Original Article

Evaluation of the Automated Nucleated Red Blood Cell (NRBC) Enumeration on Sysmex XN Analyser in Preterm and Term Neonates

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Introduction: *Increased NRBC values are associated with poor postnatal prognosis of neonates. The described study compares preterm and term neonatal NRBC counts at various levels by traditional microscopic manual counts and automated NRBC counts using the Sysmex XN-Series analyser. Furthermore, mean NRBC counts (%) at various postnatal days were determined.*

Methods: *One hundred and twenty-one samples from preterm and term neonates were included in the study and evaluated microscopically and on a Sysmex XN-Series analyser. The NRBC values from the analyser were used to determine mean NRBC counts (%) at various postnatal days for term as well as preterm neonates.*

Results: *A comparison between the manual and Sysmex XN counts for all 121 samples, assessed by Passing-Bablok regression, revealed a good correlation with regression equation $y = 0.998x + 0.201$. For samples with both manual and Sysmex Automated Hematology Analyzer XN-Series counts in the range of 0–10% (99 samples) Bland-Altman analysis showed a mean bias of -0.5% with 95% limits of agreement between -3.4% and 2.5%. The mean NRBC counts (%) at different postnatal days for term and preterm neonates showed a significant difference between these groups. A significant drop in the percentage NRBC was seen for all groups from the day of birth to 2–4 days after birth.*

Conclusion: *The results indicate that the automated NRBC counts in neonatal samples correlate well with the manual counts. The Sysmex Automated Hematology Analyzer XN-Series count is accurate and effective for the analysis of neonates' NRBC.*

Key Words   NRBC, Sysmex XN, Neonates, Enumeration, Evaluation

INTRODUCTION

Nucleated red blood cells (NRBC) can be seen in the circulating blood of term and preterm neonates. Beyond the neonatal period, in healthy humans there are no circulating NRBC, since in the steady state these immature erythrocytes, also called erythroblasts, are restricted to the bone marrow. Seven days after birth, NRBC are usually no longer detected in the peripheral blood of healthy neonates.[1,2] In preterm neonates, erythropoiesis is increased and a significant number of NRBC can be observed for a longer postnatal period. The presence of an increased NRBC count reflects an increased erythropoiesis due to a variety of reasons such as chronic hypoxia, anaemia, acute stress or postnatal hypoxia. Several studies found a correlation between elevated NRBC count and increased postnatal morbidity and mortality of neonates.[3] A fast and accurate quantification is needed allowing the use of NRBC as a prognostic marker for morbidity and mortality. Traditionally, NRBC are counted manually using a microscopic counting chamber. However newer, fully automated methods enable a faster and more accurate measurement of NRBC than the manual method. Recently, a comprehensive performance evaluation of nucleated red blood cell count of five haematological analyzers and found an excellent precision for Sysmex XN-Series with limit of quantification (LoQ) $0.029 \times 10e9/L$, a value below LoQ declared by the company.[4]

Notes: The specifications, performances and functions described here may be different depending on the regions or the countries due to the regulatory affairs, legal matters or local guidelines. For more details, please contact your regional affiliates or distributors.

The fully automated Sysmex Automated Hematology Analyzer XN-Series (Sysmex Corporation, Kobe, Japan) measures NRBC as part of the complete blood count. Only the measurement of NRBC together with a complete blood count provides a corrected WBC count. Previous studies about the analytical performance demonstrated very good correlation between automated NRBC counts from the Sysmex XN-Series and manual microscopic counts.[4,5] However, these studies were carried out only in adult populations. The primary objective of the presented study was to measure NRBC in the peripheral or cord blood of preterm and term neonates using the reference manual count and compare it with the results obtained using the Sysmex XN technology. The secondary objective of this study was to follow Sysmex XN NRBC counts (%) for term and preterm neonates at the day of birth (day 0), day 1 after birth and days 2−4 after birth and compare these values with the NRBC reference range values found in the literature.

