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Original Article

Pathology Section

A Comparative Study between Conventional Leishman Stain and a Modified Blood Stain for the Evaluation of Haematologic Elements

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ABSTRACT

Introduction: Leishman is the most common Romanowsky stain globally used for studying peripheral blood morphology. Leishman stain is a compound neutral dye using combination of either eosin Y or eosin B with partially oxidised methylene blue. Inconsistent staining reactions occurring with these dyes are attributed to the difficulty in controlling the methylene blue oxidation process.

Aim: To compare the staining qualities of Leishman with a modified blood stain called Villanueva stain which has a combination of non-oxidised methylene blue, azure II and Eosin.

Materials and Methods: Present cross-sectional study was carried out in Central Haematology laboratory of a tertiary health care centre in Southern India, from August 2017 to January 2018. Blood Sample was collected in anticoagulated vials. Two thin wedge smears were prepared from each sample and one was stained with Villanueva and other with Leishman stain. The staining characteristics were assessed and scored in terms of 5 parameters- nuclear chromatin, eosinophil granules, neutrophil granules, platelets, Red Blood Cell (RBC) staining and finally all scores were summed as poor/satisfactory/good/excellent by an experienced pathologist. The results were entered in Microsoft excel sheet and were analysed by Statistical Package for the Social Sciences (SPSS) software version 16.0.

Results: The chromatin staining was superior in Leishman staining (Measure agreement kappa = 0.028, p=0.631, McNemar test p=0.001). For platelet staining and RBC the statistical agreement between both tests were moderate and for neutrophils and eosinophil granules agreement was poor between two staining methods.

Conclusion: The modified stain can be easily prepared from chemicals cheaply available. Though modified stain gave inferior results with conventional Leishman stain, present study was helpful to know if peripheral smear can be interpreted with this modified stain in case of Leishman stain shortage. Alternative staining method deserves importance in this era of global lock down where manufacture and transportation of chemicals are adversely affected worldwide.

Keywords: Azure, Non-polychromed stain, Romanowsky, Villanueva

INTRODUCTION

Peripheral blood film examination plays a very crucial role in the diagnosis of many haematologic diseases. A well stained peripheral blood film with good morphological details is very essential for an accurate diagnosis from blood films. Hence, assuring the supply of best quality stains is one of the prime concerns of haematology laboratories all over the world. Romanowsky stains are compound neutral dyes using a partly polychromed methylene blue in combination with Eosin Y or Eosin B [1-3]. The polychroming process gives differentiation ability to the Romanowsky dyes creating wide range of hues in different types of Leucocytes and help in their easy identification in smears [2,3].

Leishman, Giemsa, Jenner, Wright etc are the members of Romanowsky family of which Leishman is the most commonly used one all over the world [4,5]. Commercial preparation of Leishman stain is done by oxidising methylene blue. But even when controlled spectrophotometrically, does not always give repeatable results. Improper oxidation can result in inconsistent staining reactions posing difficulties in morphological interpretation of cells. Another problem in polychroming of methylene blue is the presence of impurities in the dye used, which also affect the proper balancing of eosin during the combination process. The commercially prepared Leishman powder after mixing with methanol undergoes deterioration in its potency gradually [4-6]. Uninterrupted supply of good quality stain is a challenge for many laboratories especially India. In many centres running with limited resources, pathologists are forced to intend least priced chemicals and stains over the best quality ones.

Inconsistent staining results with Leishman's stain were reported in our laboratory in few occasions where we could not procure best quality dyes. This prompted us to experiment on modified stains giving comparable results like conventional methods, which can be prepared in the laboratory. Though many modified methylene blue techniques are described in literature, Villanueva method was chosen in present study due to its ease of preparation in the laboratory [7-9]. The modified stain described here was developed by Villanueva AR who used known quantities of dyes of unpolychromed methylene blue, azure II and eosin Y [8]. In addition, the precipitated powder keeps indefinitely without any appreciable loss of potency when it is dissolved in 100% Methanol or in equal parts of glycerol and methanol [7,8]. The stock solution is stable at room temperature. This study was aimed at comparing the staining reaction of the modified stain with Leishman stain.

