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A Comparative Study between Conventional and Modified Leishman Stain

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ABSTRACT

Introduction: Leishman stain has been used as the stain of choice for peripheral blood films since many decades .But it has a disadvantage of consuming 15 minutes for the procedure alone thereby increasing the turnaround time of peripheral smear reporting. In this study modified Leishman stain was made by adding phenol to conventional Leishman to reduce the staining time to 3 minutes without interfering with the quality of stain.

Aim: To study the quality of modified Leishman stain in comparison with conventional preparation on peripheral blood smears.

Materials and Methods: The present cross-sectional study was carried out in Central Haematology laboratory of a tertiary health care centre in Southern India. A pilot study was done to determine the ideal ratio of phenol to Leishman powder in preparing modified stain without compromising the staining quality. After obtaining consent, the blood sample was collected in anticoagulated vials. Sample size was calculated to be 85.Two thin wedge smears were prepared from each sample and one was stained with conventional Leishman stain and other with modified Leishman stain. The staining characteristics were assessed and scored by an experienced pathologist in terms of 6 parameters- RBC pattern, nuclear pattern, neutrophil granules, eosinophil granules, platelets and background staining. The grades were 1, 2, 3 & 4 (poor, satisfied, good and excellent). Smears each with grades 2, 3 and 4 were selected for study. The results were analysed by Statistical Package for the Social Sciences (SPSS) software version 16.0.

Result: The mean value for the staining quality using conventional method was 4.73 and for modified method was 5.39, and the total score possible is 6. Photomicrograph showed excellent results with modified Leishman stain. Thus from these values we can interpret that modified method gave much more acceptable results than that of conventional method.

Conclusion: Unlike the conventional method which requires a total of 15 minutes, to complete the staining process, modified Leishman staining techniques takes only 3 minutes. Blood films can be stained within a short period of time thus aiding in rapid diagnosis and treatment of patients.

Key words: Leishman stain, Modified Leishman stain, Phenol

INTRODUCTION

Romanowsky stain which is a combination of an acidic stain and basic stain is the most common stain used worldwide for staining blood films. [1,2] The staining technique is named after the Russian physician Dmitri Leonidovich Romanowsky (1861-1921). [3] The special property of Romanowsky stains to distinctively stain granules of each cell is due to two components Azure B (trimethyl thionine) and eosin Y (tetrabromoflourescein). [4,5] The original Romanowsky combination contained polychrome methylene blue and eosin. The azure B and eosin Y was advocated for the International Committee for Standardization in Haematology (ICSH). [6] Azure B is one of the oxidative products of methylene blue and is superior to other azures, the reason it

is more commonly employed in Romanowsky dyes. [7]

Original Romanowsky method was modified by William Boog Leishman a British pathologist and was called as Leishman's stain. [8] Many modifications have been done in Romanowsky dyes and Leishman stain occupies an intermediate position among the various modifications available.

Leishman stain is routinely used in Hematology labs to stain peripheral blood films. [2] But the procedure is time consuming and cannot meet emergency needs in very sick patients. A very few modifications have been tried in Leishman stain to reduce the time taken for staining. Since it is an era of rapid diagnostic techniques and automated machines are used for getting quick blood count results modifications are being tried in Leishmans stain to reduce staining time without compromising the quality. In modified Leishman stain, phenol an accentuating agent is used to optimize staining and to reduce the time taken. [9] Phenol which is used in Zeil Neelson staining for optimizing staining is used in modified Leishman stain expecting the same result. Phenol act by changing the pH of the staining solution thereby increasing the rate of stain uptake by the tissue. [10] Phenol also helps in ripening of the modified Leishman staining solution and thus shortens fixing and staining time. This will help in overall reduction in turn-around time (TAT) of peripheral smear reporting. This study was undertaken to compare the quality of modified Leishman stain with conventional preparation on peripheral blood smears.

MATERIALS AND METHODS

This cross sectional study was carried out in the Central hematology laboratory and Department of MLT at a tertiary care hospital in south India. It was approved by the Institutional Ethics Committee. Total two smears were collected from each patient and all patients were properly informed about this study with a duly signed consent form in both English and Malayalam.

Study population / study material

Included all patients(OP&IP) whose blood samples are collected into clean , dry penicillin bottles containing proper concentration of EDTA anticoagulant (1.5mg/ml of blood)

Inclusion criteria-Properly collected and labelled blood smears from Central hematology laboratory, Govt. Medical College Hospital.