MATERIALS AND METHODS

Blood samples

For the sake of the study 121 clinical blood specimens were collected in the Division of Neonatology and analysed in the Division of Paediatric Haematology and Oncology, both at the Department of Paediatrics, Inselspital, Bern University Hospital, and University of Bern, Switzerland. The blood samples were collected in a 200µl peripheral blood sampling Microvette tube (SARSTEDT, Nümbrecht, Germany) containing K_2EDTA. The blood samples were either of capillary or cord blood origin. NRBC were measured using an automated XN-Series haematology analyser within 2 hours of blood collection. Peripheral blood smears were made within 30 minutes after automated analysis and NRBC were counted using the reference manual microscopic method.

Patients' information

One hundred and forty-three patients were enrolled to analyse the mean NRBC counts at day of birth (day 0) and postnatal days (day 1, days 2−4 − sample measured at days

2 or 3 or 4). Out of these 143 samples, 121 were also used for the enumeration evaluation. Manual counts were not available for the remaining 22 samples, which were added to maximise the number of analysed samples. Each sample was obtained from a unique neonate and thus no neonate was measured multiple times over the course of the study. The classification and size of groups were as described in *Table 1*.

Definition of prematurity was according to the WHO classification: neonates born alive before the end of 37 weeks of pregnancy.[6] The mean gestational age of preterm neonates was 31 weeks. The health status of the neonate was examined by paediatricians and each neonate was classified as healthy or unhealthy.

Sysmex XN haematology analyser count

The haematology analyser used in this study was Sysmex XN-1000 (Sysmex Corporation, Kobe, Japan) equipped with software version 00−12, but samples were later re-analysed with software version 00−18. The automated NRBC measurement on this analyser is performed in the WNR channel. The WNR channel counts WBC and performs a differential count of basophils and NRBC. While causing complete haemolysis of red blood cells, the surfactant (Lysercell WNR) leaves the nuclei of NRBC and the cell membrane of the WBC mostly intact but penetrates the nuclear envelope of the NRBC and the cell membrane of the WBC. After this treatment, Fluorocell WNR fluorescently stains nucleic acids in the WBC and NRBC nuclei. Then the treated samples are analysed by flow cytometry and the signals of side-fluorescent light (SFL), forward-scattered light (FSC) and side-scattered light (SSC) are generated and analysed. The NRBC have a weak side-fluorescent signal and medium forward-scattered light and due to this signal combination, can be separated from WBC or cell debris in the WNR scattergram. The NRBC counts are analysed and reported with every ordered blood count.

Manual microscopic evaluation of blood smears

The peripheral blood smears were stained with Giemsa using a Hema-tek 2000 (Bayer, Leverkusen, Germany)

Table 1 *Number of samples within the different study cohorts*

	Term healthy	Term unhealthy	Preterm unhealthy
Day 0	10	15	35
Day 1	6	14	17
Day 2−4	14	17	15

slide stainer. The smears were then examined by two experienced laboratory staff, who inspected two slides using the light microscope at 100x magnification and established the number of NRBC per 200 WBC. The average NRBC numbers from the four determinations were then reported as NRBC (%) according to the H20-A2 Clinical and Laboratory Standards Institute guidelines.[7]

Manual versus automated count comparison study

One hundred and twenty-one samples from neonates (61 term and 60 preterm) were analysed in the study. The Sysmex XN count and manual count were compared by Passing-Bablok regression [8] and the Bland-Altman [9] plot was used to evaluate differences plotted against the averages of the two measurement methods in order to assess the agreement between the methods.

Statistical analysis

Statistical analysis was performed using MS Excel 2010 and MedCalc Statistical Software version 16.2.0 (MedCalc Software, Ostend, Belgium).[10] Data were compared across groups with non-parametric Mann-Whitney U-test statistical tests and a P-value < 0.05 was considered statistically significant.

