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A quick and reliable method for estimating platelet count on unstained peripheral smears in comparison to stained peripheral blood smears

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Abstract
Amongst the various methods for estimating platelet count, the manual count of platelets on stained peripheral smears is most commonly employed. However, this method has limitations and the study of platelet count on unstained smears have been mentioned in only 3 literatures. Considering the effectiveness of unstained smears for platelet counting, we planned to use it and compare its results with stained smears. On 250 samples, platelet count estimation using unstained smears were observed under 100X objective, lowered condenser and closed iris diaphragm. Average of platelets in 10 fields were multiplied by 15000. Smears were stained, counted platelets and results were compared using Intra-class Correlation Coefficient. Platelet counts obtained from unstained smears were not significantly different from stained smears (p value 0.0001). Platelet count estimation on unstained peripheral smears can help reduce the need of stained smears and automated counters in emergencies where resources are limited and workload is more.

Keywords: Platelet count, Peripheral blood smears, unstained smears.

1. Introduction
Platelets are one of the main components of blood and have an important role in normal haemostasis, by clumping together and form plugs at the site of blood vessel wall injury to prevent excessive bleeding.[1, 2] Platelets measure about 2-3µm and on Romanowsky-stained peripheral blood smear, they appear as small, anuclear cells with prominent reddish purple granules. The normal concentration of platelets in the blood is 150 – 440 x 10^9/L [2, 3].
Deficiency of platelets with below 20 x 10^9/L can result in fatal bleeding which can be prevented by prophylactic platelet transfusion. On the other hand, an increase in platelets may present as thrombotic events [4, 5]. An accurate platelet count is an important laboratory parameter to make a clinical decision, especially in conditions with severe thrombocytopenia. Therefore, a platelet count estimation is an important and routinely requested laboratory investigation [2].
The four main analytical procedures for platelet counting are: manual counting using phase contrast microscopy, optical light scatter/fluorescence analysis using various commercially available analysers, impedance analysis and immunoplatelet counting by flow cytometry.[6]
Even still, the most frequently used method is the manual counting of platelets on stained peripheral blood smears [1].
However, the manual method of using stained smears is time-consuming, subjective and tedious. In addition, there are few other limitations with suboptimal staining quality and stain precipitates that may cause difficulty in estimation of accurate platelet count [1, 2].
Automated blood cell counters have largely replaced the manual method of platelet counting for their accuracy and reduced analysis time, but it also has its own limitations like cost, quality assurance and their unavailability in small peripheral centers [1, 2]. Moreover, all the abnormal platelet values generated by the cell counters need to be verified and confirmed by manual examination of stained peripheral blood smear which further increases the Turn-Around Time (TAT) [1, 7].
Examination of unstained smears is a familiar technique to all pathologists but limited to the examination of urine, semen, vaginal smears and other body fluids [8-10].

To our knowledge, examination of platelet counts from unstained peripheral blood smears has been mentioned in only 3 literatures. Platelets in unstained peripheral blood smears appears as small, round and refractile bodies that can be counted with little expertise [1, 2, 11]. Hence, we planned to use unstained smears for platelet counting estimation which is less time consuming to assess whether it can be helpful in times of emergencies when workload is more.

The aim of the present study is to compare the platelet count on unstained and stained peripheral blood smear and the effectiveness of the method on unstained smears, as well as to compare the advantages and disadvantages of these two methods.

2. Material and Method

A total of 250 blood samples collected in Ethylene Diamine Tetra-acetic Acid (EDTA) vacutainer were received in the hematology laboratory, Akash Institute of Medical Sciences and Research Centre, Devanahalli, for performing complete blood counts were included in this study. Each sample was numbered without any personal identifying information. Thin peripheral blood smears were prepared from these samples on clean glass slides within three hours of collection. The unstained smear was air-dried and scanned under 10X objective and 40X objective to choose an ideal area at the junction of body and tail for platelet counting. A circular area of about 2-3mm in diameter was marked using a lead pencil where RBCs were uniformly spread and not overlapping. Condenser was lowered and iris diaphragm was closed to minimize the illumination. The marked area was examined under 100X objective without placing immersion oil. Platelets were counted in 10 fields and the average was taken. The same smear was stained with Leishman's stain and platelets were counted under oil immersion objective in the marked area for 10 fields by the usual method and the average was calculated. The estimate of platelet count per micro liter (µL) was calculated in both the methods by multiplying the average number of platelets per field by 15,000.

3.1. Statistical Analysis

Intra-class Correlation Coefficient (ICC) and p value.

