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Effect of Sample Storage at 2-8°C for 24 Hours on Reticulocyte Count Determined by Manual Method

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ABSTRACT

Reticulocytes are immature red blood cells that contain residual RNA and ribosomes but no nucleus. Reticulocytes count is a haematological test that assists in assessing bone marrow activity. It can be determined by manual and flow cytometric method. Reticulocytes mature in vitro within six hours of sample collection; hence the sample should be processed within this time frame for accurate results. **Objective:** To determine the difference in reticulocyte count between a fresh sample and a sample stored for 24 hours at 2-8°C by manual method. **Methods:** This quasi-experimental study included a total of 220 EDTA-anticoagulated blood samples submitted to the Hematology Department of Allama Iqbal Medical College/Jinnah Hospital, Lahore, from June 12, 2024, to December 18, 2024, for reticulocyte count analysis using non-probability consecutive sampling technique. Each sample was processed using brilliant cresyl blue stain, within one hour of collection and reported two times: within 6 hours of sample collection and after a period of 24 hours of storage. **Results:** The manual reticulocyte count showed a significant difference between freshly prepared slides and those stored for 24 hours. Mean reticulocyte count at 6 hours was 2.99 ± 0.09, while after 24 hours, it decreased to 2.41 ± 0.10, resulting in a mean difference of 0.58 ± 0.16. **Conclusions:** In regions where automated methods are not available, performing a reticulocyte count manually on a fresh sample can significantly contribute to improving healthcare quality. This simple yet essential test is crucial for diagnosing and managing various clinical conditions effectively.

INTRODUCTION

Reticulocytes are newly formed erythrocytes that originate in the bone marrow, enter the bloodstream and develop into mature red blood cells within 1–2 days. The reticulocyte count reflects the bone marrow's ability of red blood cell production and is an essential metric for diagnosing anemia, evaluating treatment effectiveness, and determining prognosis. Reticulocyte count can be measured using two primary methods: the manual method, which involves light microscopy, and the automated flow cytometric method. Although flow cytometric method is more precise but it can give spurious counts in some situations, so manual counting by light microscopy, although tedious, remains an accepted reference method especially in resource limited countries like Pakistan [1]. The manual method for reticulocyte counting is a routine hematology test that is simple to perform and widely used in laboratories [2]. Since 1940, it has been considered a traditional and standard technique due to its simplicity and cost-effectiveness [3]. A local study conducted at Aga Khan University, Karachi, confirmed that manual reticulocyte counting is both reliable and economical, making it particularly beneficial for resource-limited settings [4]. A study conducted nearly four decades ago investigated the effects of storing samples at room temperature on reticulocyte count and concluded that the results remain reliable for up to 24 hours without

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significant changes compared to baseline values [5]. Reticulocyte count is determined by microscopic examination of peripheral blood smears that are stained using supravital stains, such as brilliant cresyl blue or new methylene blue. Reticulocytes mature in vitro within six hours of sample collection, making it essential to process samples ideally within this time window. A comparison of reticulocyte count at different time intervals by George L et al., using a manual technique indicated a notable difference between freshly prepared and stored slides at 6 hours (2.92 ± 3.56) and 24 hours (2.36 ± 3.18) with a difference of 0.56 ± 0.38 [6]. For precise estimation of reticulocyte count, it must be reported in six hours if stored at room temperature, or within 72 hours if stored at 2-6°C, as the maturation process is dependent on both time and temperature [7]. Tsuda I and Tatsumi N observed an approximate 60% decrease in relative proportion of reticulocytes from its original value after 03 weeks of storage at 4°C with a strong negative correlation (r = -0.9972) [8]. Furthermore, a study conducted in Oslo, Norway, examining the stability of reticulocyte and erythrocyte parameters over six days in three diagnostic groups (iron-deficient, thalassemia and healthy subjects) revealed an unexpected increase in reticulocyte count in all groups after 03 days of room temperature storage [9]. Reticulocyte count is a fundamental haematological test that is routinely requested in the facility. Since it is affected by both temperature and time, therefore, the goal of my study is to track any change in its count that may result from sample storage at 2-8°C.

Literature on this subject is outdated, extremely scant especially in the region and since most of the existing studies show variable results, it is therefore, important to clarify the ambiguity about variation in reticulocyte count with sample storage.