RESULTS

Manual versus automated count comparison study

The correlation for all 121 tested samples determined by Passing-Bablok regression between the automated NRBC count and the reference manual technique is shown in **Fig. 1**. The demonstrated regression equation was: $y = 0.998x + 0.201$. The 121 NRBC counts determined manually were within the range of 0−393% with more than 80% of values within the range of 0−10%. Taking into consideration this wide distribution of values and the high clinical relevance in the low range, correlation between automated NRBC counts and manual NRBC counts for samples with manual count between 0−10% (N = 99) is depicted in **Fig. 2**. The regression equation is: $y = 1.000x + 0.200$ and the respective Bland-Altman plot is
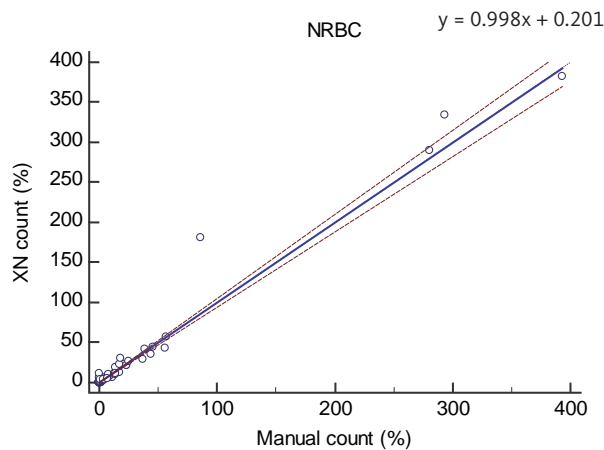

Fig. 1 *Comparison of automated nucleated red blood cell (NRBC, %) from Sysmex XN against manual microscopic counts for all tested samples (N = 121)*
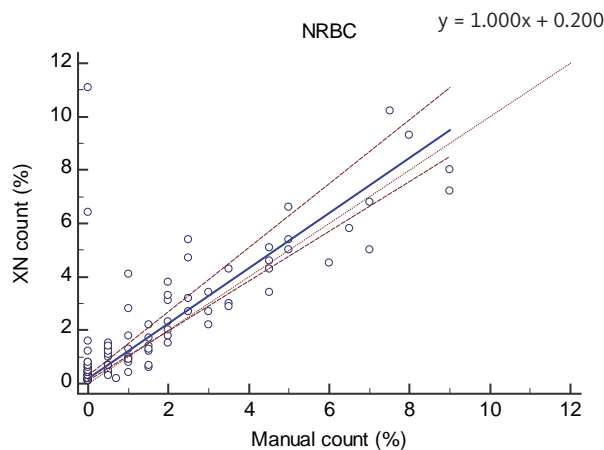

Fig. 2 *Comparison of automated nucleated red blood cell (NRBC, %) counts from Sysmex XN against manual microscopic counts for values of manual count in the range 0−10% (N = 99)*

shown in ***Fig. 3***. The mean bias was -0.5% with 95% limits of agreement between -3.4% and 2.5%. The agreement between automated NRBC counts and manual counts at different ranges is presented in ***Table 2***. The ranges were classified into 4 categories: 0.0–1.0%, 1.1%–10.0%, 10.1%–100.0% and more than 100.0%. The overall agreement rate between the manual and automated count based on this group classification was 85.1% (103/121). According to CLSI guidelines, cases with ≥ 1% NRBC are considered positive and cases with NRBC < 1% are considered negative.[7] Based on these criteria, diagnostic performance based on an automated XN count was calculated as follows: sensitivity 96.7%, specificity 78.3%, positive predictive value 81.9% and negative predictive value 95.9%.

At the low manual count range (0–10%) there were two main outliers with a very significant difference between the manual and the automated NRBC count. The two samples both had a manual count equal to 0%, while the XN counts were 6.4% and 11.1%. ***Fig. 4*** shows the corresponding scattergrams (A, B) from the WNR channel, where the NRBC are counted. The scattergrams show a good separation of the NRBC and WBC populations and revealed no obvious interference in the cluster where nucleated red blood cells are detected. ***Fig. 4*** further shows the WNR scattergram (C) and a good separation of populations for the sample with the highest observed NRBC count (manual count: 393%; automated count: 383%).
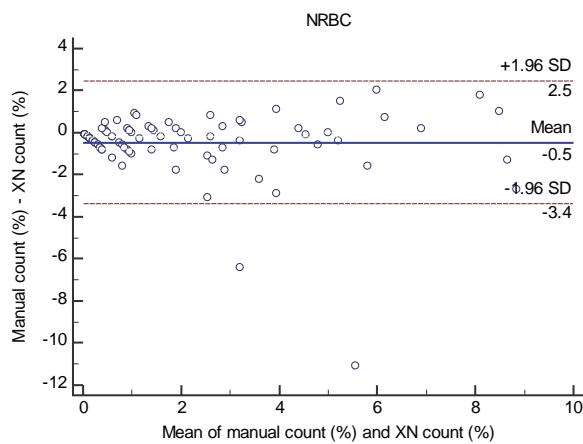

