes after initial flooding for 3 minutes. Third group were exposed to undiluted stain for 3 minutes only. The differential WBC values were determined and presence of malaria parasites and sickle cells were noted.
Results: Mean ± SEM of the results compared using SPSS software showed no significant difference in all the cells ($P>0.05$) except in monocytes values between '3minutes' and '15 minutes' procedures ($P<0.05$). Presence of sickle cells and malaria parasites were recorded in 2 slides (3.3%) and 5 slides (8.3%) respectively – in all the tested groups.
Conclusion: It could be concluded that 3 minutes and 9 minutes are enough to stain thin blood films using Leishman stain and obtain accurate results. The shortened protocol may become useful under emergency situations.

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Authors' contributions: *Muhibi M A was the project leader, Hassan A O and Nassar AS were responsible for experimental and project design. Muhibi M A, Hassan R O and Tijani B A performed most of the experiments. Collations and statistical analysis of data were performed by Muhibi M O. Muhibi M A wrote the manuscript, conceptualized and approval of the draft was done by all.*

Key words: Leishman stain, SOP, Blood film, Emergency.

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1. INTRODUCTION

The use of Romanowsky stain in haematology routine laboratory for staining peripheral thin blood films and bone marrow films had long been established. Performance of Romanowsky stains; in this regard, had been shown to be satisfactory [1, 2].

The main components are: anionic dye and cationic (basic) dye such as azure B- which binds to anionic sites and gives a blue-gray colour to nucleic acid (DNA or RNA), nucleolus, proteins, granules of basophils and weakly to granules of neutrophils. The anionic (acidic) dye such as eosin binds to cationic sites on proteins and gives orange-red colour to haemoglobin and eosinophilic granules [1, 2]. There are a number of different combinations of these dyes which include May-Grunwald-Giemsa stain, Wright's stain, field stain and Leishman stain [1, 3, 4, 5].

Leishman stain was developed by William Boog Lieshman (1865-1926) and is a mixture of acidic and basic component dissolved in concentrated alcohol [5]. Leishman stain has a combination of numerous advantages over other Romanowsky stains and is preferred to most routine haematology laboratories in Nigeria and elsewhere. The advantages include ease speed of preparation, a relatively shorter staining time (when compared with other Romanowsky stains), stability of stock stain solution and reproducibility of results [3, 6].

Briefly, the procedure requires flooding dried slides with leishman stain for 3 minutes and then diluting the stain in the slide with equal volume of buffered distilled water (pH 6.8) for additional 12 minutes [7]. The stain on slide is then washed off with buffered water and slide air dried to be observed under microscope with X10, X40, and X100 objectives [1, 5, 6, 7].

With X10 objectives, general impression of quality of the film is determined and area where red cells are evenly distributed is identified and selected to study morphology of red cells, white cells and platelet using X40 objective [5, 8]. Consequently, relative proportion of platelet is noted and all abnormal cells present are noted too [9]. Oil immersion objective (X100 lens) is eventually used to confirm impression and study fine details of the cell morphology, including presence of inclusion bodies [5, 10, 11]. Malaria parasites can also be identified to species level on a leishman stained thin film [11, 12].

Despite the fact that Leishman stain is a widely used dye in haematology routine staining techniques and its relative shorter staining period (15 minutes), some health institutions in developing countries still avoid differential cells count and blood cells morphology assessment; whereas, it would have impact in making accurate diagnosis and prognosis determination [13]. This is more common in those routine haematology laboratories and hospital side laboratories with automated haematology analysers. Although, the red cell indices provide useful information about the red cells, they can in some instances appear normal even when marked red cells abnormalities exist. For example, if a patient has populations of both microcytes and macrocytes, then it is quite possible that the mean cell volume will be within the normal range [13]. Whereas, timely generation of data from laboratory analysis has clinical importance in diagnosis and treatment of patients, there is no published data on how to further reduce Leishman staining time without compromising the quality of films produced. It is hoped that this present study will shed light on the effects of reduced staining time on quality of results produced, which will serve as a guide

for its general use by professional laboratorians and diagnostic laboratories.

