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ORIGINAL ARTICLE

A prolonged microscopic observation improves detection of underpopulated cells in peripheral blood smears

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Abstract We evaluated an extended time in the microscopic review in samples in which the potential clinical information could be increased with respect to those that could be achieved with the usual laboratory methodologies. We used samples containing nucleated red blood cells in a small amount and cytopenic samples. For these purposes for each peripheral blood smear, the timing of eye-count differential was increased up to 20 min, regardless of the final number of cells which could be counted. In addition, an automated system for digital analysis of peripheral blood smears was employed and the number of cells counted was brought up to 1000 leukocytes. In both manual and automatic light microscopy extended observation, we obtained more diagnostic information in respect to those with routine or standard methods. Both automated and manual increase systems of the timing for microscopic review are useful tools to find diagnostic information that otherwise would be lost using normal and standard procedures. So, these methods should be used especially when there is a higher pre-test probability for discovery of pathological cells.

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Introduction

Complete blood cell count (CBC) and the leukocytes differential count (LDC) in the peripheral blood (PB) are the most requested laboratory tests. Today, the newest haematological analysers offer additional extended leukocytes counting (ELC) for circulating nucleated red blood cells (nRBC) and immature granulocytes as well as flags and alarms for the hypothetical presence of abnormal or pathological cells (PC) such as blasts (BL) or atypical lymphocytes. This information from CBC and LDC represents the most frequent cause of the review by light microscopy [1].

In the daily practise, the proposed guideline for light microscopy review (LMR) [2] as well as the good practice guidance [3, 4] are not applied extensively because of the reduction of professional resources which does not allow a review based on a standard number of leucocytes/blood smear in most cases [5–7]. This represents a critical aspect especially when BL, nRBC or other PC could be in PB at low levels. As a consequence, the reduction in the number of eye-count differentials (ECD) and blood film reviews leads to an increase of false negative results in the haematological diagnostic processes [6]. Because of these, recently automated digital morphology and information systems to pre-classify circulating cells in PB were produced and more increasingly used in clinical laboratories [8, 9]. These systems allow the reading and digitization of microscopic images and show these pictures to operators in a pre-classification mode. The final classification is performed by operators by moving pictures of individual cells or groups of them.

In our study, we employed the extended timing of LMR up to 20 min as well as the pre-classification system Vision Hema (VH). With the last option, we used an increase of the cellular count up to 1000 leukocytes in order to better evaluate and quantify underpopulated cells in peripheral blood.

Methods

This study was performed on discarded remnants of PB samples collected for clinical purposes in K_2EDTA tubes (Vacutest Kima, Arzergrande, Italy). After processing on the Sysmex XN-9000 haematological analyser (Sysmex, Kobe, Japan) (XN), the samples were chosen with the following criteria:

i. Fourty-four samples with CBC and LDC in normal range and without morphological flags or alarms. After the splitting of each sample into two aliquots (A and B) of equal volume, a final concentration of nRBC theoretically ranged between 0.02 and 0.34 × 10^9/L (median 0.04) and below 0.50/100WBC (range 0.20–0.50; median 0.40) was obtained by adding to each aliquot A variable amounts of PB from five thalassaemic samples containing nRBC between 5.60 × 10^9/L and 18.44 × 10^9/L. These "nRBC true positive" samples were used for further LMR (group A samples). The corresponding 44 aliquot B were used as negative controls (group B samples);
ii. Seventy-nine cytopenic samples including 39 samples with isolated neutropenia (neutrophils count <1.20 × 10^9/L; range 0.20–1.09) and 40 samples with bi-, tri-linear cytopenia (neutropenia, range 0.45–1.1 × 10^9/L plus haemoglobin <80 g/L, range 49–80 and/or platelet count <80 × 10^9/L, range 31–80). Instrumental morphological flags just as "Blast cells?" or "Atypical Lymphocytes?" or "Giant PLTs" were present in 34 out of 79 samples. In addition, neutrophilic dysplasia was observed with preliminary LMR in 21 out of 79 samples.

Peripheral blood smears were done and stained according to May Grünwald Giemsa method with an automatic slide maker SP-1000i (Sysmex, Kobe, Japan). After the first slide review, performed according to the usual laboratory procedures by a single operator who counted up to 200 leukocytes (200LP), the selected samples were processed as follows in order to increase the probability of detecting underpopulated cells [10]:

i. According to CLSI H20-A2 standard [11] by two experienced operators (with a third in cases of disagreement) up to 200 leukocytes (total count 400 leukocytes), at ×1000 magnification (400ST counting)
ii. With VH systems pre-classification up to 400 leukocytes (400VH counting) at ×400 magnification
iii. With VH systems pre-classification up to 1000 leukocytes (1000VH counting) at ×400 magnification
iv. According to the protocol study for a maximum time of 20 min/slide or less until the observation of at least the first pathological cell whose presence was confirmed by a second opinion (extended time, ET counting) (×1000 magnification). In cases in which a first PC had been observed, the eye-count still continued up to 20 min (prolonged extended time, PET). BL, plasma cells (PL) megakaryocytes-megakarioblasts (MK/MKB) and atypical lymphocytes (ATY) including hairy cells (HC), in addition to nRBC were considered as "Pathological cells" (PC)

After pre-classification, the automated results from 400VH and 1000VH counting were screen-revised by two experienced operators who could also use the "show on slide" function for the direct microscopic observation at ×1000 magnification of a single cell wherein the instrument can move under the lens automatically, starting from the spatial coordinates of the first reading.

