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Original research article

Efficacy of modified Leishman stain in peripheral blood smears using a scoring system

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Abstract

Context: Leishman stain, one of the Romanowsky stain, is used as the stain of choice for routine staining of peripheral blood films since many years in most of the hematology laboratories worldwide. But it has a disadvantage of consuming around 15-20 minutes of time for staining alone, which can lead to increase in the turnaround time for reporting peripheral blood smears. In this study Phenol, an accentuating agent is used as the vehicle of innovation for preparing modified Leishman stain that can aid to reduce the staining time without altering the quality of smears.

Aim: To know the optimal phenol: Leishman powder ratio that is appropriate for preparing modified Leishman stain and to evaluate the staining efficacy of this modified stain over the conventional Leishman by a scoring system.

Settings and Design: It is a cross sectional study done after taking the ethical clearance from Institutional Ethical Committee.

Methods and Material: The study was conducted in the department of clinical Hematology Laboratory of a tertiary health care centre in South India from July 2019 to August 2019, on a total of 600 smears prepared from EDTA anticoagulant blood samples that were stained by both conventional as well Modified Leishman stain. These smears were then given a score based on five parameters and quality index was calculated.

Statistical Analysis: The results obtained were entered into a Microsoft excel sheet. The minimum and maximum scores for a single case ranged from 0-10. Quality index of these stains was obtained by dividing the actual score by the maximum score possible. No statistical instruments were used as the study was based on interpretation of staining characteristics.

Results: Modified Leishman stain at phenol: Leishman powder ratio of 1:5 on day 10 gave best staining quality when compared to other days at various concentrations with a quality index of 0.86 and was as good as conventional Leishman stain

Conclusions: Modified Leishman Stain prepared by adding phenol to conventional Leishman stain can give better staining results in 4 minutes unlike the conventional Leishman stain which takes 15-20 minutes of time for staining peripheral blood smears.

Keywords: Conventional Leishman stain, modified Leishman stain, peripheral blood smears

Introduction

Romanowsky stains are used worldwide in most of the laboratories to stain the peripheral blood smears. The original Romanowsky method was modified by William Boog Leishman a British pathologist and was called as Leishman's stain. [1] The advantage of Leishman stain over Giemsa staining (another Romanowsky stain used in hematology) is that it can be prepared easily, low cost and staining can be done within 10-15 minutes.[2,3,4] Few modifications in preparation of Leishman stain has been attempted that can aid in reducing the duration of staining of peripheral blood smears.

One such attempt is addition of phenol, an accentuating agent that helps to reduce the duration of staining when added to Leishman stain. The present study was thus done to assess the effectiveness of modified Leishman stain in staining the smears on day 1, 3, 5, 10 and 30 of preparing the stain.

Subjects and Methods

The study was a cross sectional study conducted in the department of clinical Hematology Laboratory of a tertiary health care centre in South India from July 2019 to August 2019, after getting the approval from the Institutional ethics committee. All the participants were informed about the study from whom these samples were collected and a duly signed informed consent was obtained.

The study was done on 100 EDTA anticoagulant blood samples that are adequate to make smears after

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completion of ordered investigations. Inadequate and non EDTA blood samples were excluded.

Preparation of peripheral blood smears: The100 EDTA anticoagulant blood samples were divided into five batches and each batch contains 20 samples. From each sample six smears are prepared. One smear was stained with conventional Leishman stain and the remaining five smears were stained using modified Leishman stain prepared by using various concentrations of liquid phenol on various days. Per batch on a particular day, 120 smears were stained and examined. Thus, a total of 600 smears were prepared and were labeled serially. These labeled smears were stained using conventional Leishman stain as well as modified Leishman stain.

Preparation of modified leishman stain: The modified leishman stain was prepared by adding liquid phenol at various concentrations to 100ml of absolute alcohol by dissolving 150 mg of Leishman powder and is shown in Table 1.

Table 1: Protocol used for preparing modified Leishman stain solution using liquid phenol

	Concentration A	Concentration B	Concentration C	Concentration D	Concentration E
Liquid phenol (ul)	30	40	50	75	150
Leishman Powder (mg)	150	150	150	150	150
Absolute alcohol (ml)	100	100	100	100	100
Phenol: Leishman powder ratio	1:5	1:3.75	1:3	1:2	1:1

Labeling of smears

Smears that are stained on day 1 are labeled as 1, 1a, 1b, 1c, 1d &1e where the numerical number indicates the day on which the smear was stained by conventional leishman stain and examined. The alphabets represent the varying concentrations of liquid phenol used for preparing modified Leishman stain. For example, smear labeled as 1a represents day 1 sample stained by modified Leishman stain at phenol concentration of 30ul, b at 40ul, c at 50ul, d at 75ul and e at 150ul.

