Original Article

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Pathology Section

Prior Incubation as a Tool to Overcome Understaining of Leishman Stain: A Cross-sectional Study

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ABSTRACT

Introduction: A well stained peripheral smear is an integral part in the diagnosis and management of many clinicohaematologic conditions. Leishman stain is recommended universally by the International Committee for Standardisation in Haematology (ICSH). However, a commonly encountered problem in tropical countries, especially during the rainy season, is under staining by Leishman stain as a result of interference by the atmospheric moisture. Hence, incubation of slides and/or buffer prior to standard Leishman staining protocol could bring out best staining qualities.

Aim: To assess the effects of prior incubation of slides and/or buffer on standard Leishman staining technique.

Materials and Methods: The cross-sectional study was conducted over a period of eight weeks in the Central Laboratory, Kempegowda Institute of Medical Sciences, Bangalore, India, in the months of October to November 2020. Blood samples were received in the central laboratory for routine haematological investigations. Total of 100 samples of left over non haemolysed Dipotassium Ethylenediaminetetraacetic Acid (K2-EDTA) anticoagulated blood irrespective of age, gender and cell counts were included in the study. Slides were stained following prior incubation of slides and/or buffer at 37°C and compared with the standard staining technique. The staining characteristics of nucleus, cytoplasm and granules of White Blood Cells (WBCs), platelets and erythrocytes were observed. One-way Analysis of Variance (ANOVA) test and p-value of <0.05 was considered statistically significant and higher f value (>104.35152) rejected the null hypothesis.

Results: Prior incubation of slides and/or buffer resulted in better stained smears which were found to be statistically significant (p-value <0.05) as compared to standard Leishman staining technique.

Conclusion: Minor modifications when applied to standard Leishman staining technique such as prior incubation of slides and/or buffer at 37°C produced best staining characteristics to overcome the effects of humidity which normally interfere with staining of slides.

Keywords: Buffer, Haematology, Humidity, Staining

INTRODUCTION

Peripheral blood smear examination is an invaluable test in haematologic work-up, even in the age of artificial intelligence, automation and molecular diagnostics, with the cornerst one in diagnosis requiring a well stained peripheral blood smear [1]. History of stains for blood films dates back to 19th century when Baeyer and Caro introduced eosin and methylene blue respectively while Ehrlich mixed it with an acidic dye to create a neutral stain which allowed differential staining of blood cells [2]. Checin'ski used eosin as the acidic dye to create the methylene blue-eosin prototype. Plehn, by altering the proportions of methylene blue and eosin widened the range of the colours. In 1891, Russia physician, Dmitri Leonidovich Romanowsky developed a staining procedure by mixing eosin Y, azure B and methylene blue [3]. The resulting dye stained the nucleus and the granules of the cytoplasm in different shades of purple [4]. Leishman's stain, a variant of the Romanowsky stain, is named after Scottish pathologist William Borg Leishman. Leishman and Karl Reuter independently developed these stains. They combined eosin with polychromed methylene blue, filtering off the precipitate and redissolving it in alcohol. Leishman used methanol and substituted eosin B for eosin Y, whereas Reuter used absolute alcohol [2]. Leishman stain is used universally for staining peripheral blood smearsand is recommended by International Committee for Standardisation in Haematology (ICSH) [5,6].

Automated haematology analysers have greatly reduced the need for peripheral blood smears. However, smear examination is still warranted when flagged forred cell fragments/schistocytes, abnormal cells/blasts or when clinically indicated as in early diagnosis of thrombotic thrombocytopenic purpura pseudo-thrombocytopenia and malaria [7,8]. Peripheral smears also aid in the conclusive diagnosis of sickle cell disease or chronic myeloid leukaemia [1].

Further, many laboratories across India still rely on manual smear examination making a well stained peripheral blood smear prerequisite for correct diagnosis. However, factors such as humidity and temperature interfere in the staining process [1]. With India being a tropical country, a commonly encountered problem is under staining of peripheral smears by Leishman stain, especially during the rainy season, as a result of interference by the atmospheric moisture [8]. Studies on utility of Leishman stain as a cytological stain too, have reported understaining of 3-dimensional cell clusters, individual cells and the cytoplasmic constituents such as granules, along with intense staining of extracellular ground substance and nucleus. Giemsa stain, another commonly used Romanowsky stain in haematology and cytology, excellently stains the cytoplasm but shows reduced staining of nucleus and cytoplasmic granules [9]. Hence, it was proposed to conduct a detailed study to assess the effects of prior incubation of slides and/or buffer on standard Leishman staining technique and to statistically evaluate the staining characteristics in comparison with standard Leishman staining technique and to reject the null hypothesis.