Vision Hema (West Medica—Perchtoldsdorf, Austria) is an automated system for scanning, digitalization and pre-classification of cells in peripheral blood smears. After the process, the cells are divided into band neutrophils, segmented neutrophils, eosinophils, basophils, monocytes, blasts, promyelocytes, myelocytes, metamyelocytes, lymphocytes, prolymphocytes, large granular lymphocytes, plasma cells, reactive lymphocytes and unknown. The final correct cell classification was obtained by moving single cells or groups from the pre-classification area to the area of interest. Among the different available models (Assist, Pro and Ultimate), we employed the intermediate model VH Pro. The statistical analysis was performed by MedCalc software version 11.4.2.0 (MedCalc, Ghent, Belgium). To test the distribution of the data, we used the D'Agostino-Pearson test: because in each group, we observed a p value <0.05 (data did not fit a normal distribution); data are presented as median, minimum and maximum. One thousand VH counting was used as gold standard to evaluate the diagnostic accuracy of the 200LP, 400ST, 400VH and ET counting and then area under the curve (AUC) was obtained from receiver operating characteristics (ROC) curve analysis; for a p value lower than 0.05 between A.U.C. obtained, the two compared areas were considered to be significantly different. The study was carried out in accordance with the Declaration of Helsinki in line with any relevant local legislation.

Results

In all LMR modes, the presence or the absence of nRBC and PC was recorded respectively as positive (POS) or negative (NEG).

Table 1 ROC curve relative to nRBC presence in PB with different LMR

LMR mode	AUC (95%IC)	Diagnostic agreement % (absolute count)
200LP	0.807 (0.709–0.833)	61.4 (17 FN)
400ST	0.875 (0.787–0.936)	75.0 (11 FN)
400VH	0.943 (0.872–0.981)	88.6 (5 FN)
1000VH[a]	1.000 (1.000–1.000)	–
ET	0.989 (0.938–1.000)	97.7 (1 FN)

[a] Classification variable

In the group A samples, the LMR was POS for nRBC presence in 27, 33, 39 and 44 out of 44 samples respectively with 200LP, 400ST, 400VH and 1000VH counting. With the ET counting, POS samples were 43 out of 44. In this last ECD mode, the timing ranged between 1:28 and 20:00 min (median 3:54). Since in each sample a variable amount of nRBC was manually added, the NEG results obtained with 200LP, 400ST, 400VH and ET counting have been considered as "false negative" (FN) results. For the same ECD modes, the incidence of FN was respectively 38.6 (17 FN), 25.0 (11 FN), 11.4 (5 FN) and 2.3 (1 FN) percent (Table 1). The differences in the FN incidence for ET in respect to the other modes were statistically significant for 200LP and 400ST ($p < 0.0001$ and $p = 0.0004$, respectively) and not significant for 400VH ($p = 0.0958$) (Table 2). The ROC curve analysis showed significant differences of the AUC with an increase of these from 200LP to ET and 1000VH (Table 1 and Fig. 1). In each sample, the false negativity did not seem to be correlated to the number of nRBC which was added previously (data not shown). nRBC were not observed with any of the LMR modes in the group B samples.

In the neutro, cytopenic samples, no pathological cells were detected with 200LP and 400VH modes of LMR whereas 29 different types of PC have been observed in a very low amount in 13 samples with 400ST, 1000VH and ET/PET as shown in Table 3. The total number of PC detected was equal to 1 with 400ST, 17 with ET (including PET) and equal to 11 with 1000VH, (delta ET/1000VH equal to 1.54). The LMR timing in ET mode ranged between 8:32 and 20:00 min for all samples and between 8:32 and 17:48 min for positive samples when the end-point was represented by the first PC observed.

Table 2 Pairwise comparison of ROC curves relative to nRBC presence in PB with different LMR

	Difference between areas	Standard error	p value[1]
200LP vs 400ST	0.0682	0.0384	0.0761
200LP vs 400VH	0.136	0.0376	0.0003
200LP vs ET	0.182	0.0367	<0.0001
400ST vs ET	0.114	0.0320	0.0004
400VH vs ET	0.0455	0.0273	0.0958

[1] p value: significance level for comparison of ROC curve

The time required for peripheral blood smears reading by using the VH for both nRBC and cytopenic samples ranged between minutes 2:48 and 7:23 (median 3:51) for the 400VH counting and between 5:45 and 13:34 (median 7:56) for 1000VH counting depending on the WBC total count and on the quality of blood smears. The average time for each operator required to observe and move the pre-classified cells was about 50 s.