Fig. 3 *The Bland-Altman plot of the difference between nucleated red blood cell (NRBC) count from Sysmex XN and manual microscopic count. The solid line and dotted lines represent the mean bias and 95% limits of agreement, respectively.*

Table 2 *The comparison of distribution of nucleated red blood cell (NRBC) counts from manual microscopic method and from Sysmex XN*

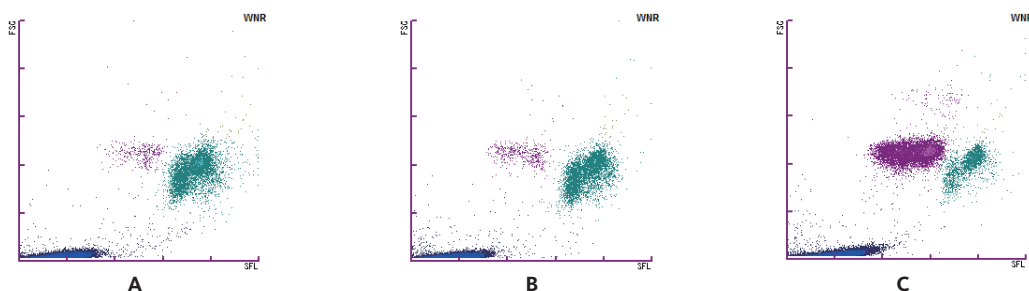| NRBC (%) range from manual microscopic count	Number of samples	NRBC (%) range from Sysmex XN count			
		≤ 1.0	1.1 – 10.0	10.1 – 100.0	> 100.0
		Number of samples			
≤ 1.0	60	47	12	1	
1.1 – 10.0	39	2	36	1	
10.1 – 100.0	19		1	17	1
> 100.0	3				3

Fig. 4 *The WNR scattergrams of three samples showing a good separation of the nucleated red blood cell (NRBC) population and WBC population. The WNR scattergrams for samples with manual count equal to zero and automated count of 6.4% (A) and 11.1% (B). The WNR scattergrams for the highest manual count of 393% and the corresponding XN automated count of 383% (C)*

NRBC count at day of birth and postnatal days

At the day of birth, the first day after birth (day 1) and postnatal days 2–4, the mean of NRBC (%) was calculated for different neonatal cohorts based on their medical records and the results are shown in **Fig. 5A–5C**. At the day of birth, the mean NRBC count was 2.35% (CI 1.14–6.30) for healthy neonates at term, 3.70% (CI 1.26–8.62) for unhealthy neonates at term and 15.50% (CI 6.94–23.40) for unhealthy preterm neonates (**Fig. 5A**). At day 1, the mean was 1.05% (CI 0.26–2.65) for healthy neonates at term, 0.90% (CI 0.28–2.25) for unhealthy neonates at term

and 5.10% (CI 3.42–11.10) for unhealthy preterm neonates (**Fig. 5B**). At days 2–4, the mean was 0.70% (CI 0.30–1.57) for healthy neonates at term, 0.50% (CI 0.30–1.20) for unhealthy neonates at term and 1.40% (CI 0.55–5.21) for neonates born preterm and identified by the responsible paediatrician as unhealthy (**Fig. 5C**). Term neonates had on average lower NRBC count than preterm neonates. The difference between all term and preterm groups was statistically significant for day 0 and day 1 (**Fig. 5A, 5B**). For days 2–4, the differences were not considered statistically significant except in the term unhealthy group in comparison to preterm unhealthy group, p = 0.049.
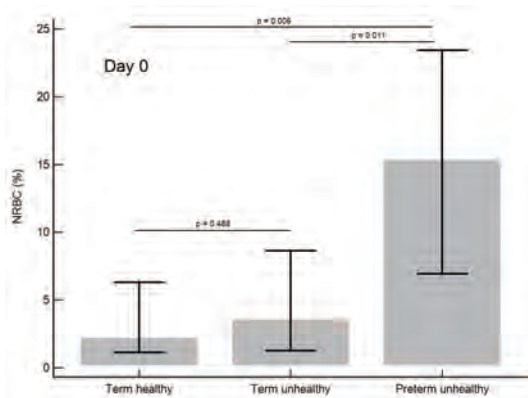

