Original Article

Analysis of Papanicolaou stain on peripheral smear compared to Leishman's stain: A prospective study

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Abstract

Aim: The aim of the study is to know the exact effect of Papanicolaou (Pap) staining on the peripheral smear while compared to Leishman's stain on the peripheral smear.

Background: Pap staining is discovered and usually used for wet-fixed cytological smears. As a differential stain and using hematoxylin and eosin as a part of the staining solutions, Pap stains also have a principle of acidic and basic pH and contents as other Romanowsky stains. One of the commonly known Romanowsky stains is Leishman's stain, which is usually designed and used for air-dried peripheral smear. This study is designed to find out the effect of wet fixation and Pap stain on the air-dried peripheral smear by comparing it to Leishman's stain.

Materials and Methods: The study included randomly selected 20 patients. With the informed consent, two peripheral smears were prepared with the blood samples. First set of samples were fixed with isopropyl alcohol and stained with Pap stain. Second set of smears were routinely air-dried and stained with Leishman's stain. Both the sets were analyzed for staining and morphological characteristics and statistically compared.

Conclusion: After the study, it was quite evident that Pap stain does not give desired results on the peripheral smear, making it difficult for examination. Hence, Pap satin is not used for peripheral smear as it causes lysis of cells, change in cellular morphology, nondifferentiable among other cells, and many other complications for a cellular examination. Leishman's stain is the best commercially available stain for the peripheral smear examination.

Keywords: Leishman's stain, morphology, Papanicolaou stain, peripheral smear, red blood cells, white blood cells

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INTRODUCTION

Blood, being the major fluid content of our body and other animals, has the most necessary functions such as delivering oxygen and nutrients and transporting many other metabolic secretions and constituents to various parts of the body. Blood is a viscous fluid containing plasma, red blood corpuscles, white blood corpuscles, and platelets. Constituting 7% of the human body weight, blood is an easy indicator for any abnormalities or change in the internal conditions of our body. Therefore, a small sample of the blood can be collected conventionally and can be used to diagnose according to the presumption.[1]

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Peripheral smear, also known as blood film, is a method used to microscopically diagnose any abnormalities in the blood. It is easily done with minimum requirements. A thin blood film is prepared and stained to view under a microscope. Different stains are used to stain the different components of the cells in the blood. This can also be preserved for further future verification. The methods of staining and the stains used differ.

A complete blood smear examination begins with a visible scrutiny for acceptable quality of smear and stain and absence of macroscopic starches. An appropriate smear is then examined under the microscope in × 10, to recheck the stain quality and to make sure that the smear is free of:

a. Clumps of platelets, white blood cells, or red blood cells (WBCs or RBCs)
b. Any sort of living microorganism
c. Rouleaux formation
d. Microfilaments
e. Protein strands.

All the areas of the smear are examined carefully. After confirmation, the slide is examined under a higher magnification such as × 50 or × 100 oil objective.

Commonly used stains are binary compound: Romanowsky such as Field's and JSB stain or alcohol-based Romanowsky such as Giemsa, Leishman's, and Wright stains. Binary compound Romanowsky stains are most liked in field settings within which there is a risk of evaporation. These settings are appropriate for staining thick blood smears. Alcohol-based Romanowsky is used for thin smears.[2]

Pap stain is a universal stain used for medical specialty and nongynecological blood smear. It is chiefly used for oral and cervical cancer screening in symptomless populations and for the follow-up patients with cancer. Pap stain was first discovered by Greek doctor George Nicholas Papanicoloau at Cornell Medical College in New York, to determine the various maturities at the cellular level and their metabolic activity in smears. The original Pap stain was brought to use in 1942, and it was later modified by Dr. Papanicoloau in 1954 and 1960.[3]

The main action of Pap stain is to differentiate the various cellular components based on their chemical nature such as acidophilic and basophilic cell components and to obtain a detailed chromatin pattern. The pap stain contains three solutions with six dyes.

- Solution 1: Hematoxylin stain - Basic nuclear stain
- Solution 2: Orange G6 with phosphotungstic acid - Acid dye for keratin
- Solution 3: EA - Polychromatic stain (light green/fast green, eosin Y and Bismarck brown Y).

Leishman's stain, a type of Romanowsky stain, is a mixture of methylene blue and eosin dye. It is prepared in alcohol and is diluted using distilled water. It stains human cells purple in color. It is one of the best methods preferred for the peripheral blood smear examination. Leishman's stain was first discovered by a British surgeon W. B. Leishman. It consists of an acidic stain eosin and a basic stain methylene blue. It is capable of fixing the smear to the slide, thereby prefixing steps can be avoided. The various methods in which Leishman's stain is used are:

a. Differential leukocyte count
b. Determination of type of anemia
c. Platelet count.