MATERIALS AND METHODS

A hospital-based cross-sectional study was conducted in the Central Laboratory, Department of Pathology, Kempegowda Institute of Medical Sciences (KIMS), Bangalore, India, over a period of eight weeks during the rainy months of October to November 2020 after obtaining approval by Institutional Ethics committee (IEC)

with the ethical clearance number KIMS/IEC/A039/M/2020. The sample size was restricted to 100 samples based on a previous study and selected by simple random sampling method [8]. The left over K2-EDTA anticoagulated blood samples received at the central laboratory for various haematology tests were included in the study irrespective of age, gender and cell counts. Haemolysed and clotted samples were excluded from the study.

Study Procedure

For each sample collected, four different peripheral blood smears were made as described below:

1. Control Slide (CS): Standard Leishman staining: Slides were directly used without any incubation. Air dried peripheral smears were placed on the staining rack and covered with undiluted Leishman stain for one to two minutes following which double the amount of buffer water was poured and kept undisturbed for 8-10 minutes [10]. The slides were then washed under running water stream and air dried.

2. Incubated Slide (IS): Smears were made on slides preincubated at 37°C for atleast one hour and stained according to the standard Leishman staining procedure.

3. Incubated Buffer (IB): Peripheral blood smears were stained similar to control slides, however buffer water priorly placed in an incubator at 37°C for atleast one hour was used.

4. Incubated Slide and Buffer (ISB): Both preincubated slide and buffer at 37°C were used in the standard Leishman staining procedure.

Thus, series of four smears were obtained for each sample (4×100=400 blood smears). Each blinded smear was observed under 1000X (oil immersion) magnification for the staining characteristics of all blood elements and scored from 0 to 2. Smears showing ghost outline of WBCs, barely visible/unstained platelets and erythrocytes showing sharply refractile area of central pallor making chromia difficult to assess were considered as poorly stained smears and scored 0. Score 2 reflecting excellent staining characteristics was awarded for smears wherein WBCs showed crisp cytoplasmic borders, well stained nucleus and clearly discernible granules; well stained granulated platelets and well-preserved erythrocyte morphology and chromia. Smears which demonstrated staining characteristics which was neither 2 nor 0 were considered as satisfactory and scored as 1. Thus, each method would get a maximum score of 200 (100×2) and minimum score of 0.

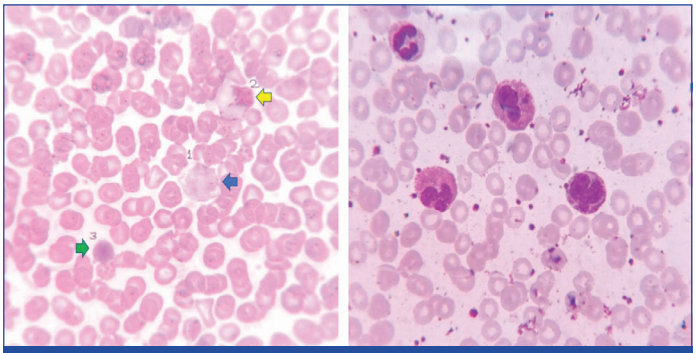
STATISTICAL ANALYSIS

The statistical analysis was done using online free software in socscistatistics.com. One-way ANOVA test and p-value of <0.05 was considered statistically significant and higher f value (>104.35152) rejected the null hypothesis.

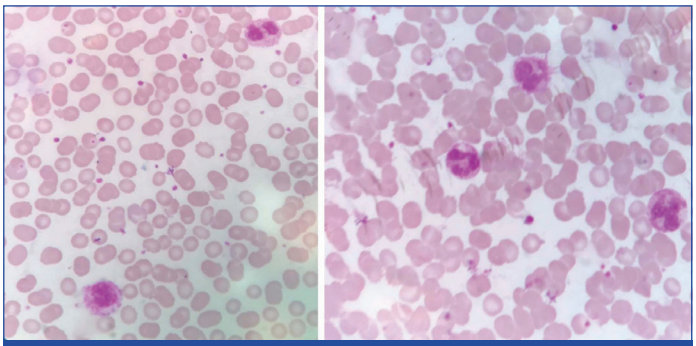
RESULTS

A total of 400 (100×4) smears were screened. The staining characteristics, such as crisp cytoplasmic borders, well stained nucleus and clearly discernible granules in WBCs; well stained granulated platelets and well-preserved erythrocyte morphology and chromia, showed poorest staining quality with standard Leishman staining technique [Table/Fig-1], while best staining characteristics were identified in smears prepared with prior incubation of both slides and buffer [Table/Fig-2]. This was followed by smears prepared with only preincubated buffer [Table/Fig-3], further followed by smears prepared on preincubated slides alone [Table/Fig-4].