METHODS

This quasi-experimental study was carried out in the Hematology Department of Allama Iqbal Medical College/Jinnah Hospital, Lahore, from June 12, 2024, to December 18, 2024 after approval by institutional ethical review board (Ref No: ERB166/4/11-06-2024/S1 ERB). Non-probability consecutive sampling technique was used. Sample size was calculated using OpenEpi software which came out to be 220 keeping 95% confidence interval, power 80% with absolute precision 0.05 and anticipated mean difference in reticulocyte count as 0.56±0.38. Samples older than 06 hours or without mention of time of collection, insufficient sample quantity (less than 3cc) and clotted samples were excluded. Each blood sample was processed using brilliant cresyl blue stain within an hour and reported at two time points: within six hours of collection and following 24 hours of storage. The brilliant cresyl blue stain highlights the RNA as blue color against an otherwise pink cell. Light microscopic examination of stained peripheral blood films was done to determine the number of reticulocytes among 1000 RBCs and expressed as a percentage. Prior consent was taken from all patients, authorizing use of their personal information for research purpose. Data were entered and analyzed using SPSS version 27.0. Qualitative variables were shown as frequency and percentage while quantitative variables were reported as mean ± standard deviation. To assess the significant difference in reticulocyte count between the baseline (6 hours) and 24 hours post-storage, a paired t-test was applied, with a p-value of < 0.05 regarded statistically significant. Following stratification, a t-test was conducted as well. This was based on findings by George L *et al.*, who reported a difference of 0.56 ± 0.38 in a similar setting [6]. Prior to this, normality of data was assessed using the Shapiro-Wilk test.

RESULTS

The subjects in this study ranged in age from 15 to 60 years, with mean age of 38.05 ± 10.09 years (Table I). The study comprised 116 males (52.73%) and 104 females (47.27%), yielding a male-to-female ratio of 1.1:1.

Table 1: Patient Distribution Based on Age (n=220)

Age (Years)	Number of Patients Frequency (%)
15-40	141 (64.09)
41-60	79 (35.91)
Total	220 (100)
Mean ± SD	38.05 ± 10.09 Years

A comparison of reticulocyte counts at various time intervals using the manual method showed a significant difference between freshly prepared and stored sample slides (Table 2).

Table 2: Reticulocyte Count: Fresh Versus 24-Hour Stored Sample (Manual Method)

Reticulocyte Count	Mean ± SD
Reticulocyte Count within 6 Hours	2.99 ± 0.09
Reticulocyte Count at 24 Hours	2.41 ± 0.10
Difference	0.58 ± 0.16

Table 3 stratification of the mean difference in reticulocyte count based on age groups and gender respectively.

Table 3: Stratification of Mean Difference in Reticulocyte Count with Respect to Age Groups

Age Groups	Difference in Reticulocyte Count Mean ± SD	p-Value
15-40	0.57 ± 0.16	0.111
41-60	0.60 ± 0.15	

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Table 4: Stratification of Mean Difference in Reticulocyte Count with Respect to Gender

Gender	Difference in Reticulocyte Count Mean ± SD	p-Value
Male	0.61 ± 0.15	0.011
Female	0.55 ± 0.17	