Exclusion criteria-Unlabelled and improperly collected smears are excluded.

Study period

A period of six months after the approval of ethics committee.

Sample size calculation

Formula,

$$\mathbf{N} = \frac{\{z1-\alpha/2\sqrt{2p(1-p)}+z1-\beta\sqrt{p1(1-p1)+p1(1-p1)}\}}{(p1-p2)^2}$$

Where; $\mathbf{P} = \frac{P1+P2}{2}$

P1: Proportion in the first group= 80%, P2: Proportion in the second group= 60%, α: Significance level = 5%

1-β: Power =80%

N = 85 in each group

A pilot study was conducted to get optimum concentration of phenol by preparing stains using various ratios of phenol and Leishman powder in acetone free methanol.

Components of Modified Leishman Stain;

Component ingredients of modified Leishman stain are Leishman powder, absolute methanol, and phenol crystals.

Determination of Phenol: Leishman Powder Ratio/100ml of Leishman

Weighed 30mg, 37·5mg, 50mg, 75mg and 150mg of phenol crystals with 150mg of commercially-prepared Leishman powder into different brown screw- capped bottles to get phenol: Leishman powder in

the ratios 1:5, 1:4, 1:3, 1:2 and 1:1 respectively and this was dissolved in 100ml of absolute methanol to get 100ml of modified Leishman solution. The preparation was done at room temperature and the stain was kept protected from direct sunlight.

The blood films were assessed after staining with modified stains in the ratio 1:3 and became the reference phenol: Leishman powder ratios with optimal staining performance. Inadequate fixing and improper staining times of staining process resulted in under-staining or over-staining of blood films. This caused wrong differential count results. The fixing and staining times of staining process were determined by experimenting the procedure time thus leading to development of new staining procedures.

Collection of blood - Blood samples were collected by venipuncture from the median cubital vein.

Blood smear preparation

Two separate smears were prepared from each patient; those smears were allowed to air dry. One smear used for conventional Leishman staining and the other smear was used for modified Leishman staining.

STAINING PROTOCOL

For conventional method

- Took an air dried smear prepared by manual wedge method and covered the smear with undiluted stain, kept for 1-2 minute.
- Added twice volume of buffered water (pH 6.8) to dilute the stain.
- Mixed the water with stain by gently blowing with a plastic bulb pipette or with a straw. Allowed to stain for 10-12 min
- Washed off stain with tap water. Flooded the smear with buffered water, pH 6.8 for 2 minutes and rinsed with water and wiped the back of the slide and kept for drying.

For modified method

- Covered the smear with undiluted modified Leishman stain for 25 seconds
- Added twice volume of buffered water to dilute the stain.
- Allowed to stain for 50 seconds.
- Washed off the stain with water. Flooded the smear with buffered water with a pH6.8 for 2 minutes and rinsed with water.
- Wiped the back of the slide and kept for drying.
- The morphological features of RBC, Neutrophil, Eosinophil and Platelets stained with conventional and modified Leishman stain in oil immersion(100X) is depicted in fig 1, 2, 3 & 4

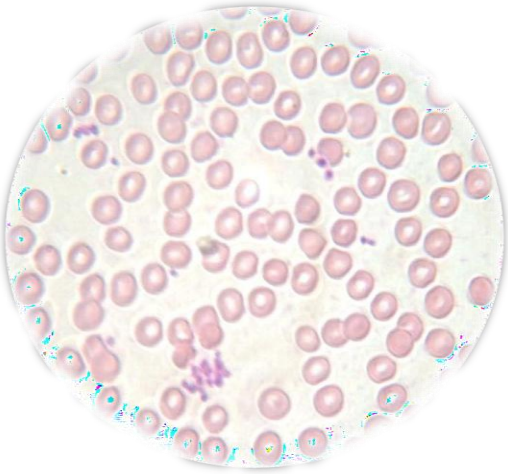

Fig 1 RBC pattern stained with conventional preparation, 100x.

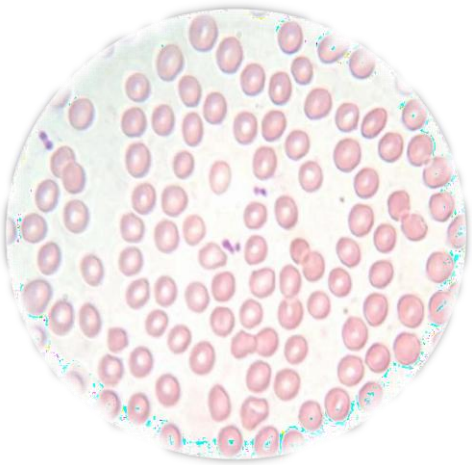

Fig 1 RBC pattern stained with modified Leishman stain, 100x.