Thus, each slide was scored on a scale of 0-2 for the different staining characteristics such as WBC nuclear staining, crisp cytoplasmic borders and granule staining quality and overall smear quality which was inclusive of Red Blood Cells (RBCs) and platelet morphology. The scores obtained for each staining techniques was summed up. The smears prepared following prior Incubated Slide and Buffer (ISB) obtained highest scores. Smears prepared with only preincubated buffer scored marginally better than smears prepared using preincubated slides alone. All the methods scored higher than CS [Table/Fig-5].



[Table/Fig-1]: Standard Leishman staining. Under stained neutrophil (blue arrow), eosinophil (yellow arrow) and a lymphocyte (green arrow) with unstained platelets and erythrocytes showing sharp refractile area of central pallor in the background.
[Table/Fig-2]: Incubated Slide and Buffer (ISB). Crisp cytoplasm, well-demonstrated granule and nuclear features in WBCs with well-stained platelets and erythrocytes. (Images from left to right)



[Table/Fig-3]: Incubated buffer alone (IB). Better stained granules of neutrophils, better stained platelets and erythrocytes than standard staining techniques.
[Table/Fig-4]: Incubated slide alone (IS). Better stained granules of neutrophils, better stained platelets and erythrocytes than standard staining techniques. Nucleus appears hazy. (Images from left to right)

Blood elements	Score 0	Score 1	Score 2	Sum of scores
WBC nucleus				
CS	63	30	7	44
IS	9	67	24	115
IB	4	59	37	133
ISB	0	7	93	193
WBC cytoplasm				
CS	52	43	5	53
IS	8	68	24	116
IB	6	67	27	121
ISB	0	17	83	183
WBC granules				
CS	50	43	7	57
IS	3	65	32	129
IB	2	64	34	132
ISB	0	7	93	193
Overall smear quality				
CS	58	38	4	46
IS	1	73	26	125
IB	0	69	31	131
ISB	0	5	95	195

[Table/Fig-5]: Score obtained in different staining techniques. n=100; max score: 200

Statistical analysis to compare the staining characteristics of the nucleus, cytoplasm and granules of WBCs was performed. Prior incubation of both slides and buffer showed a higher value and was statistically significant with p-value <0.05 as shown in [Table/Fig-6-8].

WBC nucleus	Leishman stain	IS	IB	ISB	Total
Total number of samples	100	100	100	100	400
Sum of the scores (ΣX)	44	115	133	193	485
Mean	0.44	1.15	1.33	1.93	1.213
Standard deviation	0.6247	0.5573	0.5515	0.2564	0.7408

[Table/Fig-6]: Statistical analysis for smear quality of WBC nucleus.
One way ANOVA; f-value=140.61343; p-value=<0.001; The result is significant at p<0.05

WBC cytoplasm	Leishman stain	IS	IB	ISB	Total
Total number of samples	100	100	100	100	400
Sum of the scores (ΣX)	53	116	121	183	473
Mean	0.53	1.16	1.21	1.83	1.183
Standard deviation	0.5938	0.5453	0.5374	0.3775	0.6931

[Table/Fig-7]: Statistical analysis for smear quality of WBC cytoplasm.
One way ANOVA; f-value=104.35152; p-value <0.001; The result is significant at p<0.05

WBC granules	Leishman stain	IS	IB	ISB	Total
Total number of samples	100	100	100	100	400
Sum of the scores (ΣX)	57	129	132	193	511
Mean	0.57	1.29	1.32	1.93	1.278
Standard deviation	0.6237	0.5183	0.5101	0.2564	0.6904

[Table/Fig-8]: Statistical analysis for smear quality of WBC granules.
One way ANOVA, f value=125.84194, p-value <0.001; The result is significant at p<0.05

The overall smear quality, which was scored based on staining characteristics of WBCs, erythrocytes and platelets, too demonstrated the best staining characteristics with prior incubation of both slides and buffer. The mean scores were 1.95, 1.31 and 1.25 for ISB, Incubated Buffer (IB) and Incubated Slide (IS) respectively as shown in [Table/Fig-9]. The standard Leishman technique had a mean score of 0.46. The f-ratio value was 185.007788 and the results were statistically significant with p-value <0.001.

