Avaliação de Valores de Normalidade da Contagem de Reticulócitos Utilizando o Contador Hematológico Cell-Dyn 3500

Evaluation of Normal Values for Reticulocyte Counts Using the Hematological Cell Counter Cell-Dyn 3500

Ketrin Goetz Müller[a]; Anna Paula de Borba Batschauer[a]; Railson Henneberg[b]; Aguinaldo José do Nascimento[c]; Henrique Ravanhol Frigeri[d]; Paulo Henrique da Silva[b]*

[a]Universidade do Vale do Itajaí, Curso de Especialização em Hematologia, SC, Brasil
[b]Universidade Federal do Paraná, Departamento de Patologia Médica, PR, Brasil
[c]Universidade Federal do Paraná, Programa de Pós-Graduação em Ciências Farmacêuticas, PR, Brasil
[d]Pontifícia Universidade Católica do Paraná, Escola de Saúde e Biociências, PR, Brasil

*E-mail: paulo.hs@ufpr.br

Resumo

O objetivo deste trabalho foi determinar os valores de referência para a contagem automatizada de reticulócitos para a população atendida pelo Laboratório de Análises Clínicas KG, Joinville (SC, Brasil), utilizando como contador hematológico o aparelho Cell-Dyn 3500 (Abbott). Este aparelho utiliza como corante o azul de metileno novo. Foram coletadas amostras sanguíneas de 290 indivíduos normais, sendo 126 do sexo feminino e 164 do sexo masculino e com idade entre 11 e 60 anos. Todos os indivíduos estavam fazendo exames periódicos e as coletas foram feitas com EDTA tripotássico e o hemograma de todos os pacientes foi considerando normal para a idade e sexo. As contagens de reticulócitos variaram de 0,85 a 2,80 %, em valores relativos e de 37 a 129 x 10⁹/L, em valores absolutos. Quando o perfil reticulocitário foi avaliado de acordo com o sexo, obtiveram-se valores mais elevados para o sexo masculino, somente em valores absolutos. Os valores de normalidade obtidos no presente trabalho foram mais elevados do que os encontrados na literatura, o que demonstra a necessidade do estabelecimento de valores de referência para uma população especifica, considerando condições próprias de instrumentação do laboratório.

Palavras-chave: Reticulócitos. Valores de Referência. Contagem de Reticulócitos.

Abstract

The aim of this study was to determine reference values for automated counting of reticulocytes for the patients from the Clinical Laboratory KG, Joinville (SC, Brazil), by using device the hematological counter Cell-Dyn 3500 (Abbott), and new methylene blue as staining agent. Blood samples were collected from 290 normal patients, being 126 female and 164 male, and aged between 11 and 60 years. Periodic examinations had been performed for all individuals, and samplings were made using tripotassium EDTA. The CBC (complete blood count) was considered normal for age and sex. Reticulocyte counts varied from 0.85 to 2.80%, in relative values, and 37-129x10⁹/L, in absolute values. When the reticulocyte profile was evaluated according to sex, higher values were obtained for males, but only in absolute values. Normal values obtained in this study were higher than those found in the literature, which demonstrates the need to establish reference values for specific population, considering proper conditions of local laboratory instrumentation.

Keywords: *Reticulocyte. Reference Values. Reticulocyte Count.*

1 Introduction

Reticulocyte is a non-nucleated red blood cell that enters the blood with residual detectable amounts of RNA. The number of reticulocytes in a volume of blood allows for an estimate of marrow erythrocyte production and thus, it is useful in evaluating the pathogenesis of anemia by distinguishing inadequate production from accelerated destruction (KAUSHANSKY *et al*., 2011; GREER *et al*., 2008). Reticulocyte counts also provide information about the bone marrow capacity to synthesize and release red cells in response to a physiologic challenge (ENGLAND *et al*., 1998). It is very important to estimate the recovery of bone marrow transplantation when compared with platelet or neutrophils counts (LAZARUS *et al*. (1992).