Similarly, other smears were also labeled as 3, 3a-3e on day 3; 5, 5a-5e on day 5 smears; 10, 10a -10e for day 10 smears and 30, 30a-30e for day 30 smears.

Staining of the smears using conventional leishman stain

The peripheral blood smears were prepared manually by wedge method, air dried, covered with conventional Leishman stain and was kept for 1 minute, after which buffered water was added to cover the slide entirely. The slide is allowed to stain for around 15-20 minutes and then washed with tap water.

Staining of the smears using modified leishman stain

For staining the labeled smears, we require Modified Leishman stain and buffered water at a pH of 6.8. The stain with various concentrations of liquid phenol as well as buffered water was taken in separate coplin jars. The labeled air-dried blood smears were placed in modified Leishman stain for 1 minute. After 1 minute, slides were removed from the stain & placed in coplin jar containing buffer water (pH 6.8) for 3 minutes. After 3 minutes slides were dried on slide rack.

Numbering & Preparation of master copy

After staining the labeled smears, all the 600 slides were mixed and were renumbered from 1-600. These numbers were entered into the Microsoft excel sheet and a master copy of the smear numbers and corresponding day (1, 1a-1e etc.) was maintained. These smears were observed by two pathologists independently and a score was given.

Scoring of smears

The labeled and stained smears were given a quality index score based on five parameters which included overall staining quality, nuclear morphology, red blood cell staining cytoplasmic staining and platelet staining. The scores for each parameter ranged from 0-2. Score 0 indicates unsatisfactory/ unstained pattern of staining, Score-1 indicates satisfactory pattern of staining and score 2 indicates excellent staining. The overall score range for each slide ranges between 0-10.

These smears were observed by two pathologists independently. In case of discrepancies in assigning the scores, an expert and experienced pathologist opinion was taken.

Calculation of quality index

The maximum score for a single case was calculated taking into account all the 5 parameters and it is 10. Quality index of the stained smears is calculated by diving the final score obtained/ maximum score possible. The quality index ranges from 0-1.

The efficacy of staining using modified Leishman stain was assessed based on quality index which depends on the staining characters of the smears.

The quality index score obtained from the smears stained with modified Leishman stain will be

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compared with the quality index obtained from the conventional Leishman stained smears.
Prior to the study, a pilot study was done on 20 samples with 120 smears. All the smears were prepared in a day. As the study was done on various days (day 1, day 3, day 5, day 10 and day 30) after preparing the modified leishman stain, the preservation of unstained peripheral blood smears without degenerative changes for 30days was difficult.
Hence, the study was done on the freshly prepared peripheral blood smears.

Results

The results of the stained smears were entered in Microsoft excel sheet, tabulated and analyzed.
A total of 600 peripheral blood smears prepared from 100 EDTA blood samples with no gender and age specification were included in the study.
The scores obtained by using Conventional and modified Leishman stain of the smears on day 1, 3, 5, 10 and 30 were tabulated and displayed in tables 2, 3, 4, 5 and 6.

Table 2: Total scores obtained using Conventional Leishman stain and modified Leishman stain on Day 1

	Overall staining	Nuclear morphology	Red blood cell staining	Cytoplasmic staining	Platelet staining
Conventional	32	38	40	40	38
1a	18	18	32	30	28
1b	26	29	34	25	30
1c	28	24	36	30	30
1d	30	32	34	28	28
1e	26	34	34	32	32

Table 3: Total scores obtained using Conventional Leishman stain and modified Leishman stain on Day 3

	Overall staining	Nuclear morphology	Red blood cell staining	Cytoplasmic staining	Platelet staining
Conventional	35	38	40	40	38
3a	26	26	21	21	20
3b	20	27	29	23	22
3c	36	20	26	23	20
3d	22	23	28	20	20
3e	20	21	23	22	25

Table 4: Total scores obtained using Conventional Leishman stain and modified Leishman stain on Day 5

	Overall staining	Nuclear morphology	Red blood cell staining	Cytoplasmic staining	Platelet staining
Conventional	35	38	40	40	38
5a	22	28	22	24	22
5b	23	30	26	31	31
5c	23	2	26	28	36
5d	20	24	25	26	28
5e	21	25	29	24	37

Table 5: Total scores obtained using Conventional Leishman stain and modified Leishman stain on Day 10

	Overall staining	Nuclear morphology	Red blood cell staining	Cytoplasmic staining	Platelet staining
Conventional	36	38	40	40	39
10a	35	38	32	38	30
10b	30	31	35	35	37
10c	20	33	27	40	33
10d	18	36	26	33	35
10e	22	33	23	32	37

