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Temporal trends in reticulocyte count with Methylene blue staining: A comparative study to assess reliability of reticulocyte count as a quality control parameter

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Abstract
Introduction: The New Methylene Blue method for staining reticulocytes was introduced by Brecher[1] in 1949. This method has found wide acceptance. It is recommended for reticulocyte counting. Although there is no literature on the exact interaction allowing staining to take place, it has been suggested that New Methylene Blue localizes at a ribosomal site. By the new methylene-blue method normal reticulocyte count ranges from 0.5 to 2.5%.
Aim: To study Variation in reticulocyte count and staining quality of blood smears stained by New methylene blue over four weeks.
Methods: 50 EDTA anti-coagulated blood specimens were collected by simple random sampling. Reticulocyte count were determined by the new methylene-blue method weekly for four weeks and statistical analysis done by Paired t-test.
Results: Total number of 50 cases in our department were assessed. Ages ranged from 8 to 85 years. Mean age is 46.5 years +/- a standard deviation of 19.5 years. Total twenty eight male (56%) and 22(44%) female patients were studied. Mean retic value on day 1 is 1.6 %, day 8 is 1.4 %, day 15 is 1.1 % and day 21 is 0.87 %.
Conclusions: The mean retic count of the samples had decreased to 0.874 on day 21 when compared with 1.6 on day 1. The difference in mean retic count was highly statistically significant as per paired t-Test ($P<0.01$). Therefor reticulocyte count may not be reliable as a quality control parameter for external quality assurance programe.

Keywords: Reticulocyte count, new methylene blue

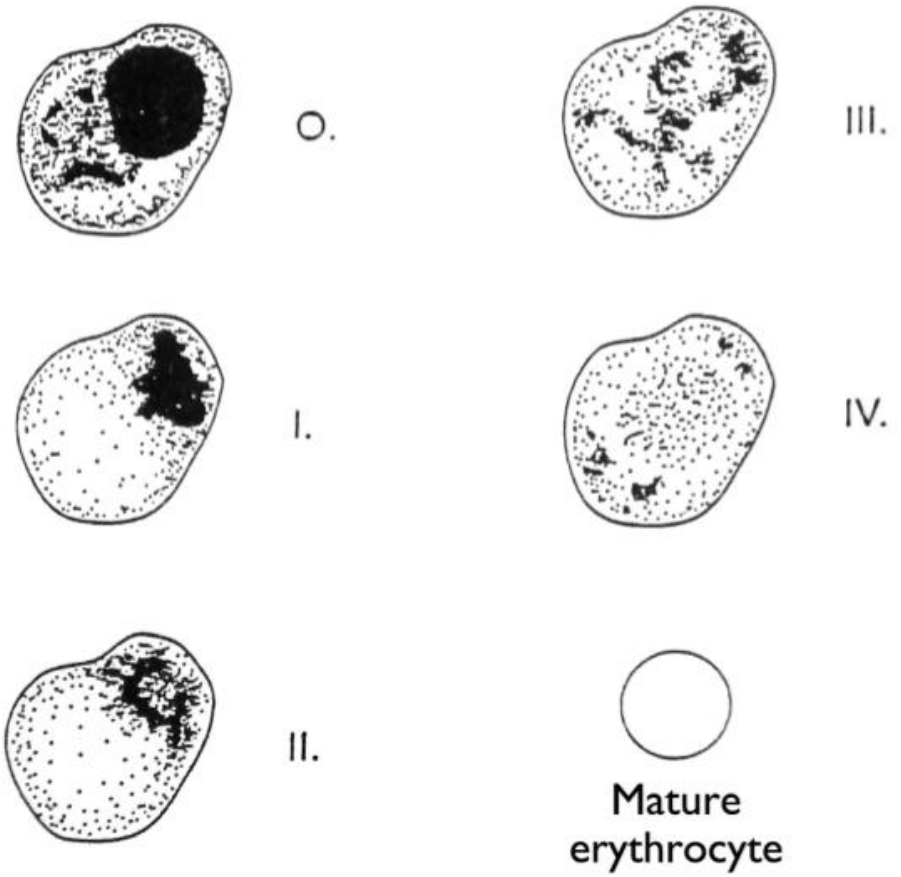
Introduction
Reticulocytes are immature red blood cells (RBCs). Reticulocytes originate in the bone marrow from the orthochromatic normoblast through nuclear exclusion. They are released into the peripheral blood after a period of maturation in the bone marrow and undergo further differentiation into mature RBCs [4]. In the laboratory, the differentiation of the reticulocyte from the mature RBC is based on the presence of RNA and other substances in the reticulocyte, which are lost during differentiation into the mature RBC. Manual counting of reticulocytes by light microscopy with supravital dyes for RNA was developed in the 1940s and remains the standard method of reticulocyte enumeration [5]. However, automated methods of reticulocyte enumeration developed during the past decade are much more accurate, precise, and cost-effective than manual counting, and are increasingly being performed in the clinical laboratory. In addition, the newer techniques provide a variety of reticulocyte-related parameters, such as the reticulocyte maturation index and immature reticulocyte fraction, which are not available with light microscopy [6].