The stain must be diluted with a phosphate buffer to achieve very accurate results. The ideal pH would be 6.8 for morphological studies of blood cells, while a pH of 7.2 is required for parasitic studies.[4]

Morphology of a normal red blood corpuscle is a biconcave disc shaped with a diameter measuring about 7–8 μm, with a central pallor. To perform its function to the fullest, a mature RBC lacks a nucleus. RBCs are stained in pink with Romanowsky stain as the hemoglobin picks up eosin stain.[5]

Early in precursor development in the marrow, cells destined to be leukocytes of the granulocytic series, neutrophils, eosinophils, and basophils, synthesize proteins and store them as cytoplasmic granules. The synthesis of primary or azurophilic granules defines the conversion of the myeloblast, a virtually agranular, primitive cell that is the earliest granulocyte precursor identifiable by light microscopy into the promyelocyte, which is rich in azurophilic granules. Synthesis and accumulation of secondary or specific granules follow. The appearance of specific granules marks the progression of the promyelocyte to neutrophilic, eosinophilic, or basophilic myelocytes. Thereafter, the cell continues maturation into an amitotic cell with a segmented nucleus, capable of amoeboid motility, phagocytosis, and microbial killing. The mature granulocytes also develop cytoplasmic and surface structures that permit them to attach to and penetrate the wall of venules. The mature granulocytes enter the blood from the marrow, circulate briefly, and move to the tissues to carry out their major function of host defense.[6]

MATERIALS AND METHODS

Peripheral blood collection

Peripheral blood samples were collected from randomly 20 patients who were taking their routine blood examination

at the Clinical Laboratory in Saveetha Dental Hospital in Chennai, after obtaining the consent from the respective individuals. The criterion for selecting the sample was the clinical request for hemoglobin and complete blood count. No restriction was made for age, sex, or clinical history of each patient under clinic care. All the procedures were carried out in the Clinical Laboratory of Saveetha Dental College, Saveetha Institute of Medical and Technical Sciences, with proper personal protection.[7]

Peripheral blood smear preparation

Forty slides were taken to prepare a set of two slides of smear for each blood sample to compare the different staining. The slides were labeled with a glass pencil by the name of the patient from whom the blood was taken. Just above the name, a small drop of blood was placed for the smear preparation. A separate clean slide was taken and held at 45° angle from the slide containing the blood drop about 1 mm above the blood drop. The slider was pulled back over the blood drop and waited until the blood spread over the full edge of the slide. After this, the slider was pushed forward until the other end of the slide to achieve an evenly spread tongue-shaped smear. Any excess blood on the sides was wiped off with a clean tissue paper. One labeled slide was placed inside a bottle of 80% isopropyl alcohol while the other was left to air dry.[8]

Leishman's staining

The air-dried labeled slide is taken and placed on the staining rack with the right side facing up. Commercially available Leishman's stain was filtered and taken in a dispensing bottle. Few drops of Leishman's stain was poured on the slide to cover the blood smear from the head to the tail end of the slide and left for 2 min. After 2 min, sufficient drops of distilled water was poured on the slide to cover the whole smear and left for 10 min. After 10 min, the slide was taken and washed under running tap water by holding the palm over the head end and directing the water on the slide, concerning to not lose the contents of the slide with the flow of the water. The slide was later air-dried. A drop of cedar wood oil was placed on the slide and viewed under a microscope in ×100.[9]

Rapid Papanicolaou staining

The labeled slide placed inside a bottle of 80% isopropyl alcohol is taken after 20 min and placed on the staining rack with the right side facing up. Equal quantity of rapid Pap cytoplasmic stain (OG-6 solution) and rapid Pap cytoplasmic stain (light green SF-eosin) is mixed together in a separate bottle labeled "working cytoplasmic stain" and kept aside. The labeled slides are washed under running tap water for 3–5 min. After washing, the slides are placed on the rack again. Excess water from the slide is carefully blotted with a tissue paper. A few drops of nuclear stain rapid Pap is poured covering the whole area of the slide and left for 60 s. After 60 s, the slide is washed under running tap water. 3–5 drops of wash buffer was added and left for 20 s. Any excess water was carefully blotted with from the slide. After 20 s, the slide was dehydrated using rapid PAP dehydrant for 60 s. After dehydration, a few drops of working cytoplasmic stain was poured on the slide to cover the smear and left for 60 s. After 60 s, the slide was washed under running tap water. Excess tap water was blotted out carefully. The smear was again dehydrated with rapid PAP dehydrant for 60 s and later was rinsed with xylene. The slide was covered with a drop of DPX and covered with a cover slip to view under the microscope.[10,11]

RESULTS AND DISCUSSION

The labeled slides were carefully analyzed under a microscope under various categories such as:

1. Structural maintenance
2. Staining technique
3. Morphology of RBCs
4. Morphology of WBCs
5. Morphology of platelets
6. Differential count.