MATERIALS AND METHODS

The study was a cross-sectional study carried out in Central Haematology laboratory of a tertiary health care centre in Southern India. The study period was six months, from August 2017 to January 2018, after getting approval from Institutional Ethics Committee (HEC.No.07/102018/MCT). Two separate smears were prepared from each patient's sample after obtaining informed consent. The

study population included all patients (outpatient and inpatient) of all age groups, whose blood samples were collected in Ethylene Diamine Tetra Acetic acid (EDTA) (1.5-2mg/ml) anticoagulated bottles, sent to the Central Haematology Laboratory, Unlabelled, haemolysed, clotted and lipemic specimens were excluded from the study. The sample size was calculated from the following formula sample size, $N=\frac{2\left(z_{1-\frac{\alpha}{2}}+z_{1-\beta}\right)^2 s^2}{\delta^2}$, $s^2=\frac{s_1^2+s_2^2}{2}$ (S_1: standard deviation in the first group, S_2: standard deviation in the second group, δ: Mean difference between the groups, α: Significance level = 5%, 1-β: Power = 80%, N= 128 in each method).

For conventional Leishman staining we used commercially available Leishman powder (manufacturer Merck). The Components of Villanueva stain powder are Methylene blue (CI No. 52015), Azure II (NA 0443), Eosin Y (CI No. 45380) and we prepared this stain in the laboratory. All stains we used in present study were manufactured by Merck. For preparing Villenueva stain, 0.85 g Eosin Y (C.I. No. 45380) was first thoroughly mixed in 47.4 ml distilled water in a beaker, slowly, with constant stirring. 0.5g of Methylene blue (CI No. 52015) and 0.35g Azure II (NA 0443) in dissolved 47.4 ml distilled water in a beaker. The precipitated mixture was covered with an opaque paper to reduce the action of light for 24 hours. The precipitate is taken by filtration methods and dried in an oven at 50°C-60°C. When completely dried it is pulverised by means of mortar and pestle and then placed in dessicator for 24 hours. The powdered stain is then transferred to into a tightly covered bottle for storage. The stain solution can be prepared by thoroughly grinding 0.2 gm dried powder using a motor and pestle and then dissolving in absolute methanol [8,9].

The samples collected in the laboratory are from venipuncture. The large median cubital and cephalic veins are usual choice. The collected blood was immediately added to the anticoagulated bottles. The anticoagulant of choice is EDTA (1.5-2mgEDTA/ml of blood). A number of potential artifacts may arise if blood cells remain in EDTA for more than five hours. This includes spherocytes, Echinocytes, vacuolated neutrophils and monocytes etc. Two separate smears were prepared from each patient sample; one for conventional Leishman stain and other used for modified blood stain. Manual wedge method is preferred for film preparation on both slides. Also, ensure that the slides are dry and free from scratches, dust, lint, and fat. The slides should measure 75x25 mm, and approximately 1 mm thickness. The spreader of choice must be smooth and free from chipped edges, and the spreader need to be narrower than the slide where blood films were made and should be wiped carefully and dried before and after each use.

Procedure for Preparing Smears [10-12]

Slide is placed on a flat surface. A drop of blood, approximately 2 mm in diameter is placed about 1cm from the end of slide. Using the dominant hand the spreader is held in front of the drop and it is moved back against the drop, allowing the blood to spread eventually along the width of spreader slide by capillary action. The angle between the spreader and the specimen slide is 45°. With a steady motion the spreader slide is pushed forward. A thin bullet shaped film of blood forms on slide surface. In the case of anaemic sample a wider angle is preferred and for polycythemia sample narrower angle. Film is labelled immediately after spreading and allowed to air dry.

Staining Procedure [10-12]

For conventional staining, air dried smears were flooded with Leishman stain and allowed to stand for 1-2 minutes, during which fixation takes place. After that twice amount of working buffer pH (6.8) is added and mixed gently by blowing and stain buffer mixture is kept undisturbed for 7-10 minutes staining of blood cells occur with this step and adequate staining result in the formation of metallic scum on the smear surface. The stain was washed with distilled water drained and air dried. Villanueva staining follows similar steps like conventional method, except lesser time in stain buffer mixture (5-6 minutes).

For each case two slides were prepared, One of them stained using Leishman stain and another with Villanueva stain The staining quality was assessed by an experienced Pathologist evaluating features like chromatin, granules of neutrophil and eosinophil, RBCs and platelets, in each set of smear. Peripheral smear examination followed systematic methods by assessing overall qualities in scanner objective, followed by 10x, 40x, 100x objectives for better magnification. Minimum 10 high power fields (hpf)/slide was selected for assessing parameters of this study. Five parameters are assessed for each staining techniques- Chromatin staining, neutrophil granules, eosinophil granules, platelet and RBC staining. Depending on the quality of staining sores 1 to 4 was given. The scoring system we used was a modified version used by Villanueva and many previous researches comparing staining qualities between different Romanowsky stains [Table/Fig-1,2] [8,13,14].