Table 6: Total scores obtained using Conventional Leishman stain and modified Leishman stain on Day 30

	Overall staining	Nuclear morphology	Red blood cell staining	Cytoplasmic staining	Platelet staining
Conventional	35	38	40	40	38
30a	19	27	17	27	36
30b	13	24	17	24	37
30c	18	25	16	24	37
30d	15	23	14	26	36
30e	13	19	16	19	32

The optical staining results of the peripheral blood smears were obtained at the phenol to leishman powder ratio of 1:5. The Quality index of the peripheral blood smears calculated by using modified Leishman stain at phenol: Leishman stain at 1:5 on day 10 was 0.86, whereas on day 1 it was 0.63, day 3 it was 0.57, day 5 was 0.59 and on day 30 it was 0.63. The overall staining, cytoplasmic staining, nuclear morphology, red cell staining and platelet staining was better on day 10 as compared to day 1 and day 5. It was close to scoring of normal smears (0.95).

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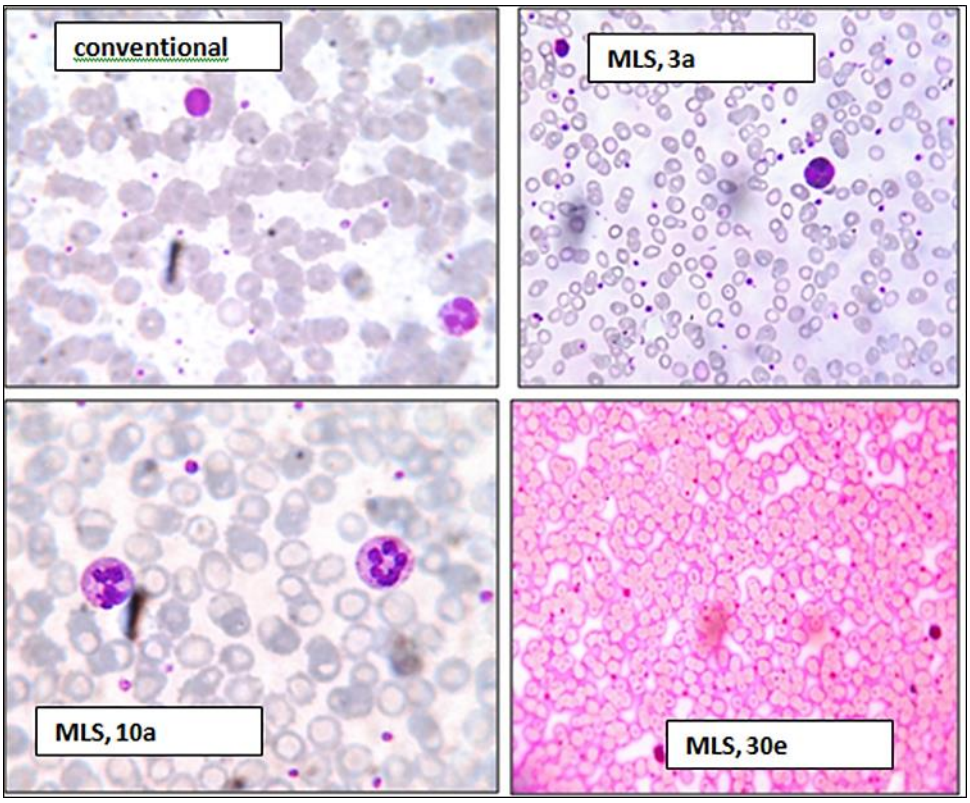
The quality index calculated on various days and using conventional Leishman stain and by Modified Leishman stain (MLS) at various concentrations are tabulated in table 7.

Table 7: Total scores obtained and Quality Index (QI) calculated using Conventional Leishman stain and Modified Leishman stain at different concentrations on various days

	Day 1		Day 3		Day 5		Day 10		Day 30	
Stain used	**Total score obtained**	**QI**	**Total score obtained**	**QI**	**Total score obtained**	**QI**	**Total score obtained**	**QI**	**Total score obtained**	**QI**
Conventional	188	0.94	191	0.95	191	0.95	193	**0.96**	191	0.95
MSL at concentration a	126	0.63	114	0.57	118	0.59	173	**0.86**	126	0.63
MSL at concentration b	144	0.72	121	0.60	141	0.70	168	0.84	112	0.56
MSL at concentration c	148	0.74	125	0.62	141	0.70	153	0.76	120	0.60
MSL at concentration d	152	0.76	112	0.56	123	0.61	148	0.74	113	0.56
MSL at concentration e	158	0.79	111	0.55	136	0.68	147	0.73	100	0.50

Figure 1 shows the stained peripheral blood smears using conventional Leishman stain and modified Leishman stain on day 3a, day10a and day 30e.