4. Result

In unstained smears, the platelets were seen as small round refractile bodies while in Leishman's stained peripheral smears, they appeared as purple bodies. (Fig 1)

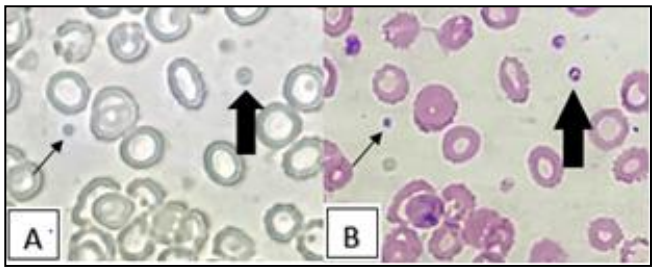

Fig 1: Platelet (arrows) in unstained (A) and corresponding area of Leishman's stained (B) smears (x1000). Normal sized platelet (wide arrow) and micro platelet (thin arrow) are seen.

Based on the average number of platelets per 10 field (x 1000 magnification) obtained in stained smears, 250 samples were divided into four classes (Table 1).

Table 1: Class distribution according to the average number of platelets in the stained smears per 10 fields under oil immersion.

Class	Average platelets on stained slides	No. of cases	Percentage of cases
1	0 – 10	82	32.8%
2	11 – 20	88	35.2%
3	21 – 30	20	8%
4	> 30	60	24%
	Total	250	100%

On the basis of the present division, the p-value and agreement between unstained and stained peripheral smears were assessed using ICC (Table 2).

Table 2: Intra-class correlation coefficient and p-value for four classes in unstained and stained smears.

Class	ICC	p value
1	0.915	0.0001
2	0.799	0.0001
3	0.997	0.0001
4	0.999	0.0001

A p-value of less than 0.05 was considered statistically significant. With platelet size in consideration, normal sized platelets were identifiable but micro platelets were not recognized easily on unstained smears (Fig 1). Platelet clumping and fragment of white blood cell being counted as aggregates of platelets were also appreciated in some of the cases of unstained smears (Fig 2).

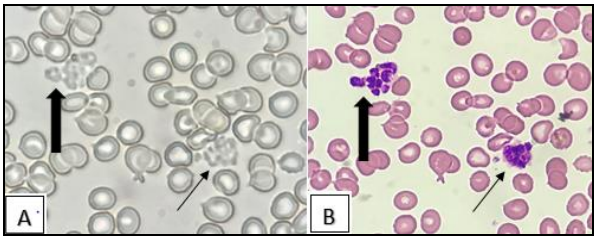

Fig 2: Platelet aggregate (wide arrow) and fragments of white blood cell appearing like platelet clumping (thin arrow) in unstained (A) and corresponding area of Leishman's stained (B) smears (x1000).

Cases with discrepant values of estimating platelet count on unstained smears in comparison to stained smears are in Tables 3 and 4.

Table 3: Difference in number of platelet count per 1000X in unstained smears in comparison to stained smears.

Class	Higher number of platelets in unstained smears (No. of cases)	Lower number of platelets in unstained smears (No. of cases)
0 – 10	5	7
11 – 20	6	15
21 – 30	2	3
> 30	2	1
Total	15/250 cases	/250 cases

Table 4: Difference in number of platelet count per 1000X in unstained smears in comparison with stained smears.

Difference in the number of platelet count/1000x between stained and unstained smears	Higher number of platelets in unstained smears (no. of cases)	Lower number of platelets in unstained smears (no. of cases)
1	1	1
2	3	7
3	1	4
4	5	7
5	2	2
6	0	4
7	1	1
8	2	0
Total	15/250 cases (6%)	26/250 cases (10.4%)

Out of 250 cases, in unstained peripheral smears 15 cases had higher and 26 cases had lower platelet count in comparison to stained peripheral smears.

The advantages and disadvantages of estimating platelet count in unstained and stained peripheral smears are given in Table 5 [1, 2, 11]

Table 5: Difference between unstained and stained peripheral smears for estimating platelet count.