Reticulocyte counts are performed manually using supravital staining with methylene blue (ENGLANG *et al*., 1998), but nowadays this method has been replaced by automated methods, which are incorporated into a high-volume hematology analyzers[1]. Standardization of staining procedures, data analysis, and quality control are important factors, since the reticulocyte RNA content is a continuous variable without clearly separation on positive and negative populations, which may be a problem for manual reticulocyte counting (GILMAER JUNIOR; KOEPKE, 1976).

A major advantage of automated reticulocyte counting is improved accuracy and precision (DAVIS; BIGELOOW, 1994; RILEY *et al*., 2002), especially in the low-normal range, when the manual method is inaccurate due to the small number of counted reticulocytes (VAN HOUTE; BARTELS; SCHOORL, 1994). Most of the newest automated hematology analyzers have automated reticulocyte counting as part of the testing capabilities and allow reticulocyte to be included with complete routine of blood count parameters (GREER *et al*., 2008). They provide percent and absolute numbers, besides evaluating other parameters, such as

reticulocyte hemoglobin content and the proportion of immature reticulocytes; thus, they can also distinguish between early and late reticulocytes (STIENE-MARTINS; LOTSPEICH-STEININGER; KOEPKE, 1997).

Automated methods for reticulocyte count are based either on the principle of fluorescence emitted by the reticulocyte, under fluorochroms with affinity for reticulocyte RNA such as auramine O, or on the light absorption changes by the reticulocyte stained with substances with high affinity for RNA, such as new methylene blue (OLIVEIRA, 2007). These methods count and classify the reticulocyte according to their maturity degree, based on the content of reticulocyte RNA as follows: low maturity being those with the lowest content; medium maturity with intermediate content; and high maturity, with the highest content of reticulocyte RNA (LEWIS; BAIN; BATES, 2006). The current trend is to express the reticulocyte count with low and medium maturity as immature reticulocyte fraction – IRF (FAILACE, 2009). Immature reticulocyte fraction, when interpreted together with the percentage and absolute count of reticulocyte, is a useful parameter to monitor the response to treatment with erythropoietin in chronic renal failure, and to detect recovery of bone marrow in the treatment of aplastic anemia or after chemotherapy (BAIN, 2007).

Reference ranges differ slightly among manual and automated methods (BUTTARELLO *et al.*, 2001). They should be re-evaluated when introducing an automated reticulocyte method, since methodology-dependent variations in reticulocyte count have been observed (VAN HOUTE; BARTELS; SCHOORL, 1994).

The aim of this study was to establish reference ranges of automated reticulocyte method using the Cell-Dyn 3500 Abbott analyzer, and new methylene blue as staining agent, in the KG Clinical Laboratory, Joinville, SC, Brazil.

2 Material and Methods

Blood samples of 290 people, 126 female and 164 male, aged from 11 to 60 years were collected in EDTA

K_3 anticoagulants. All individuals of this study were considered to be clinically healthy, had normal blood counts, and no clinical history of anemia. They were attended at KG Laboratório de Análises Clínicas of Joinville, Santa Catarina, SC, Brazil.

Samples were prepared by placing 5 µl of whole blood-EDTA into a tube containing 925 µl of reticulocyte reagent Cell-Dyn (new methylene blue). The mixture was incubated for 30 minutes at room temperature and then it was processed by flow cytometry, using the Cell-Dyn Hematology 3500 counter (Abbott Diagnostic Division). All the technical procedure occurred within the first 8 hours post-collection.

Reticulocyte profile included reticulocyte percentage (RET %), absolute reticulocyte count (RET N), low reticulocyte fraction (LRF), medium reticulocyte fraction (MFR), and high reticulocyte fraction (HRF).