Overall stain quality	Leishman stain	IS	IB	ISB	Total
Total number of samples	100	100	100	100	400
Sum of the scores (ΣX)	46	125	131	195	497
Mean	0.46	1.25	1.31	1.95	1.243
Standard deviation	0.5759	0.4578	0.4648	0.219	0.6927

[Table/Fig-9]: Statistical analysis for overall stain quality.
*One way ANOVA; f-ratio value: 185.007788; p-value <0.001; The result is significant at p<0.05

Thus, the overall scores for each method were summed up to yield a maximum of 800 and a minimum of 0. Prior incubation of IS and IB alone scored 485 and 507 respectively. The incubation of both slide and buffer (ISB) yielded highest score of 764 with f-ratio value of 539.2215 and was found to be statistically significant with p-value is <0.001. The CS yielding lowest score of 200 as shown in [Table/Fig-10].

	Mean score	Total score obtained (out of max score 800)	SD
CS	0.5	200	0.604
IS	1.212	485	0.522
IB	1.292	507	0.517
ISB	1.91	764	0.286

[Table/Fig-10]: Comparative scores of all slides.
*One way ANOVA; f-ratio value=539.2215, p-value <0.001; The result is significant at p<0.05

DISCUSSION

Peripheral smear examination forms an integral part of haematologic work-up with a well stained peripheral smear being an absolute prerequisite. Several pioneers such as Caro, Ehrlich and Romanowsky have contributed to present days' blood staining protocol. ICSH recommends Leishman stain made up of Azure B (oxidized methylene blue dye) and Eosin Y which provides good contrast, is inexpensive and readily available [6]. The azures, containing the alkylamino substituents, are basic and bind to the acid nuclei forming a blue-purple colour while the acid dye eosin, containing a carboxyl group, binds to the alkaline cytoplasm forming red colouration.

Many laboratories in the present day are equipped with automated haematology analysers to evaluate complete blood counts. However, manual verification by checking blood films is often necessary for clinical/haematologic diagnosis as in case of malaria, schistocyte quantification in early diagnosis of thrombotic thrombocytopenic purpura and as per the guidelines recommended by the International Consensus Group for Haematology Review [11,12]. Further, according to Clinical and Laboratory Standards Institute (CLSI) H_2O-A22 (formerly NCCLS H_2O-A), peripheral smear examination is recommended for the assessment of leukocyte differentials as a part of quality control and verification of automated analysers [13].

Thus, a well stained peripheral blood smear is imperative to correct diagnosis. However, an ideal peripheral blood smear is dependent on many factors which can be classified into:

1. Technical factors: time since collection of blood samples, blood drop size, angle and pressure of spreader, speed of smearing, reagents stability, absoluteness of methanol, staining time and washing of the slides.
2. Environmental factors: temperature, humidity and pH of the buffer [14,15].

Technical factors can be appropriately managed by adequate staff training and maintenance of quality assurance programs while the environmental factors act as natural confounders which affect the staining quality of smears. Excessive acidic pH causes excessive pinkish discoloration of smear while excessive basic pH causes bluish discoloration. One should even avoid pipetting from the stock solution so as to minimise exposure of moisture which can deteriorate the quality of Leishman stain [8]. Furthermore, pH, temperature and humidity are interdependent on one another. With India being a tropical country, humidity in the air influences the staining quality resulting in understaining of the slides especially during rainy months [8].

The staining procedure with ISB showed excellent staining characteristics with clear nuclear features, crisp cytoplasm and well appreciated granules similar to a study by Sareen R et al., who demonstrated best morphological features in peripheral blood smears following prior incubation of glass slides and buffer solution [8]. The process of incubation provides for controlled, contaminant-free environment by regulating conditions like temperature and humidity. However, in the present study, comparing the effects of only IS and IB, overall better staining characteristics were experienced with prior IB as against Sareen R et al., who found improved the staining qualities of blood smear with prior slide (IS) incubation technique [8]. The Leishman stain and buffer solution and staining time was retained the same as in routine staining procedure except that the same buffer solution was priorly incubated in IB and ISB technique in this study. It was observed that overall prior incubation of either slide/buffer or both resulted in better results than routine Leishman staining technique (CS) similar to Sareen R et al., [8].

Furthermore, in this study, p-value was found to be highly significant (<0.001) in assessment of all the cell characteristics. The results also showed a higher f-value, which rejected the null hypothesis and underlines a positive effect of incubation on the Leishman staining quality similar to the findings by Sareen R et al., who showed a higher f-value when comparing the three modified Leishman staining procedures [8].

Limitation(s)

The study did not assess the utility of incubation on other Romanowsky stains such as giemsa, which is also associated with understaining of the smears. Further, these findings are pertinent to tropical countries with high humidity. Hence, studies involving large population groups are needed to verify the findings.

CONCLUSION(S)

Peripheral blood smears, being an important part of haematologic work-up, a well stained smear is for paramount importance. Minor modifications like prior slide and/or buffer incubation results in simple, yet effective solution to understaining of slides in tropical countries like India which can be easily adopted in routine laboratory practice.

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