Parameters	Unstained smear	Stained smear
TAT	2-5 minutes	15-20 minutes
Cost Effectiveness	No staining required, therefore cost-effective	Comparatively costly
Stain Artifacts	No stain artifacts	Presence of stain artifacts and poor staining quality may lead to poor preservation of morphology of cells
Reliability	Useful in emergency circumstances where the platelet count is urgently required It can be used when there are problems with staining and to crosscheck the platelet values from automated analyzers In thrombocytopenic disorders, it gives reliable values	Clearly differentiates platelets and non-platelet particles in contrast to unstained peripheral smears and most automated counters The original smear can be preserved and re-evaluated
Labour Intensiveness	Easy to perform, hence less labour intensive	Tedious as staining of smears is required Repeat smear and staining is required in smears with staining artifacts
Usefulness	With short TAT and less labour intensive, it can be used as a screening test in certain situations like in dengue outbreaks and bleeding disorders where the platelet count is the basic and a mandatory investigation Number and morphology of platelets, giant platelets, platelet clumping and platelet superimposed on RBCs as well as fragments of red and white blood cells can be recognized with expertise Its usefulness is much required in rural centers where resources are limited	In well stained smear, platelet clumping and micro platelets can be easily differentiated from other dust particles Fragments of red and white blood cells can be easily recognized As a gold standard, any low values obtained on automated counters should be cross-checked using stained smears
Other Drawbacks	In the beginning, Pathologist requires training and may face eye-strain and headaches till he/she gets accustomed to recognize the platelets as refractile bodies	Difficult to use in circumstances with large sample load when seen in dengue outbreak and in setups with limited staining procedure

5. Discussion

The manual method of platelet counting is time consuming and tedious and therefore has been replaced by automated haematology analyzers. However, this method of manual counting of platelet has a significant role by verifying the platelet count results obtained from automated blood cell counters in cases of thrombocytopenic blood disorders or where platelet clumping is present, even though the manual method has certain drawbacks such as inter-observer variability [2, 11, 12].

The method on unstained smears has been limited to examination of urine, vaginal smears, semen and other body fluids. The advantages of using unstained smears for platelet counting are it has a short TAT and avoids the drawbacks of stained peripheral smears with improper staining and staining artifacts that requires repeated smear preparation. It also carries an advantage in setups where lack of resources are limited [1].

In our study, 15 (6%) out of 250 cases showed falsely low platelets and 26 (10.4%) out of 250 cases were overestimate in unstained smears. This was similar to the study done by Dhakar S *et al.* [1] where out of 500 cases, 5% were falsely high cases and 9% were falsely low cases on unstained smears.

With the underestimated counting in some cases of unstained smears, this could be due to the presence of micro platelets which were not easily recognizable as well as those platelets that were superimposed on RBCs in a few cases. The reason in cases with overestimated value, could be due to the counting of refractile dust particles as well as counting the fragments of white blood cells in some cases. However, expertise on examining the unstained smears may

help to reduce the number of cases with discordant results between unstained and stained peripheral smears. In our study platelet clumping in unstained smears was appreciated in most cases.

Umashankar T *et al.* [2] in their study, found similar issue with identifying micro platelets, platelets superimposed on RBCs and large platelets confused with WBC in unstained smears causing spurious low counts. Large granules released from the ruptured eosinophils and RBC fragments were counted as platelets and had led to a higher platelet counts in unstained preparation.

A study done by Muthu S *et al.* [11] have mentioned that the platelet count were underestimated in most cases and the difference was within 15000 in thrombocytopenic cases and within 30000 in cases with adequate platelet count. They also obtained platelet levels to be overestimated in unstained smears with a maximum difference of only 12000. These variations are acceptable in this type of counting method and concluded that platelet count estimation on unstained smears is a good preliminary method where round the clock laboratory facilities are not available, particularly in rural areas.

According to Muthu S *et al.* [11] since this technique does not need any staining, every undergraduate medical student and treating clinician can be trained to curb situations when there are limited resources in rural areas and in urban setups as an emergency measure in planning the management early. This technique can also be used to crosscheck the platelet values from automated counters till the problems with the stain such as improper staining and staining artifacts are corrected. However, in spite of the simplicity of the procedure, short TAT and comparable degree of accuracy, this would not be recommended as a routine alternative to stained peripheral smears as many findings in the red blood cell and white blood cells can be missed in unstained peripheral smears. This technique shall be used as a backup tool when the staining procedure is limited and as a preliminary tool by the treating clinician to gain vital information earlier.

In the present study, we found that platelet count by unstained peripheral smear had a short TAT of less than 7 minutes as compared to the usual 15-20 minutes by stained smear technique. Therefore, unstained smears may be used as an initial screening method in situations with large sample load especially seen during dengue outbreak. There were also some limitations faced in this present study that the estimation of platelet count on unstained smears requires training and more expertise to give an accurate result in comparison to stained smears.

6. Conclusion

To conclude, platelet count estimation using unstained smears yields results similar to stained peripheral smears. Platelet counts obtained from unstained smears were not significantly different from stained smears (p value 0.0001). Owing to their small size and refractile nature, platelets can be recognized in the unstained smear from freshly prepared smears. Unstained smear examination is rapid, cost effective, reliable, and requires less manpower. The problems faced due to poor staining quality are also curtailed. Although, the evaluation of unstained peripheral smears needs more practice and expertise to give better results.

7. Acknowledgments

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