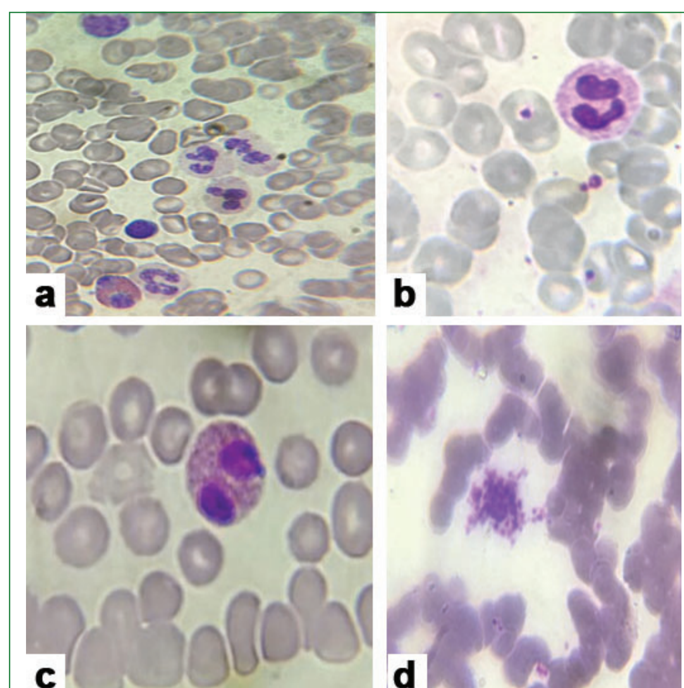
Chromatin	Neutrophil granules	Eosinophil granules	Platelet	RBC	Score
Purple	Red-purple	Purple red	Pale purple	Dull pink	1
Purple-blue	Blue-purple	Dull red	Purple	Grey pink	2
Blue-purple	Purple	Orange-red	Purple	Pink	3
Blue	Bright lilac	Bright orange red	Violet-purple	Salmon pink	4

[Table/Fig-1]: Staining patterns of Leishman stain.

Chromatin	Neutrophil granules	Eosinophil granules	Platelet	RBC	Score
Unstained	Red	Pink	Pink	Pale pink	1
Pale blue	Reddish purple	Orange	Pale purple	Pink	2
Light purple	Purple	Orange red	Purple	Pink	3
Dark purple	Bright lilac	Brick red	Bright purple	Deep pink	4

[Table/Fig-2]: Staining patterns of Villanueva stain.

The morphologic features of blood cells in Leishman and modified Villanueva stain in high power objective (40x)/Oil immersion (100x), is depicted in [Table/Fig-3].



[Table/Fig-3]: Staining of blood cells in Conventional and Villanueva stains: a) Villaneuava stain 40x- Eosinophils, Neutrophils and RBCs; b) Leishman stain 100 x neutrophils and RBCs; c) Leishman stain 100x -Eosinophils and RBCs; d) Villaneuva stain 100X-platelet

For each smear, the five parameters were individually scored from 1-4 as in [Table/Fig-1,2]. The individual scores of each parameters were compared between two staining methods. The final score of each staining method for all slides were calculated by summing up individual scores of all five parameters. The total scoring was categorised into I to IV (poor/satisfactory/good/excellent) based on the total scores we got from each staining method [Table/Fig-4]. The overall staining qualities between two staining methods were compared by comparing the final scores.

Category	Subjective rating of stains for diagnostic purpose	Total score
I	Poor	1-5
II	Satisfactory	6-10
III	Good	11-15
IV	Excellent	16-20

[Table/Fig-4]: Total score.

STATISTICAL ANALYSIS

The results were analysed by SPSS version 16. Tests applied were McNemar test, measurement agreement kappa.

RESULTS

The present study evaluated the comparison between conventional Leishman stain and Villanueva stain. One hundred and twenty eight samples were assessed for both the methods. Five parameters were statistically evaluated separately and finally overall staining characteristics were compared. Each parameter was compared using scoring system. The chromatin pattern, in Leishman Staining, and Villanueva is showed in [Table/Fig-5]. (Measure agreement kappa = 0.028, p=0.631, McNemar test p=0.001). There was a poor agreement between the two test methods indicating there was a significant difference between the two test results. Leishman method was comparatively better than Villanueva [Table/Fig-6].

Chromatin	Leishman		Villanueva	
	Frequency	Percent	Frequency	Percent
Poor	8	6.3	12	9.4
Satisfactory	32	25.0	60	46.9
Good	80	62.5	48	37.5
Excellent	8	6.3	8	6.3
Total	128	100.0	128	100.0

[Table/Fig-5]: Chromatin staining comparison between Leishman and Villanueva.