The following are considered valid clinical indications for performance of a reticulocyte count:

- Anemia without known cause in a patient with no prior CBC results available to determine whether there is a bone marrow erythropoietic response to the anemia
- Decrease of hemoglobin of greater than 1.5 g/dL without a known cause
- Distinguishing hyper-regenerative and hypo regenerative anemias
- A neonate with anemia

- Monitoring development of premature infants
- Patients with suspected hemolytic anemia
- Monitoring the development of induced myelo suppression
- Monitoring the effect of hematinic therapy
- Determination of bone marrow engraftment or recovery from myelosuppression[3]

The manual reticulocyte count has been replaced by semi-automated and fully automated methods in laboratories of nearly all developed nations, except for small-volume laboratories and office laboratories in which they are not as cost effective. The eye count method remains the reference method.



Group 0: nucleated erythrocyte (orthochromatic normoblast), stained strongly for reticulin and the nucleus. This cell type is not included in the reticulocyte count. Group I: non-nucleated red cells, appearing with a dense clumped reticulum; they comprise 0.1% of the population of reticulocytes in normal individuals. Group II: extended network of loose reticulum; they comprise 0.7% of the reticulocyte population in normal individuals. Group III: scattered granules with residual reticulum network; they comprise 32% of the reticulocyte population in normal individuals. Group IV: scattered granules; they comprise 61% of the reticulocytes in normal individuals[4]

Fig 1: Maturation stages of reticulocytes according to Heilmeyer classification

Aims and Objectives

- To study variation in reticulocyte count and staining quality of blood smears stained by New methylene blue over four weeks.

Materials and Methods

This descriptive study was conducted in the department of Pathology, Sree Mookambika Institute of Medical Sciences, Kulasekharam. 50 EDTA (Ethylene diamine-tetraacetate)-anticoagulated blood specimens were collected by simple random sampling cases and reticulocyte count was done by the new methylene-blue method. The same slide was then reassed on day eight, day fifteen and day twenty one. Single group is studied by using convenient Sampling. Software used for statistical analysis used is Paired t-test and analysis done by SPSS Software Trial Version 20.0.

New methylene blue solution is prepared as follows: New methylene blue 1.0 gm along with Sodium citrate 0.6 gm, Sodium chloride 0.7 gm and Distilled water 100 ml. Reagent should be kept stored in a refrigerator at 2-6°C and filtered before use.

Steps

- Take 2 drops of new methylene blue in cuvette.
- Add equal amount of blood and mix well.
- Keep the mixture at room temperature for 15 minutes.
- After mixing, place a drop from mixture on slide, prepare a thin smear, and allow to dry.
- Examine under microscope using oil-immersion objective. Mature red cells stain pale green-blue. Reticulocytes show deep blue precipitates of fine granules and filaments in the form of a network
- Count 1000 red cells and note the number of red cells that are reticulocytes. Counting error is minimized if size of the microscopic field is reduced. This is achieved by using a Miller ocular disk inserted in the eyepiece [3].

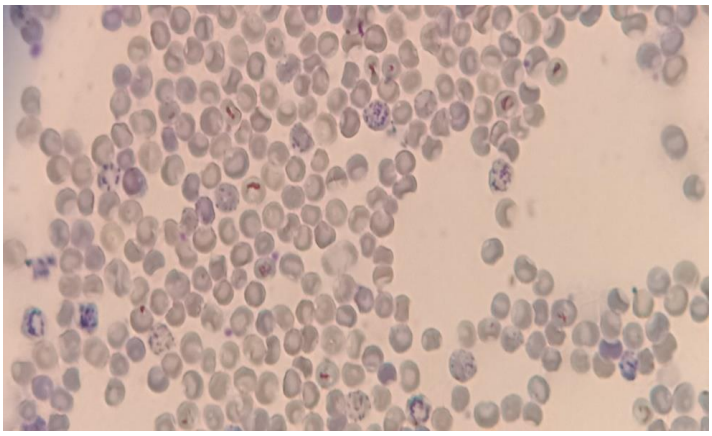


Fig. 2: New methylene blue stained slide showing reticulocytes100 X

Observations and Results

Total number of 50 cases were included in this study. Total twenty eight male (56%) and 22(44%) female patients were studied. Ages ranged from 8 to 85 years. Mean age is 46.5 years +/- a standard deviation of 19.5 years.