Reference ranges were obtained from 2.5 and 97.5 accumulated percentiles in normal distributions. The data were also analyzed by t-test for two independent samples. Analyses were carried out using an Excel spread sheet (Microsoft) and the statistical package Statistica 10.0 (StatSoft). The criterion for statistical significance was set at the nominal probability (p) level of $p \leq 0.05$. This study was approved by the Ethical Committee of the University of Vale do Itajaí – UNIVALI (Register number: 136/11a; CAAE: 0620.0.000.223-11).

3 Results and Discussion

The population studied is from a convenience sample, without sample size calculation, and, as such, may not reflect the characteristics of the population in general. However, it is of representative size for patients attended at the Laboratory and may have alleviated the possible selection bias.

Values of percentage and absolute reticulocytes reference ranges from normal adults (male and female) are described in Table 1. Values of reticulocyte counts (%) stratified by fraction size and sex are described in Table 2.

Table 1: Reticulocytes counts from normal adults

| | Reticulocyte Counts | | | | | |
	Percentil	Absolute	%	Parameters	Absolute	%
Totals	2.5th	37	0.85	Mean	70.68	1.48
	97.5th	129	2.80	Std. dev.	22.98	0.46
Male*	2.5th	42	0.87	Mean	74.52	1.49
	97.5th	129	2.72	Std. dev.	21.59	0.42
Female*	2.5th	37	0.85	Mean	65.67	1.46
	97.5th	126	2.86	Std. dev.	23.84	0.52

* - Significant statistical differences in averages for absolute counts (t=3.31; p=0.001)
Sample size: 290. The unit for absolute counts was x 10^9/L.

Table 2: Reticulocyte Counts (%) stratified by fraction size and sex.

| | Reticulocyte Counts | | | | | |
| | High | | Medium* | | Low* | |
Parameters	Male	Female	Male	Female	Male	Female
Minimum	0.30	0.60	8.90	10.80	58.70	63.60
Maximum	9.00	9.40	33.20	32.00	89.30	86.60
2.5%	1.10	1.24	14.33	12.81	66.05	67.74
97.5%	7.58	7.20	29.00	26.73	83.98	85.23
Median	3.20	3.05	21.10	19.55	75.15	77.25
Mean	3.61	3.57	21.34	19.32	75.05	77.10
Std. dev.	1.96	1.88	4.60	4.29	5.22	4.78
Std. err.	0.15	0.17	0.36	0.38	0.41	0.43

* - Significant statistical differences in averages for percentages (t-test 3.81 and 3.43 for medium and low, respectively; $p=0.000$). Sample size: male, 164; female, 126

Manual reticulocyte counts are expressed as a percentage or in absolute number per liter. The reference range for adults in some laboratories is 0.5 to 1.5%, whereas in others, a broader range of 0.5 to 2.0% is used (STIENE-MARTIN; LOTSPEICH-STEINEINGER; KOEPKE, 1997). The range may be slightly higher in women (DEISS; KURTH, 1970) and in newborns (2.0-6.0%), but this value drops to near adult levels within 1 to 2 weeks of birth (LOWENSTEIN, 1959). In absolute numbers, the reference range is approximately 10 to $110 \times 10^9/L$[9].

The reference range differs among manual and automated methods (BUTTARELLO e*t al*., 2001). In the manual method, it is not possible to evaluate the proportion of immature reticulocytes (STIENE-MARTIN; LOTSPEICH-STEINEINGER; KOEPKE, 1997) and the inter-observers variation coefficients vary from 25 to 50%[1], which is considered a big analytical variation.

Because of the large number of cells counted, automated reticulocyte counts are more accurate and reproducible than manual counts. However, the automated count is changed by factors such as choice of the fluorochrome, blood exposure time to fluorochrome, temperature at which the sample is maintained after mixture, and the threshold position - superior threshold to delete the fluorescent nucleated cells, but inferior to delete the background autofluorescence. Reference limits from automated reticulocyte count are therefore specific to the instrument and to the method and may vary considerably. This demonstrates the need for establishing reference values for a specific population, considering the laboratory instrumentation conditions. Ideally, the automated and manual counts must present good correlation (GREER *et al*., 2008).