Chromatin		Villanueva				Total
		Poor	Satisfactory	Good	Excellent	
Leishman	Poor	4	4	0	0	8
	Satisfactory	4	12	16	0	32
	Good	4	40	28	8	80
	Excellent	0	4	4	0	8
Total		12	60	48	8	128

[Table/Fig-6]: Chromatin staining comparison between Leishman and Villanueva and measuring kappa.
Measure agreement kappa= 0.028; p=0.631; McNemar test p=0.001

The neutrophil granules, in Leishman staining and in Villanueva is shown in [Table/Fig-7] respectively. There was poor measurement of agreement between two test method (0.089, 0.067) [Table/Fig-8].

The grading of eosinophil granules as poor, satisfactory, good and excellent in Leishman staining and in Villanueva staining is shown in [Table/Fig-9] respectively. There was slight measurement of agreement between two methods (0.243, <0.001) [Table/Fig-10].

Neutrophil granule	Leishman		Villanueva	
	Frequency	Percent	Frequency	Percent
Poor	12	9.4	24	18.8
Satisfactory	28	21.9	60	46.9
Good	68	53.1	36	28.1
Excellent	20	15.6	8	6.3
Total	128	100.0	128	100.0

[Table/Fig-7]: Neutrophil granule staining comparison between Leishman and Villanueva.

Neutrophil granule		Villanueva				Total
		Poor	Satisfactory	Good	Excellent	
Leishman	Poor	0	12	0	0	12
	Satisfactory	4	16	8	0	28
	Good	16	24	24	4	68
	Excellent	4	8	4	4	20
Total		24	60	36	8	128

[Table/Fig-8]: Neutrophil granule staining comparison between Leishman and Villanueva and measuring kappa.
Mc Nemar test p<0.001; Measure agreement kappa = 0.089; p=0.067

Eosinophil granule	Leishman		Villanueva	
	Frequency	Percent	Frequency	Percent
Poor	16	12.5	24	18.8
Satisfactory	40	31.3	60	46.9
Good	28	21.9	32	25.0
Excellent	44	34.4	12	9.4
Total	128	100.0	128	100.0

[Table/Fig-9]: Eosinophil granule staining comparison between Leishman and Villanueva.

Eosinophil granule		Villanueva				Total
		Poor	Satisfactory	Good	Excellent	
Leishman	Poor	12	4	0	0	16
	Satisfactory	12	28	0	0	40
	Good	0	24	4	0	28
	Excellent	0	4	28	12	44
Total		24	60	32	12	128

[Table/Fig-10]: Eosinophil granule staining comparison between Leishman and Villanueva and measuring kappa.
McNemar test p<0.001
Measure agreement kappa = 0.243; p<0.001

For platelet staining, Leishman staining and in Villanueva is shown in [Table/Fig-11] respectively. There was moderate measurement of agreement between two methods (0.500, <0.001) [Table/Fig-12].

Platelet	Leishman		Villanueva	
	Frequency	Percent	Frequency	Percent
Poor	8	6.3	16	12.5
Satisfactory	40	31.3	56	43.8
Good	72	56.3	52	40.6
Excellent	8	6.3	4	3.1
Total	128	100.0	128	100.0

[Table/Fig-11]: Platelet staining comparison between Leishman and Villanueva.

RBC staining in Leishman staining in Villanueva is shown in [Table/Fig-13]. There was moderate measurement of agreement between two methods (McNemar test p<0.001, Measure agreement kappa = 0.254, p<0.001 [Table/Fig-14].

Platelet		Villanueva				Total
		Poor	Satisfactory	Good	Excellent	
Leishman	Poor	8	0	0	0	8
	Satisfactory	8	32	0	0	40
	Good	0	20	48	4	72
	Excellent	0	4	4	0	8
Total		16	56	52	4	128

[Table/Fig-12]: Platelet staining comparison between Leishman and Villanueva and measuring kappa.
McNemar test p<0.001; Measure agreement kappa = 0.500; p<0.001

RBC	Leishman		Villanueva	
	Frequency	Percent	Frequency	Percent
Poor	12	9.4	28	21.9
Satisfactory	48	37.5	60	46.9
Good	60	46.9	36	28.1
Excellent	8	6.3	4	3.1
Total	128	100.0	128	100.0

[Table/Fig-13]: RBC staining comparison between Leishman and Villanueva and measuring kappa.