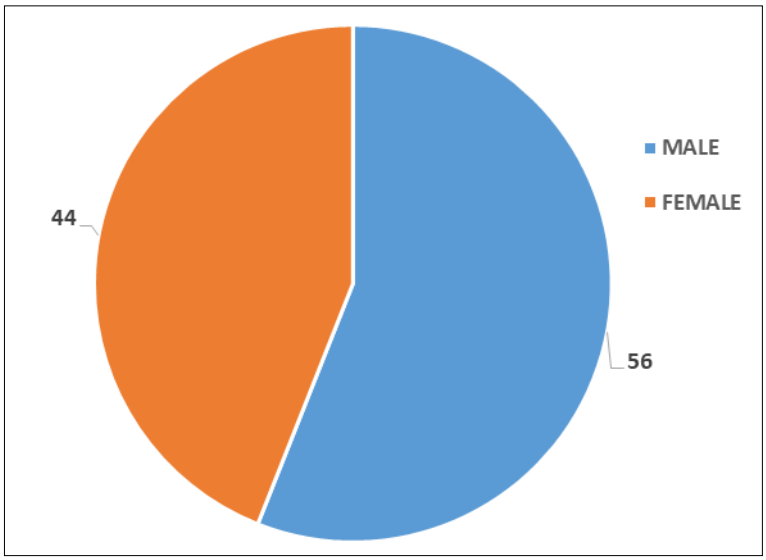


Fig 3: Gender distribution

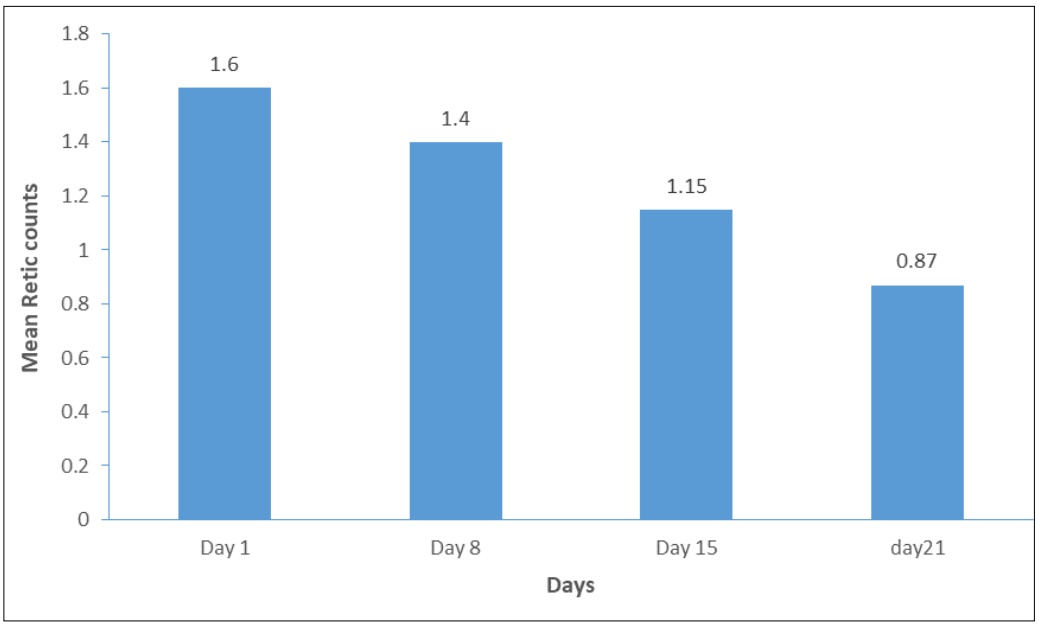


Fig. 4: Comparison of means from Day 1 to Day 28

Mean retic value on day 1 is 1.6 %, day 8 is 1.4 %, day 15 is 1.1 % and day 21 is 0.87 %. The mean retic count of the samples had decreased to 0.874 on day 21 when compare to 1.6 on day 1. The difference in mean retic count was highly statistically significant as per paired t- Test (P<0.01).