Simionatto *et al.*, 2009; 2010) used the Cell Dyn 3500 SL equipment (Abbott) and found good correlation between manual and automated methods. The differences between both methods were small, with systematic error of 0.4% and random error of 3.9%, proving that the manual count can be used whether is technically well done.

It was observed that the frequency distribution profile of the data obtained by the automated Cell-Dyn 3500 was very similar to that obtained by van den Bossche *et al.* (2002) using Cell Dyn 4000. On the other hand, Buttarello *et al.* (2001) used the same Cell-Dyn 4000 equipment but found a slight difference at the lower limit, and van Houve, Schisano e Brace (2000) showed gender differences.

Although the Cell-Dyn 3500 and 4000 belong to the same manufacturer (Abbott), they use different dyes to perform the reticulocyte count. Cell-Dyn 3500 utilizes a non-fluorescent dye, new methylene blue, while Cell-Dyn 4000 uses fluorescence dye, 530 CD4K. When evaluating other equipment that uses the same non-fluorescent dye in Cell-Dyn 3500, the reference values also differ. In a study by Abbott with 200 American employees, the reference value obtained for the Cell-Dyn 3500 equipment was 0.88 to 2.37%. Butarello *et al.* (1996) used Coulter Maxm, and new methylene blue as a non-fluorescent dye, and found reference values of 0.37 to 1.80 %. With Sysmex R-1000 equipment, the reference values varied from 0.60 to 1.95%.

Arai *et al*. (2011) confirmed the largest erythropoietic activity in children with sickle cell anemia (HbSS) in relation to children with SC hemoglobinopathy (HbSC), using fractions of the reticulocyte counts (low, medium and high). This stratification of reticulocyte population proved to be useful for a better evaluation of the activity of the bone marrow. Children with hemoglobin SC showed a reticulocyte profile with predominance of low reticulocyte fluorescence, thus greatly reinforced the lesser erythropoietic activity in this hemoglobinopathy when compared to the SS group. The conclusion of this study was that the reticulocyte profile (absolute, relative count and reticulocyte fractions) should be established for each hemoglobinopathy.

In the present study, the reference range was established as 0.85-2.80%, and 37 to $129 \times 10^9/L$ in absolute numbers. Fractions of reticulocyte profile showed high value of low fluorescent fraction for the hematology counter Cell-Dyn

3500 utilizing a non-fluorescent dye, new methylene blue, in a population serviced by KG Clinical Laboratory of Joinville, SC, Brazil, and can be used as reference values for this population.

4 Conclusion

Based on the present study, we conclude that the reference range of 0.85 to 2.80% for the reticulocyte counts is higher than that found using the manual method. The same is observed in absolute numbers, which were 37 to 129x 10^9/L. When the group was evaluated in terms of gender (male/female) there was a difference as far the absolute count is concern (t-test, p=0.001), but none for percentage counts (t-test, p=0.647). It was observed that the profile of reticulocyte fractions showed high value of low reticulocyte fractions (Table 2). No difference among male and female was observed when the profile was evaluated as high, medium and low fractions, in percentage of reticulocyte counts (t-test, p>0,05).

Normal values obtained in this study were higher than those found in the literature, which demonstrates the need for establishing reference values for a specific population, considering proper conditions of local laboratory instrumentation.

References

ARAI, M. *et al*. Reticulocitograma em pacientes com anemia falciforme e hemoglobinopatia SC. Publication UEPG Biol. Health. Sci., v.17, n.1, p.53-58, 2011.

Bain BJ. Células sanguíneas: um guia prático. Porto Alegre: Artmed, 2007.