Fig. 5A *The bars show mean NRBC (%) in different groups of neonates at the day of birth (day 0). The respective error bars represent 95% confidence intervals for mean.*
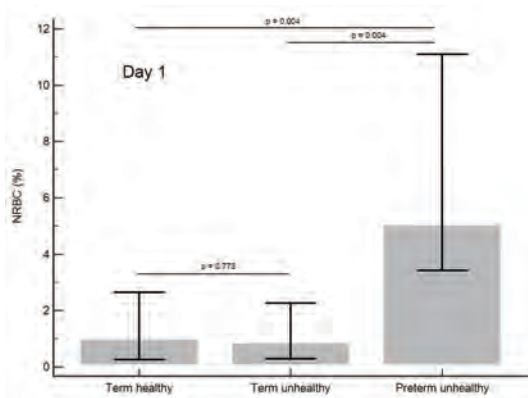

Fig. 5B *The bars represent mean NRBC (%) in different groups of neonates at postnatal day 1. The respective error bars represent 95% confidence intervals for mean.*
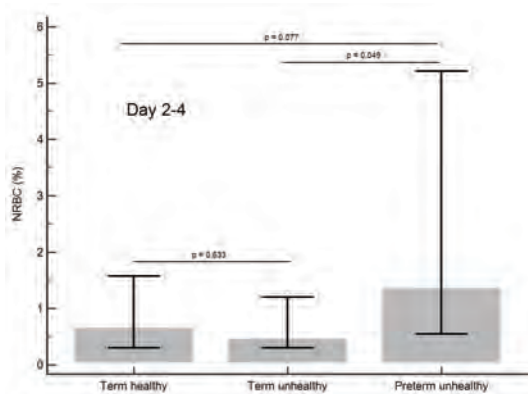

Fig. 5C *The bars represent mean NRBC (%) in different groups of neonates at days 2–4. The respective error bars represent 95% confidence intervals for mean.*

An analysis of the mean counts for different neonatal cohorts over time (day of birth, day 1, days 2–4) shows a general trend with a significant decrease in NRBC count between day 0 and day 1 in neonates born at term (healthy as well as unhealthy), while the difference between day 1 and days 2–4 was much smaller: term healthy neonates: 2.35%, 1.05%, 0.70% (**Fig. 6A**); term unhealthy neonates: 3.70%, 0.90%, 0.50% (**Fig. 6B**). On the other hand, for preterm unhealthy neonates the large drop in NRBC values happened between day 0 and day 1 as well as between day 1 and days 2–4: 15.50%, 5.10%, 1.40% (**Fig. 6C**).
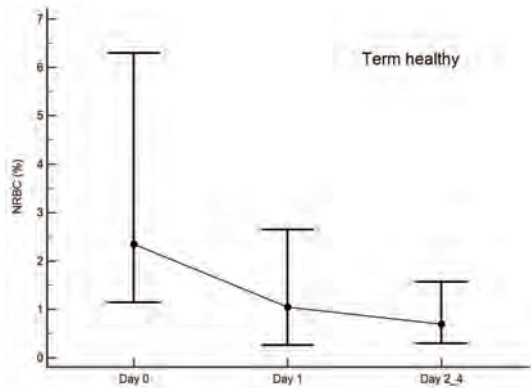

Fig. 6A *The bars represent mean NRBC (%) for different time points at or after birth for term healthy neonates. The respective error bars represent 95% confidence intervals for mean.*
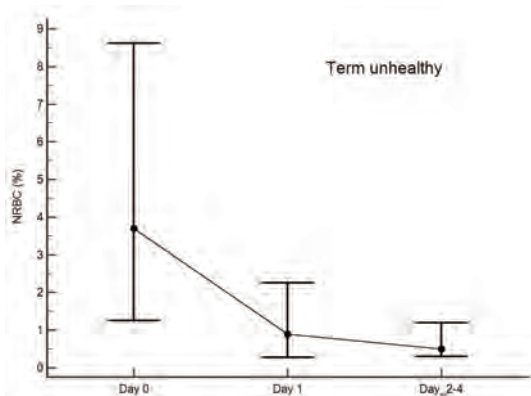