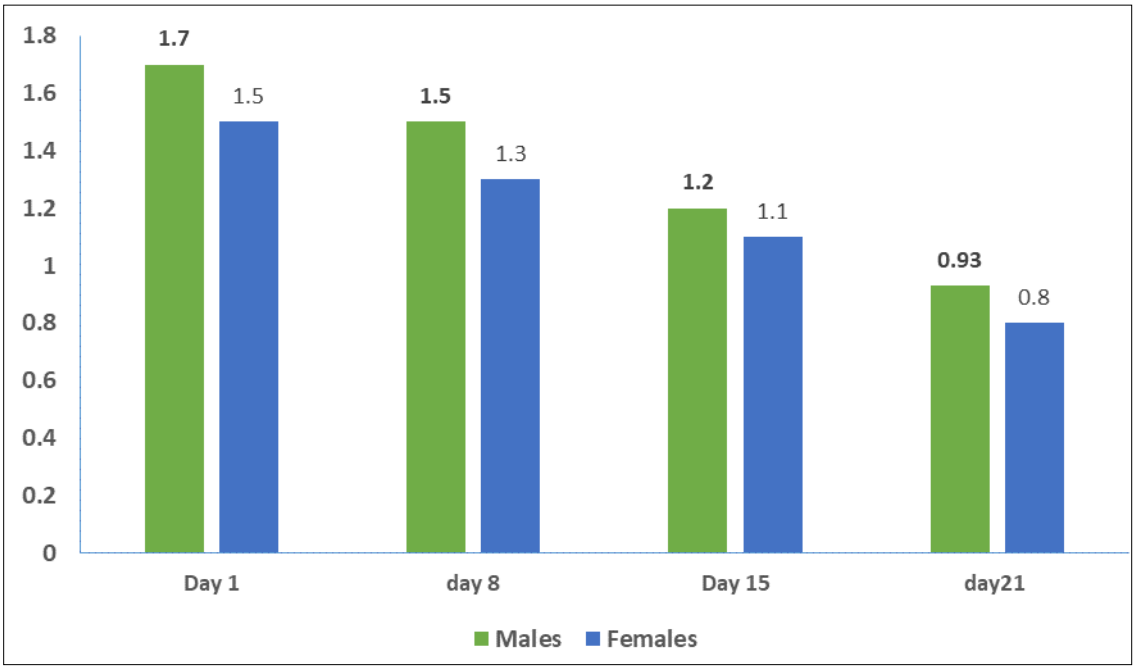


Fig 5: Gender differences in mean retic counts over Day 1 to Day 21

Discussion

In present study total 50 cases selected randomly and done new methylene blue staining for counting reticulocyte count .For all fifty cases first day retic count was noted. On the eight day same slides were reviewed and reticulocytes counted, similarly on day fifteen and twenty one also the slides were reviewed and reticulocyte count repeated. All four days values are different, with counts decreasing every week .The mean values of day one when compared to day twenty one was higher and the difference is statistically significant.

Wainwright *et al*, (2002) [7] states that New Methylene Blue used as a routine stain for reticulocytes. Although there is no literature on the exact interaction allowing staining to take place, it has been suggested that New Methylene Blue localizes at a ribosomal site. In recent testing of New Methylene Blue as part of a series of Methylene Blue derivatives in a melanoma cell line, New Methylene Blue exhibited high photo cytotoxicity but low dark (inherent) toxicity at micro molar levels.

Andrew diss *et al* [2] states that new methylene blue, like brilliant cresyl blue, stains siderotic granules in siderocytes. Therefore, in the presence of large numbers of circulating siderocytes, such as may occur following splenectomy, the reticulocyte count by either method may be erroneous. Under these circumstances, reticulocytes are best enumerated on preparations which have been first stained for reticulum and then counterstained with the Prussian blue stain

Conclusions

Reticulocyte count is an excellent screening method for the diagnosis of anemia of different etiologies [8]. Reticulocyte indices and count are now available on most hematology analyzers, but still new methylene blue stained smear is commonly used [9, 10]. In present study shows that retic count decreases with time so reticulocyte count may not be a reliable parameter for external quality assurance program, as the slides may reach various center only after a delay of days to weeks. Even when used as a quality control parameter, the authors feel that the smears should be covered by cover slips to prevent deterioration of staining with time

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Conflict of interest: None declared

Ethical approval: The study was approved by the Institutional Ethics Committee

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