The analyzed slides were graded accordingly following the below-mentioned grade system [Table 1].

Structural maintenance

The slides with smear that were immersed into 80% isopropyl alcohol for Pap staining had alterations in the structural morphology of the cell and smear and had made it difficult to analyze the smear after staining. The motive of fixing the stain was not achieved completely, and thereby, discrepancies were seen in the smear. However, the air-dried smear used for Leishman's stain[12] had no such alterations or morphological changes and thereby gave a more clear image under the microscope [Tables 2 and 3].

Staining technique

The effect of the Leishman's stain on the peripheral smear for the purpose of analysis was better and showed clearer results compared to the staining of Pap stain. The cells were more evident and recognizable in Leishman's stain. The Pap stain had also stained enough to make the cells visible while the proper anatomy was not appreciated [Tables 4 and 5].

Table 1: Likert's three-tier grading system

Grades
0 - Not possible
1 - Poor
2 - Average
3 - Good

Morphology of red blood cells

The morphology of RBCs was maintained properly in the slide stained with Leishman's stain. Disc-shaped round cells with center pallor were appreciated. They were easily differentiable from other cells in the blood. On the other hand, the RBCs in the slides stained with Pap stain had mostly lysed and lost their morphological structure. Hematin filaments were formed giving a mesh-like appearance [Tables 6 and 7].

Table 2: Grading for processing analysis on Leishman's stain and Papanicolaou stain

	Structural maintenance	
Sample number	Leishman's stain	Papanicolaou stain
1	3	1
2	3	1
3	3	1
4	3	1
5	3	1
6	3	1
7	3	1
8	3	1
9	3	1
10	3	1
11	3	1
12	3	1
13	3	1
14	3	1
15	3	1
16	3	1
17	3	1
18	3	1
19	3	1
20	3	1

Table 3: Statistical analysis of morphological analysis

Pairwise multiple comparison procedures (Tukey test)		
Difference of ranks	q	$P<0.05$
2510	8.084	Yes

Table 4: Grading for the quality of the stains on peripheral smear

	Staining technique	
Sample number	Leishman's stain	Papanicolaou stain
1	3	2
2	3	2
3	3	2
4	3	2
5	3	2
6	3	2
7	3	2
8	3	2
9	3	2
10	3	2
11	3	2
12	3	2
13	3	2
14	3	2
15	3	2
16	3	2
17	3	2
18	3	2
19	3	2
20	3	2

Morphology of white blood cells

The morphology of WBCs was maintained properly in the slide stained with Leishman's satin. The different types of leukocytes were effortlessly found out. There were not any discrepancies that affected the analysis of the smear. The WBCs in the slide that was stained with Pap stain were appreciable.[13] The different leukocytes were able to be located based on the shape and the number of lobes in the nucleus [Tables 8 and 9].

Morphology of platelets

The morphology of platelets was not at all visible in the slides with Pap satin. It was difficult to recognize platelets. On the contrary, the morphology of the platelets was well defined in the slides stained with Leishman's stain [Tables 10 and 11].

Differential count

It was difficult to perform a differential count test in a slide stained with Pap stain as there were any recognizable differences between the different cells in the slide as every cell looked almost similar. However,

Table 5: Statistical analysis of staining quality

Pairwise multiple comparison procedures (Tukey test)		
Difference of ranks	q	$P<0.05$
1521	4.899	Yes

Table 6: Grading for morphology of red blood cells on both stains

	Morphology of RBC	
Sample number	Leishman's stain	Papanicolaou stain
1	3	2
2	3	2
3	3	1
4	3	1
5	3	1
6	3	1
7	3	1
8	3	2
9	3	2
10	3	2
11	3	2
12	3	1
13	3	2
14	3	2
15	3	1
16	3	1
17	3	1
18	3	1
19	3	1
20	3	2

RBC: Red blood cell

Table 7: Statistical analysis of red blood cell morphology

Pairwise multiple comparison procedures (Tukey test)		
Difference of ranks	q	$P<0.05$
2141	6.896	Yes

Table 8: Grading for white blood cell morphology in both stains

Morphology of WBC		
Sample number	Leishman's Stain	Papanicolaou stain
1	3	2
2	3	2
3	3	2
4	3	2
5	3	2
6	3	2
7	3	2
8	3	2
9	3	2
10	3	2
11	3	2
12	3	2
13	3	2
14	3	2
15	3	2
16	3	2
17	3	2
18	3	2
19	3	2
20	3	2

WBC: White blood cell

Table 9: Statistical analysis of white blood cell morphology

Pairwise multiple comparison procedures (Tukey test)		
Difference of ranks	*q*	*P*<0.05
1690	5.443	Yes

Table 10: Grading for the platelet morphology in both stains

Morphology of platelets		
Sample number	Leishman's stain	Papanicolaou stain
1	3	0
2	3	0
3	3	0
4	3	0
5	3	0
6	3	0
7	3	0
8	3	0
9	3	0
10	3	0
11	3	0
12	3	0
13	3	0
14	3	0
15	3	0
16	3	0
17	3	0
18	3	0
19	3	0
20	3	0

with the slide stained with Leishman's stain, it was easier to perform differential count tests. The structural configuration of the cells was visible, thereby making it easier to differentiate between different types of cells [Tables 12 and 13].