Discussion

Leishman stain, which is a group of Romanowsky stains, is a combination of acidic and basic dye. The basic dye is positively charged and stains nucleic acids and granules of basophils in gray color. The acidic dye that is negatively charged stains the hemoglobin and eosinophilic granules in red orange color [5, 6].

In conventional Leishman stain preparation, both the acidic and basic dyes are dissolved in acetone free methyl alcohol and it takes about 15-20 minutes of time to stain the peripheral blood smears. However, the usage of automated hematology analyzers and rapid diagnostic methods has created a need to reduce this duration of staining the peripheral blood smears especially in cases of sepsis and thrombocytopenia.

Hence, the present study was undertaken to develop a modified stain that can reduce the staining time of peripheral blood smears without comprising the staining quality. The results obtained were comparable to the other similar studies.

John William Field, in 1941 started using rapid stains in diagnostic laboratory for staining thick blood films to identify hemoparasites [7].

Fasakin *et al.* [8] modified the Leishman stain by adding phenol to conventional Leishman stain. This modification has aided in rapid staining of peripheral blood smears. He also concluded that modified Leishman stain can be used to reduce the staining time of peripheral blood smaers with overall reduction in turnaround time (TAT) of reporting the smears. In the study done by Fasakin *et al.* [8], modified Leishman stain was prepared by adding phenol to leishman powder at various ratios as 1:5 1:4 1:3 1:2

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and 1:1 respectively in 100 ml of staining solution and concluded that 1:5 and 1:3 ratio of modified Leishman stain gave better morphology than the conventional Leishman stain.
Phenol which is critical in Ziehl Neelsen stain, acts as an accentuating agent [9]. Phenol when added to leishman stain and dissolved in methanol can influence the fixation, staining and can facilitate in rapid analysis of blood cells without altering the cellular morphology [10].
In the study done at Premkumar *et al.* [10], the smears were stained used modified leishman stain on day 1, day 5 and day 10 and a quality indicator was used to compare and to assess the staining quality of the parameters such as overall staining, leucocyte staining (nuclear and cytoplasmic), erythrocytes staining and platelet staining and was compare with conventional leishman stained smear and was similar to the present study. The study done by Premkumar *et al.* [10] concluded that modified Leishman stain used on day 10 of preparation gave better results as compared to day 1 and day 5.
Modified Leishman stain at phenol to leishman powder ratio of 1:3 gave better results according to the study done by Rizwana Abdul Hye *et al.* [11].
In the study done by Aiswarya Anithakumari Manmadhan *et al.* [12], Villanueva stain was used, which was a modification of leishman stain and concluded that Chromatin patterns and neutrophil granular staining was poor with Villanueva compared to Leishman.
The present study has been compared with the results of similar studies and are tabulated in table-8.

Table 8: Comparison of the present study with similar studies

	Present study [2022]	**Rizwana Abdul Hye *et al* [11]. [2021]**	**Aiswarya Anithakumari Manmadhan *et al*. [12] [2020]**	**Premkumar *et al*. [10] [2019]**	**Fasakin *et al*. [8] [2014]**
No. of samples	100	85	128	101	-
Total no. of peripheral blood smears examined	600	170	256	404	-
Modifications done to conventional leishman stain	By adding Phenol	By adding Phenol	Villanueva staining	By adding Phenol	By adding Phenol
Phenol: leishman ratio	1:5, 1:3.75, 1:3, 1:2, 1:1	1:5, 1:4, 1:3, 1:2 and 1:1		1:3	1:5, 1:4, 1:3, 1:2 and 1:1
Optimal ratio	1:5 on day 10	1:3		1:3 on day 10	1:5 and 1:3
Parameters examined	Overall staining, Nuclear morphology, Red blood cell staining, Cytoplasmic and platelet staining	RBC pattern, nuclear pattern, neutrophil granules, eosinophil granules, platelets and background staining	Nuclear chromatin, granules of neutrophil and eosinophil, RBCs and platelets	Overall staining, cytoplasmic staining, nuclear morphology, red cell staining and platelet staining	Red blood cells, leukocytes including the nuclei and cytoplasmic granules (specific for each granulocytic leukocyte) and platelets
Scoring system used	Quality Index	Grading of the peripheral blood smears	Scoring system	Quality Index	-

Conclusion

Modified Leishman Stain can be prepared easily by adding phenol to conventional Leishman stain preferably at a concentration of 1:5 and can be use for staining the peripheral blood smears thereby reducing the turnaround time.

Conflict of Interest: None.
Funding Support: Nil.

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