2.0 MATERIALS AND METHODS

2.1 Film preparation

Sixty blood samples of clients (male=21, female=39) who came to haematology routine laboratory of Ladoke Akintola University of Technology Teaching Hospital (LTH), Osogbo, Osun state of Nigeria, for Full Blood Count on April 07, 2011 were collected into K_3EDTA bottles (1ml of blood/1.5mg of anticoagulant) as recommended by Dacie and Lewis[5] ; and Mc-Shine *et al* [9]. Thin blood films were made within 1 hour of sample collection in triplicate and labeled A, B, and C for each serial number. That is 1A, 1B, and 1C; 2A, 2B and 2C through 60A, 60B and 60C (where $1 \leq N \leq 60$).

2.2 Staining protocol

Air-dried slides of group A were flooded with Leishman stain solution (1.5g/L of acetone free alcohol; source KIRAN LIGHT LABORATORIES, LOT NO 120305K) for 3 minutes and stain was diluted with buffered distilled water (pH 6.8) for additional 12 minutes before the stain was washed off. This is in accordance with Ladoke Akintola University of Technology Teaching Hospital Haematology Laboratory Standard Operating Procedure [14]. For group B (trial 1), slides were flooded with Leishman stain solution for 3 minutes and diluted with buffered distilled water for 6 minutes. Group C slides (trial 2) were flooded with stains (undiluted) for initial 3 minutes without further dilution. Thus, total staining periods for groups A, B, and C were 15 minutes, 9 minutes, and 3 minutes respectively.

The slides were maintained at ambient temperature of 27^o c and humidity level of 25%.

2.3 Microscopy

Stained slides were then air-dried and observed independently by 3 principal medical laboratory scientists who routinely do the job at Ladoke Akintola University of Technology Teaching Hospital Haematology Laboratory. Differential white blood cell count (in %) and cell morphology of the samples were determined using battlement method as described by Baker and Silverton (1985) [13]. Averages of the results obtained per slide by the 3 microscopists were obtained and compared using the one-way analysis of variance. P value was set at 0.05.

3.0 RESULTS

The results of differential white blood cells counts obtained from standard operating procedures (SOP) (staining period-15 minutes) when compared to trial 1 procedure (staining period-9 minutes) revealed no significant difference($p>0.05$) (Table 1). However, when SOP was compared with trial 2 procedure (staining period-3 minutes), significant difference ($p<0.05$) was noted in monocytes count only (Table 2). Also, no significant difference ($p>0.05$) was observed when the outputs generated by both Trials 1 and 2 procedures were compared (Table 3).

The proportion of red cells cytoplasmic inclusions (malaria parasites) and predominant abnormal shapes (sickle cells), as observed in the slides stained using various methods are shown in Table 4. While only 2 of the 60 (3.3%) samples processed revealed presence of drapatocytes

in all the groups; 5 out of 60 (8.3%) samples had *Plasmodium falciparum* (Table 4).

Table 1: Comparison of Results between SOP and Trial 1

	Group	Mean ± S.D.	P- Value
Neutrophil	Standard (15 mins) Trial 1(9 Mins)	54.58 ± 17.886 53.70 ± 18.820	.793 (P > 0.05)
Lymphocyte	Standard (15 mins) Trial 1(9 Mins)	43.37 ± 18.225 43.98 ± 17.740	.851 (P > 0.05)
Eosinophil	Standard (15 mins) Trial 1(9 Mins)	1.07 ± 2.007 1.15 ± 1.830	.813 (P > 0.05)
Basophil	Standard (15 mins) Trial 1(9 Mins)	0.10 ± .303 0.15 ± .360	.412 (P > 0.05)
Monocyte	Standard (15 mins) Trial 1(9 Mins)	0.90 ± 1.053 0.75 ± 1.230	.474 (P > 0.05)

Table 2: Comparison of Results between SOP and Trial 2

	Group	Mean ± S.D.	P- Value
Neutrophil	Standard (15 mins) Trial 2 (3 mins)	54.58 ± 17.886 54.53 ± 17.257	.988 (P > 0.05)
Lymphocyte	Standard (15 mins) Trial 2 (3 mins)	43.37 ± 18.225 43.53 ± 17.062	.959 (P > 0.05)
Eosinophil	Standard (15 mins) Trial 2 (3 mins)	1.07 ± 2.007 1.28 ± 1.823	.537 (P > 0.05)
Basophil	Standard (15 mins) Trial 2 (3 mins)	0.10 ± .303 0.13 ± .343	.573 (P > 0.05)
Monocyte	Standard (15 mins) Trial 2 (3 mins)	0.90 ± 1.053 0.50 ± .893	.027 (P < 0.05)