Fig. 6B *The bars represent mean NRBC (%) for different time points at or after birth for unhealthy term neonates. The respective error bars represent 95% confidence intervals for mean.*
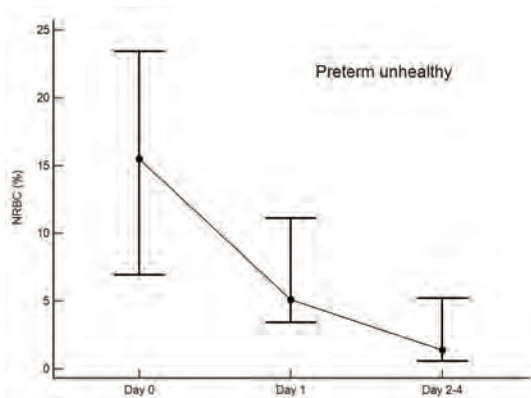

Fig. 6C *The bars represent mean NRBC (%) for different time points at or after birth for unhealthy preterm neonates. The respective error bars represent 95% confidence intervals for mean.*

DISCUSSION

In this study, we evaluated the performance of NRBC count in a very wide range of neonatal blood samples on the Sysmex XN analyser and compared it with the manual count. The data showed very good correlation between manual and automated counts over a wide range. Compared to previous studies in adult populations, where manual NRBC counts were compared to those obtained with the automated Sysmex XN-Series [4,5], in this study the samples originated from healthy and unhealthy neonates. The overall results showed high concordance with manual count over four reference intervals including very low and very high counts. Even for samples with a manual count of zero but higher automated counts (samples with disagreement), the analysis of scattergrams indicate that NRBC cells were detected and clearly separated from other cell populations. However, we did not investigate the discrepancy in cell count further and thus we cannot prove the correct cell count. On the whole, we found a low number of false negative samples and a diagnostic performance with a high sensitivity (96.7%) and specificity (78.3%). As NRBC count is part of CBC test in Sysmex XN-Series, the routine implementation of automated NRBC counts improves laboratory costs, efficiency and workflow also in labs handling blood samples from neonates.

Nucleated red blood cells are very rarely present in the peripheral blood of healthy children and adults. However, NRBC are sometimes found in the blood of neonates just after birth. Hypoxia is known to be a reason for an increased NRBC count due to both stimulated erythropoiesis [11-13] and release from the storage pool [14]. Many recent studies have demonstrated a strong association between elevated NRBC counts and unfavourable perinatal outcome including intraventricular haemorrhage [15-17], necrotizing enterocolitis [15], idiopathic intra-uterine growth retardation [18], brain injury [19] and mortality. Unfortunately, [3] the knowledge on reference ranges in different neonatal cohorts is quite sparse, especially for NRBC as a percentage of WBC. Perrone et al. categorized 695 neonates into four groups according to gestational age and determined reference values for NRBC counts in these term and preterm neonates. [20] Other authors have also determined reference values for healthy neonates depending on time after birth and gestational age. [21-24] Generally, the authors studied only a limited sample number (< 100) and reported reference values as NRBC count. We found highly significant differences between term and preterm cohorts especially for day 0 and day 1. There is also quite a significant drop between day 0 and day 1 for term neonates (both healthy and unhealthy) compared to a lesser drop from day 1 to day 2–4. For unhealthy preterm neonates a significant drop is seen between day 1 and day 2–4. This is in contrast with the study of Kil et al., where the authors investigated the NRBC in very low birth weight infants. They observed a significant drop in the NRBC counts up to two weeks after birth. [25] Due to this trend of fast initial drop in NRBC (%) count, our data suggest that NRBC count at day 0, measured immediately after the birth, is likely to be very important for prognosis of a patient's health outcome.

In conclusion, the Sysmex XN analyser accurately and effectively enumerates NRBC counts in neonates and our study suggest that the automated count can be used to replace the subjective, time consuming and expensive manual microscopic NRBC count, including highly pathological samples obtained from sick preterm neonates. Moreover, in this study we suggest ranges for NRBC (%) for different neonatal cohorts.

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