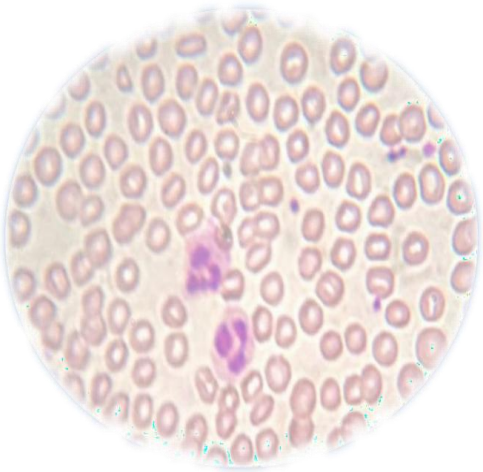

Fig 2 Neutrophils stained with conventional Leishman stain, 100x.

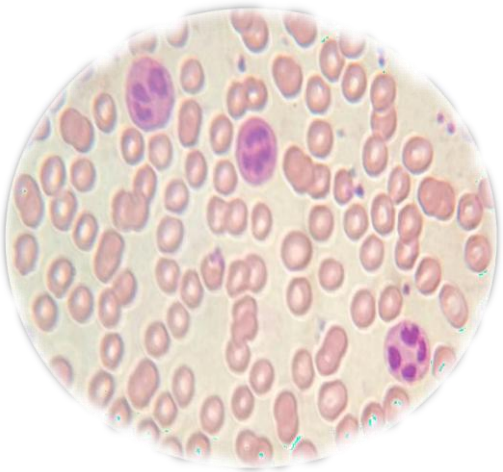

Fig 2 Neutrophils stained with modified Leishman stain, 100x.

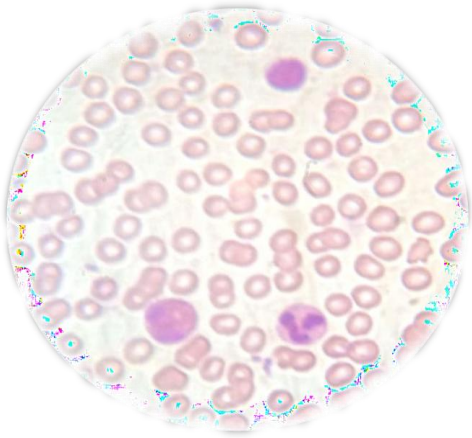

Fig 3 Eosinophil stained with conventional Leishman stain, 100x.

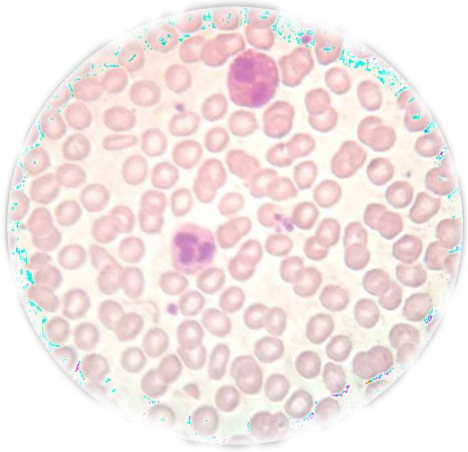

Fig 3 Eosinophil stained with modified Leishman stain, 100x.

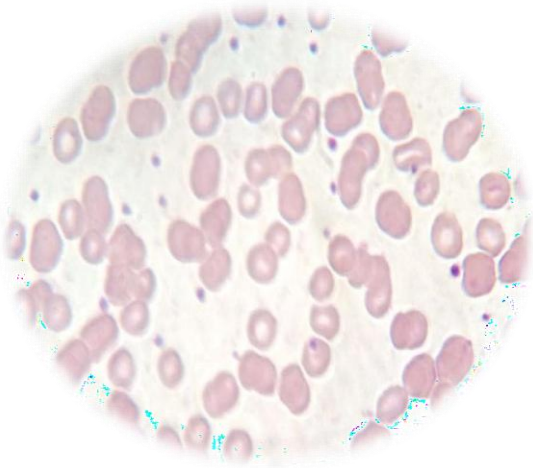

Fig 4 Platelets stained with conventional Leishman stain, 100x.