RBC		Villanueva				Total
		Poor	Satisfactory	Good	Excellent	
Leishman	Poor	8	4	0	0	12
	Satisfactory	20	28	0	0	48
	Good	0	28	28	4	60
	Excellent	0	0	8	0	8
Total		28	60	36	4	128

[Table/Fig-14]: RBC staining comparison between Leishman and Villanueva and measuring kappa.
McNemar test p<0.001;Measure agreement kappa = 0.254; p<0.001

Regarding overall staining: Out of the total slides 46.9% (n=60) were good for Leishman stain and 43.8% (n=56) for Villanueva stain, 34.4% (n=44) were excellent for Leishman stain and 9.4% (n=12) were excellent for Villanueva stain (McNemar test p<0.001, Measure agreement kappa= 0.074, p=0.176) [Table/Fig-15-17].

Overall staining	Leishman		Villanueva	
	Frequency	Percentage	Frequency	Percentage
Poor	0	0	0	0
Satisfactory	24	18.8	60	46.9
Good	60	46.9	56	43.8
Excellent	44	34.4	12	9.4
Total	128	100	128	100

[Table/Fig-15]: Overall staining comparison between Leishman and Villanueva

Overall staining		Villanueva			Total
		Satisfactory	Good	Excellent	
Leishman	Satisfactory	20	4	0	24
	Good	36	20	4	60
	Excellent	4	32	8	44
Total		60	56	12	128

[Table/Fig-16]: Overall staining comparison between Leishman and Villanueva **and** measuring kappa.
McNemar test p<0.001; Measure agreement kappa = 0.074; p=0.176

Overall staining			
Type of Stain	Total	mean	SD
Leishman	128	13.34	2.96
Villanueva	128	11.34	2,79

[Table/Fig-17]: Overall staining comparison between Leishman and Villanueva.

DISCUSSION

Rowmanowsky stains are widely employed for blood and marrow smears. Rowmanowsky is a generic description of Azure B or Methylene blue and Eosin stain family. Oxidation of metheylene blue results in formation of Azure compounds during commercial production of Leishman stain [15,16]. Nearly, all laboratories use Azure B rather than pure compound. The exact composition and particular amount of azure B varies among manufactures and this is the major reason behind poor staining qualities seen in some brands of Leishman stain. Starting from ancient reports that rare samples of methylene blue where apparently sufficiently contaminated with azures to give red plasmodial and red purple nuclear chromatin in chenzinky type methylene blue eosin stains [17]. Numerous modifications of Rowmanowsky stains available in the market [7,17,18]. Most of them include polychromed methylene blue with related thiazine dyes and eosin. The use of un polychromed methylene blue is to minimise contamination of oxidation product of methylene blue such as Azure A, Azure C, Thionin [7,8,18].

The present study was done to develop a modified blood stain using known quantities of unpolychromed methylene blue, Eosin and Azure II [6]. For chromatin, in the case of Leishman stain is superior to Villanueva stain. The results were in concordance with the observations done by Villanueva AR in bone marrow aspirate smears and peripheral blood films [8]. Eosinophil granules and platelets staining were comparable with both methods in present study and this observation was in concordance with Villanueva study in haematologic elements [8].

Rowmanowsky stain and its variants originally described or modified by numerous authors laid the foundation for modern haematology. They provided the basis of recognising and categorising normal and pathologic cells of blood and marrow. The two advantageous features of Rowmanowsky are, it can provide technically straight forward polychrome staining and can visualise entities at or below the limits of optical resolution under a microscope [1,2].

The staining properties of Rowmanowsky dyes are greatly influenced by the relative proportion of basic dyes [19,20]. Inconsistent staining qualities from different manufacturers can cause diagnostic difficulties in laboratories like ours where we are forced to quote least priced chemicals. Developing alternate staining methods gains importance in this sceneraio, where an easily producible stain within the laboratory can solve such problems to some extent.

LIMITATION(S)

The major limiting factor of present study was staining characteristics in pathologic conditions, like blood parasites, immature myeloid erythroid elements like myeloblasts/lymphoblasts, lymphoma infiltrates etc., were not included in the study.

CONCLUSION(S)

The poor quality of certain brands of conventional stain was the driving force behind this research. The three advantages of the modified method is easy availability of ingredients within the laboratory, cheaper ingredients and lesser staining time than conventional method. Chromatin patterns and neutrophil granular staining was poor with Villanueva compared to Leishman. Though overall staining was better in Leishman staining, present study was done with modified stain which helped us to develop an alternative staining method using chemicals available in the laboratory. We consider research on alternative staining methods is relavant especially in crisis situations like Covid, where manufacture and transportation of chemicals are affected worldwide. Assuring the continuous supply of good quality stains is a big challenge for many laboratories especially where government run ones all over India. Hence, substitutes to conventional methods which can be used in emergency situations will be an asset for all laboratories.

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