BUTTARELLO, M. *et al.* Flow cytometric reticulocyte counting. Parallel evaluation of five fully automated analyzers: an NCCLS-ICSH approach. *Am. J. Clin. Patho.*, v.115, n.1, p.10-111, 2001

BUTTARELLO, M. *et al*. Reticulocyte quantification by Coulter MAXM VCS (volume, conductivity, light scatter) technology. Clin. Chem., v.42, n.12, p.1930-1937, 1996.

DAVIS, B.H.; BIGELOW, N.C. Automated reticulocyte analysis. Clinical practice and associated new parameters. *Hematol. Oncol. Clin. North Am.*, v.8, n.4, p.617-630, 1994.

DEISS, A.; KURTH, D. Circulating reticulocytes in normal adults as determined y the new methylene blue method. *Am. J. Clin. Pathol*., v.53, n.4, p.481-484, 1970.

ENGLAND, J.M. *et al.* Proposed reference method for reticulocyte counting based on the determination of the reticulocyte to red cell ratio. The Expert Panel on Cytometry of the International Council for Standardization in Haematology. *Clin. Lab. Haematol.*, v.20, n.2, p.77-79, 1998.

FAILACE, R. Hemograma: manual de interpretação. Porto Alegre: Artmed, 2009.

GILMER JUNIOR, P.R.; KOEPKE, J.A. The reticulocyte:an approach to definition. *Am. J. Clin. Pathol.*, v.66, n.1, p.262-267, 1976.

GREER, J.P. *et al.* Wintrobe's clinical hematology. Philadelphia: Lippincott Williams & Wilkins, 2008.

KAUSHANSKY, K. *et al*. Williams hematology. New York: McGraw-Hill Professional, 2011.

LAZARUS, H.M. *et al.* Kinetics of erythrogenesis after bone marrow transplantation. *Am. J. Clin. Pathol.*, v.97, n.4, p.574-583, 1992.

LEWIS, S.M.; BAIN, B.J.; BATES, I. Hematologia prática de Dacie e Lewis. Porto Alegre: Artmed, 2006..

LOWENSTEIN, L.M. The mammalian reticulocyte. International *Review of Cytology*, v.8, n.1, p.134/174, 1959.

OLIVEIRA, R.A.G. Hemograma como fazer e interpretar. São Paulo: Livraria Média Paulista Editora, 2007.

RILEY, R.S. Reticulocyte analysis by flow cytometry and other techniques. *Hematol. Oncol. Clin. North Am.*, v.16, n.2, p.373-420, 2002.

SIMIONATTO, M. *et al*. Analysis of manual reticulocyte counting in clinical laboratories of Ponta-Grossa and Campos Gerais, PR, Brazil. *Rev. Bras. Hematol. Hemoter.*, v.31, p.315-320, 2009.

SIMIONATTO, M. *et al*. Manual and automated reticulocyte counts. *Hematology*, v.15, p.406-409, 2010.

STIENE-MARTIN, E.A.; LOTSPEICH-STEININGER, C.A.; KOEPKE, J.A. *Clinical hematology*: principles, procedures, correlations. Philadelphia: Lippincott Williams &Wilkins, 1997.

VAN DEN BOSSCHE, J. *et al.* Reference intervals for a complete blood count determined on different automated haematology analysers: Abx Pentra 120 Retic, Coulter Gen-S, Sysmex SE 9500, Abbott Cell Dyn 4000 and Bayer Advia 120. *Clin. Chem. Lab. Med.* v.40, n.1, p.69-73, 2002

VAN HOUVE, L.; SCHISANO, T.;BRACE, L. Anemia diagnosis, classification, and monitoring using cell-dyn technology reviewed for the new millennium. *Labor Hematol*, v.6, n.1, p.93-108, 2000.

VAN HOUTE, A.J. *et al*. Methodology-dependent variations in reticulocyte counts using a manual and two different flow cytometric procedures. *Eur. J. Clin. Chem. Clin. Biochem.*, v.32, n.11, p.859-863, 1994.