Table 3: Comparison of Results between Trial 1 and Trial 2

	Group	Mean ± S.D.	P- Value
Neutrophil	Trial 1(9 Mins) Trial 2 (3 mins)	53.70 ± 18.820 54.53 ± 17.257	.801 (P > 0.05)
Lymphocyte	Trial 1(9 Mins) Trial 2 (3 mins)	43.98 ± 17.740 43.53 ± 17.062	.888 (P > 0.05)
Eosinophil	Trial 1(9 Mins) Trial 2 (3 mins)	1.15 ± 1.830 1.28 ± 1.823	.690 (P > 0.05)
Basophil	Trial 1(9 Mins) Trial 2 (3 mins)	0.15 ± .360 0.13 ± .343	.796 (P > 0.05)
Monocyte	Trial 1(9 Mins) Trial 2 (3 mins)	0.75 ± 1.230 0.50 ± .893	.205 (P > 0.05)

Table 4: Prevalence of sickle cells and malaria parasites from films stained by the 3 procedures.

Variables	SOP(15 min) N=60		Trial 1 (9 mins) N=60		Trial 2 (3 mins) N=60	
	No	prevalence (%)	No	prevalence (%)	No	prevalence (%)
Sickle cells	2	3.3	2	3.3	2	3.3
P. *falciparum*	5	8.3	5	8.3	5	8.3

DISCUSSION

Our study has demonstrated that leishman stained peripheral blood film remains a very useful tool in hematological assessment. Attention to details in slide preparation, staining and standard criteria for cell estimates, morphology and differential reporting enhance the value of results provided to the physician [5]. Promptness in generation of results will aid effective treatment and consequently reduce morbidity and mortality. While automation provides for prompt generation of WBC differentials among others, most laboratories in the developing countries cannot afford same. Hence, alternative being the Leishman stain was noted to have several advantages over other Romanowsky stains in most British Laboratories [4, 15].

In this study, while comparing the shortened procedures with standard operating procedures, data generated from trial 1 where staining period was shortened to 9 minutes was found not to be different from those generated from standard operating procedures. With respect to trial 2, monocytes count shows significant differences from SOP while other 4 parameters (basophils, eosinophils, lymphocytes, and neutrophils) show no difference. There is dearth of data on varying fixing and staining periods of Leishman staining of blood smears for differential white cells counts, although such data exist on chromatographic techniques and cytogenetic studies [16, 17].

We also studied the usefulness of the two trial methods (shortened procedures) in detecting intra-cytoplasmic inclusions (plasmodium species) and red cell anomaly (sickle cells). Leishman stained smear microscopy was reported to be the gold-standard in the diagnosis and identification of malaria parasites to specie level; whereas, the cost of polymerase chain reaction(the supposed gold standard) remains prohibitive in most parts of the worlds [1,12,18]. Red cells morphology is also better appreciated as the cells are stained with acidic components of the stain as reported by several authors [1, 6, 7 and18].

Generally, apart from using blood films for clinical quantitative and/or qualitative analysis, Leishman stained thin film are now being used as well for quality control/quality assurance purposes and for evaluation of automated instrument-based methods [19, 20].

CONCLUSION

Significance of Blood film assessment in making diagnosis and monitoring treatment in developing countries cannot be over

emphasized and turnaround time is critical. It could be concluded from this study that, if expertise and materials required are available, Leishman stained smear could generate precise and accurate differential WBC values with 9 minutes as the staining period. Red cells morphology and inclusions could also be accurately accessed if staining and fixing period with leishman stain is shortened to as low as 3 minutes. These conditions apply where skilled laboratorians handle the staining procedure and trained and certified microscopists handle the film reporting. These shortened procedures are recommended under emergency situations. Further studies in other laboratories should be conducted to determine the reproducibility of the results of the current study.

Conflict of interest:

We hereby declare that there was no conflict of interest involved in this research work.

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