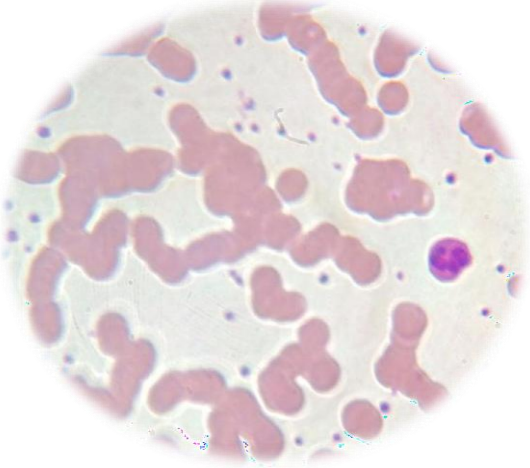

Fig 4 Platelets stained with modified Leishman stain, 100x.

Evaluation of staining quality:

Interpretation of staining quality was assessed by looking and scoring the parameters like RBC pattern, nuclear pattern, neutrophil granules, eosinophil granules, platelets and background staining as per score given.

Table1 Scoring for quality of staining

RBC pattern	Acceptable =1 Well preserved morphology with pink colour	Unacceptable = 0 Poorly preserved morphology with pale Staining
Nucleus	Acceptable =1 Brilliant violet colored and well differentiated from cytoplasm	Unacceptable = 0 Smudgy pale blue nucleus without enough contrast between nucleus and cytoplasm.
Neutrophil granules	Acceptable =1 Reddish lilac small granules	Unacceptable = 0 Colourless undifferentiated granules.
Eosinophil granules	Acceptable =1 Bright red to orange red large granules.	Unacceptable = 0 Colourless undifferentiated granules.
Platelets	Acceptable =1 Violet purple coloured bodies clearly differentiated from stain deposits	Unacceptable = 0 Unable to differentiate from stain deposits
Background	Acceptable =1 Clear without any stain deposits.	Unacceptable = 0 Obscured with stain deposits.

Staining quality was assessed and the slides were grouped into following categories.

GRADE 1 = POOR = 0-1
GRADE 2 = SATISFIED = 2
GRADE 3 = GOOD = 3-4
GRADE 4 = EXCELLENT = 5-6

Slides under 2nd 3rd and 4th category were used.

STATISTICAL ANALYSIS

The results were analysed by Statistical Package for the Social Sciences (SPSS) software version 16.0.

RESULTS

The present study evaluated the comparison between conventional and modified Leishman staining method. 85 samples were assessed for both methods. Six parameters were statically evaluated separately. Each parameter was compared using the scoring system.

1. RBC pattern

Table 2

RBC PATTERN	Conventional method		Modified method	
	N	%	N	%
Unacceptable	11	12.9	16	19
Acceptable	74	87.1	69	81
Total	85	100.0	85	100

McNemar test p=0.424

The observed p value is 0.424 and there is no significant difference between modified and conventional method in the aspect of RBC pattern in staining.

2. Nuclear pattern

The observed p value is < 0.001 and there is no significance difference between modified and conventional method in the aspect of nuclear pattern in staining.

Table 3

NUCLEUS	Conventional Method		Modified method	
	N	%	N	%
Unacceptable	24	28.2	6	7
Acceptable	61	71.8	79	93
Total	85	100.0	85	100

McNemar test p < 0.001

3. Neutrophil granules

Table 4

NEUTROPHILGRANULES	Conventional method		Modified method	
	N	%	N	%
Unacceptable	12	14.1	1	1
Acceptable	73	85.9	84	99
Total	85	100.0	85	100

McNemar test p=0.003

The observed p value is 0.003 and there is no significance difference between modified and conventional method in the aspect of neutrophil granules in staining.

4. Eosinophil granules

Table 5

EOSINOPHIL GRANULES	Conventional method		Modified method	
	N	%	N	%
Unacceptable	17	20.0	2	2
Acceptable	68	80.0	83	98
Total	85	100.0	85	100

McNemar test p<0.001

The observed p value is <0.001and there is no significance difference between modified and conventional method in the aspect of eosinophil granules in staining.

5. Platelet

Table 6

PLATELET	Convential Method		Modified method	
	N	%	N	%
Unacceptable	17	20.0	8	9
acceptable	68	80.0	77	91
Total	85	100.0	85	100

McNemar test p=0.078

The observed p value is 0.078 there is no significance difference between modified and conventional method in the aspect of platelets in staining.