The data were all entered in an Excel spreadsheet to easily compare between the results of the two stains that were

Table 11: Statistical analysis for platelet morphology

Pairwise multiple comparison procedures (Tukey test)		
Difference of ranks	*q*	*P*<0.05
3220	10.371	Yes

Table 12: Grading for differential count in both stains

Differential count		
Sample number	Leishman's stain	Papanicolaou stain
1	3	0
2	3	0
3	3	0
4	3	0
5	3	0
6	3	0
7	3	0
8	3	0
9	3	0
10	3	0
11	3	0
12	3	0
13	3	0
14	3	0
15	3	0
16	3	0
17	3	0
18	3	0
19	3	0
20	3	0

Table 13: Statistical analysis of differential count

Pairwise multiple comparison procedures (Tukey test)		
Difference of ranks	*q*	*P*<0.05
3220	10.371	Yes

used on the smear. Pairwise multiple comparison procedure Tukey test was performed on the data compared under the same heading/category. Kruskal–Wallis one-way analysis of variance on ranks was used to run Shapiro–Wilk normality test on the various ranks obtained [Table 14], H = 233.920 with 11 degrees of freedom ($P \leq 0.001$). It shows that there is a significant difference between the two methods and Air dry fixed Leishman stain stained smears shows a better results than the wet fixed Papaniculaou stain stained smears. The difference is also statistically significant ($P \leq 0.001$); there is a statistically significant difference ($P \leq 0.001$)

The Pap stain is designed and discovered, especially for the cytological smears[14] and for the cervical smears. The Leishman's stain is designed for the peripheral blood smear for routine analysis. The composition of both the stains has acidic and basic dyes which act commonly for the pH variations in the cellular contents. As the Romanowsky stains, the Pap stain works with the pH principle. The only variation in the fixation and processing among the two stains causes morphological and structural difference in the smears. [15] In this study, we compared all parameters which will be affected by the fixation technique and staining

Table 14: One-way analysis of parameters

Kruskal-Wallis one-way analysis of variance on ranks

Saturday, December 21, 2019, 09:05:35
Data source: Data 1 in Notebook 1
Normality test (Shapiro-Wilk) Failed (P<0.050)

Group	Number	Missing	Median	25%	75%
Structural maintenance - Leishman's stain (column 1)	20	0	3	3	3
Staining technique - Leishman's stain (column 2)	20	0	3	3	3
Morphology of RBC - Leishman's stain (column 3)	20	0	3	3	3
Morphology of WBC - Leishman's stain (column 4)	20	0	3	3	3
Morphology of platelets - Leishman's stain (column 5)	20	0	3	3	3
Differential count - Leishman's stain (column 6)	20	0	3	3	3
Structural maintenance - Papanicolaou stain (column 7)	20	0	1	1	1
Staining technique - Papanicolaou stain (column 8)	20	0	2	2	2
Morphology of RBC - Papanicolaou stain (column 9)	20	0	1	1	2
Morphology of WBC - Papanicolaou stain (column 10)	20	0	2	2	2
Morphology of platelets - Papanicolaou stain (column 11)	20	0	0	0	0
Differential count - Papanicolaou stain (column 12)	20	0	0	0	0

RBC: Red blood cell, WBC: White blood cell

methods on the peripheral smear. This is shown in Table 14 as the *P* values for all the compared parameters are favoring the maximum difference in the stains and fixation on the peripheral blood smear. We found that there is a significant difference between the two stains and fixation technique statistically. It is well correlated with our study. In a study done by Doddagowda *et al.*,[16] they showed that a cocktail of Giemsa with Leishman's stain has a good morphological maintenance in the cytological smears than in the Pap stains smears.

CONCLUSION

As this is the first study on Leishman's stain and Pap stain comparison on the peripheral blood smear, we found and graded the structural maintenance scores and morphological scores for the two staining and fixation techniques. After the study, it was quite evident that Pap satin does not give desired results on the peripheral smear, making it difficult for examination. Hence, Pap satin is not used for peripheral smear as it causes lysis of cells, change in cellular morphology, nondifferentiable among other cells, and many other complications for a cellular examination. Leishman's stain is the best commercially available stain for peripheral smear examination.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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