DISCUSSION

Reticulocytes are developing red blood cells that contain residual RNA and ribosomes but lack a nucleus. Upon entering circulation, they shed their RNA within a day while continuing hemoglobin production despite the absence of a nucleus [10]. Reticulocytes take about 1 to 3 days to develop in the bone marrow, and once they are released into the bloodstream, they become mature red blood cells in 1 to 2 days. The Reticulocyte Count (RC) is a simple hematological test used to assess the bone marrow activity [11]. It serves as a useful diagnostic tool for identifying anemia and provides critical information for monitoring and managing anemic patients. An elevated reticulocyte count, or reticulocytosis, is often seen after blood loss, in hemolytic anemia, recovery from nutritional anemia, and hemoglobin disorders [12]. In contrast, a low reticulocyte count, or reticulocytopenia, can result from conditions such as aplastic anemia, pure red cell aplasia, bone marrow failure, chronic kidney disease, anemia of chronic disease, blood transfusion and alcoholism. Reticulocyte counting in clinical laboratories is performed using two primary methods: the conventional manual technique and the automated approach [3, 4]. The introduction of automation has significantly enhanced the accuracy and reliability of reticulocyte measurement [6]. A study conducted in Sri Lanka found that automated analyzers are increasingly being used in urban and high-volume laboratories, gradually replacing the traditional manual or visual method for reticulocyte counting. However, resource-limited laboratories, particularly in rural areas, continue to rely on the manual approach [13]. I have conducted this study to determine the mean difference in reticulocyte count between a fresh sample slide and a slide prepared after 24 hours of sample storage at 2-8°C by manual method. In my study, reticulocyte count comparison at various times using manual approach showed that there was a substantial difference between the fresh sample and stored sample slides at 06 and 24 hours with a mean of 2.99 ± 0.09 and 2.41 ± 0.10 with difference of 0.58 ± 0.16. George L *et al.*, conducted a study in Southern India and observed a similar finding between the fresh and stored slides at 06 and 24 hours with a mean of 2.92 ± 3.56 and 2.36 ± 3.18 with difference of 0.56 ± 0.38 [6]. Schapkaitz *et al.*, examined the impact of sample storage at room temperature and found that reticulocytes remained stable for up to 12 hours [14]. In contrast, Pintér E *et al.*, reported that reticulocyte counts became unstable, showing a significant reduction over 24 hours [15]. Additionally, de Baca *et al.*, observed that reticulocyte percentage and absolute values remained stable for four days when analyzed using an automated hematology analyzer [16]. Most studies in the literature indicate either no change in Reticulocyte Count (RC) or a reduction with sample storage. A study conducted in Oslo, Norway, examined erythrocyte and reticulocyte parameter stability over six days in three diagnostic groups i.e. iron-deficient, thalassemic and healthy subjects and found unexpected results. Reticulocyte counts increased in all groups after three days of storage at room temperature [9]. There was a statistically significant difference in the mean manual Reticulocyte Count (RC) between males and females (P = 0.01) observed in the study. The existing literature offers mixed findings when comparing RC between genders, but it does not specifically compare the methods used [17, 18]. This suggests that both manual and automated techniques are appropriate for determining mean RC; however, the manual method may be preferable as it is cost-effective. Various studies have reported differing results when comparing mean RC values obtained through manual and automated methods. For instance, Osgood *et al.*, did not observe a remarkable variation in the mean RC %age among individuals between 4–13 years age, whereas Jain P *et al.*, observed a notable decrease in RC in the elderly population. Bukhari and Zafar reported a notable difference in reticulocyte count (P < 0.05) [19-21]. Likewise, Tarallo P *et al.*, found no statistically significant difference in RC among girls and boys aged 4–19 years, but observed higher values in males compared to females over the age of 20 [17]. Lacombe *et al.*, studied the stability of reticulocyte percentage after sample storage at room temperature and at 4°C for different time intervals. Their study found a marked decrease in the percentage of reticulocytes after 48 hours of storage at room temperature, whereas no reportable change was observed after storage at 4°C for same duration. Additionally, they reported that the Immature Reticulocyte Fraction (IRF) parameter significantly decreased after sample storage for 08 hours at both room temperature and 4°C [22]. Similarly, Cavill *et al.*, examined the in vitro stability of reticulocytes and found that there was no notable decrease in reticulocyte count at either temperatures i.e. room temperature and 4°C. However, a reduction was noted in samples that had reticulocytosis within the first 24 hours at room temperature, with no such decline observed at 4°C [23]. Brugnara *et al.*, compared three types of automated methods with the manual technique and assessed their agreement using intraclass correlation coefficients. Their findings showed intraclass correlation coefficients between Manual/Flow Cytometry,

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Manual/SYSMEX R-2000 and Manual/H*3 Analyzer of 0.755, 0.538 and 0.610, respectively. Based on the findings, they concluded that manual reticulocyte counting technique cannot be regarded as comparable to the three types of automated methods [24]. The manual method, however, has certain limitations, including low reproducibility, the evaluation of a smaller number of cells, and the need for significant time and effort. It also requires skilled personnel for accurate reporting and depends on the observer's visual acuity and patience. Despite these challenges, it remains a cost-effective option for smaller laboratories and is more affordable for patients. On the other hand, automated reticulocyte counting can be affected by various interferences, such as platelet clumps, giant platelets, fragmented or abnormal WBCs, nRBCs, and RBC inclusions like basophilic stippling, Howell-Jolly bodies and Heinz bodies [25]. These interferences can be identified and addressed through manual examination, making the visual method valuable in assessing ineffective erythropoiesis.

CONCLUSIONS

This study found that reticulocyte counts varied significantly over time when using the manual method. A notable difference was observed between freshly prepared slides and slides stored for 6 and 24 hours, with mean results of 2.99 ± 0.09 and 2.41 ± 0.10, respectively, resulting in a difference of 0.58 ± 0.16. Based on these findings, it was suggested that the manual reticulocyte counting method was utilized in areas where automated systems are unavailable. As a fundamental diagnostic tool, it can play a crucial role in enhancing healthcare quality by aiding in the diagnosis and treatment of various medical conditions.

Authors Contribution

Conceptualization: MG
Methodology: AK, HA
Formal analysis: AK, MG, AM, RB, HA
Writing, review and editing: AK, MG, AM, RB, HA

All authors have read and agreed to the published version of the manuscript

Conflicts of Interest

All the authors declare no conflict of interest.

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