6. Background clarity

Table 7

BACKGROUND	Conventional method		Modified method	
	N	%	N	%
Unacceptable	27	31.8	19	22
acceptable	58	68.2	66	78
Total	85	100.0	85	100

McNemar test p=0.200

The observed p value is 0.200 there is no significance difference between modified and conventional method in the aspect of background clarity in staining.

Total score

Table 8

Total score	Conventional method		Modified method	
	N	%	N	%
Poor	5	5.9	0	0
Satisfied	5	5.9	5	5.9
Good	15	17.6	8	9.4
Excellent	60	70.6	72	84.7
Total	85	100.0	85	100.0

Mean value

Table 9

	N	Total score		t	P
		Mean	SD		
Conventional	85	4.73	1.577	3.394	.001
Modified	85	5.39	1.092		

Thus from these values we can interpret that modified method gave much more acceptable results than that of conventional method.

DISCUSSION

Leishman stain which is a combination of basic dye (Methylene blue or Azure B) and acidic dye (Eosin Y) has been used since decades to stain peripheral blood films. The basic component binds to anionic site and gives a blue grey color to nucleic acids and granules of basophils. Acidic dye due to its negative charge binds to cationic site and gives orange red color to hemoglobin and eosinophil granules. [11,12] This staining process takes 15 minutes to complete. This often creates hindrance to rapid diagnosis of medical emergencies like sepsis and leukemias. Even though blood counts are obtained very rapidly using automated counters, the morphological diagnosis is delayed due to the increased turnaround time for peripheral smear reporting using conventional Leishman stain.

As this is an era of rapid diagnostic methods there arouse a need for rapid staining of peripheral smears. Use of rapid stains in staining was started by John William Field who introduced the fields stain for thick blood films to identify hemoparasites. [13] This has led to the need of modifications in Leishman stain so that the time taken for staining process is reduced. Here came the importance of adding Phenol, the accentuator to reduce the staining time. Phenol and methanol both are organic compounds with terminal hydroxyl functional groups. [14] Phenol facilitates the staining process and thus reduces the fixation and staining time. Fasakin et al [15] tried modifying Leishman stain by adding phenol. He concluded that modified Leishman stain significantly reduces turnaround time on peripheral blood smear reporting. He prepared different ratios of phenol: Leishman stain and concluded that 1:5 and1:3 ratios gave better morphology to the cells than conventional Leishman stain.

We did a pilot study to determine the ideal phenol: stain concentration and concluded that 1:3 ratio gave the optimal result .the study sample size of 85 samples were selected and both the conventional and modified Leishman staining was done. It was clearly observed that the modified Leishman staining procedure, gave better staining characteristics and morphological pictures of blood cells in peripheral blood

films than the conventional method. This was comparable to results by Fasakin et al. [15]

A total of 3 minutes were taken for the whole procedure of modified Leishman stain. Whereas the conventional method recommended by the WHO takes a total of 15 minute. As the staining quality is being interpreted, emphasis was on blood samples with normal parameters.

The scoring parameters assessed were RBC pattern, Nuclear pattern, Neutrophil granules, Eosinophil granules, Platelets and Background of staining. Eosinophil granules were clearly identified and better differentiated from Neutrophil granules compared to that of the conventional method. Nuclear pattern was also well appreciated in modified method. Here, the RBC also showed better staining results, with excellent central pallor. Platelets were quite better visible than conventional method of Leishman stain. The Background score also were comparable with that of the conventional method.

CONCLUSION

Leishman stain is the stain of choice for peripheral blood films. Modified stain optimizes staining by a different approach by using the accentuating property of phenol. Phenol alters the pH of the modified Leishman stain increasing its permeability and shortening the overall time required for staining. Both phenol and methanol are polar, organic compounds with terminal hydroxyl functional groups which facilitate their reactivity.

In this study peripheral blood smear samples were stained with modified and conventional Leishman stains and results were compared. It was observed that the smears stained with modified stain provided better results as compared with conventional preparation based on the parameters include RBC pattern, nucleus, neutrophil & eosinophil granules, platelets, and background pattern. Also, modified technique is rapid, economical and reliable.

Modified Leishman stain and its application will significantly affect the practice of diagnostic hematology as this modified method is simple and cost effective. With the advent of this new technique, problems arising from late peripheral smear report are reduced in very sick patients.

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