Discussion

Due to the good analytical performances provided by haematological analysers, the microscopic reviews of peripheral blood smears in a general way are not performed to confirm LDC, but to acquire additional qualitative information as suggested by the presence of various flags and alarms or less frequently by the clinical information [3]. The ELC provided by most of the haematological analysers is a new tool for additional information such as nRBC and Immature granulocytes that allow a more oriented LMR [12]. The availability of this ELC in all samples showed that in an unsuspected group of individuals, low levels of circulating nRBC in PB are present with otherwise normal CBC parameters. In most of these, there is no evidence of increased erythropoiesis or of pathologic bone marrow processes [13, 14]. In addition, in

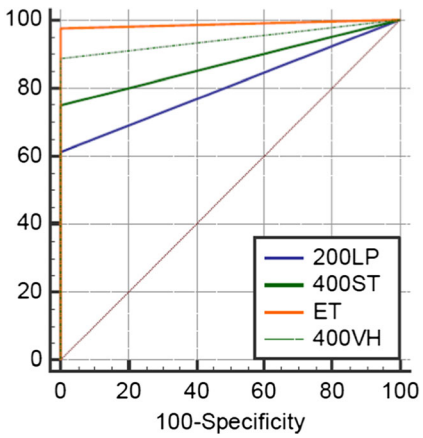

Fig. 1 Receiver operating characteristic (ROC) curve analysis. The figure shows the ROC curves of nRBC presence in PB with different LMR

Table 3 Pathological cells detected in cytopenic samples with various mode of microscopic review

Sample number/ET or PET timing	BL (mode)	nRBC (mode)[d]	MK (mode)	PL (mode)	Other (mode)	Flags/preliminary LMR
09/11:34	0	1(ET)+1 (PET)	0	0	0	Blasts?
14	1(VH)	0	0	0	0	NO
21/13:12	1(ET)	0	0	0	0	NO
30/8:32	1 (VH)+1(PET)	1(ET)	0	0	0	Blasts?/dysplasia
31/9:46	0	0	0	0	1(ET)[a]+1(PET)[a]	Blasts?
39	0	1(ST)+1(VH)	0	0	0	NO/dysplasia
44/14:23	0	1(ET)	1(PET)[b]	0	0	Giant PLT NO/dysplasia
51/9:44	1(ET)+1(VH)	1(VH)	0	0	0	NO
55/17:48	0	0	1(ET)	0	0	Giant PLT
56/11:44	0	0	0	1(ET+2(VH)	0	NO
67/12:16	1(ET)+1(VH)	0	0	0	0	Blasts?
68/9:02	1(PET)+1(VH)	1(ET)+1(VH)	0	0	0	Blasts?/dysplasia
71/11:31	1(ET)	1(ET)	1(ET)+1(VH)[c]	0	0	Giant PLT
Total ET+PET	6	6	2	1	2	
Total 1000VH	5	3	1	2	0	

ET extended timing, *PET* prolonged extended timing, *VH* prolonged counting up 1000 cells with Vision Hema, *ST* according to CLSI H20-A2 standard, *BL* blast, *nRBC* nucleated red blood cells, *MK* megakariocyte, *PL* plasma cells

[a] Hairy cells

[b] Megakaryocyte' naked nucleus

[c] Micro-megakaryocyte

[d] Not present in the instrumental count

many haematological malignancies [15] as well as in other non-haematological conditions [15–18], variable amounts of circulating nRBC can be observed. In many of these cases, the number of circulating nRBC are very low (<0.5/100WBC or $<0.1 \times 10^9$/L) just as in our experimental evaluation but their clinical significance—even potential—makes the confirmation of their presence mandatory, regardless of their amount [19].

Uni-, multi-linear cytopenia is a common clinical sign in lots of haematological disorders and may occur in any phase of the disease. When present, cytopenia can be associated with instrumental alarms which indicate the possible presence of PC or other significant morphological anomalies. Unfortunately, in these cases, the light microscope confirmation can be difficult and underpopulated cells could appear falsely absent by using the usual procedures of review.

Although the rules for microscopic review have been suggested by the International Council for Standardization on Hematology (so-called 41 rules) [2], the percentages of microscopic review in various laboratories are very different but in a general way lower in respect to those that could be obtained by using the "41rules" [20–24]. This depends on numerous variables such as the general population, the mixture of cases and the rules employed. The organizational conditions must also be considered such as increased intra- and inter-laboratory consolidation processes and reduced money resources [7]. Anyway, the quality of clinical information should not be questioned and it cannot be conditioned by economic and organizational needs.

Our research suggest that the timing of light microscopic observation should not be driven by standard procedures only but also by the reliability of the pre-test information associated together with "intuition" and professional competence. By the results obtained, we demonstrated that a prolonged timing ECD of LMR up to the confirm of the presence of suspected cells or more certainly of their absence increases the quantity of information that can be found in peripheral blood compared to other routine or standard procedures. So, although a statistical significance cannot be assigned to our results in the 79 cytopenic samples studied with ET or PET modes, these procedures showed a higher detection of PC in samples which had a putative major pre-test probability based on the presence of morphological flags or dysplasia (9 out of 13 positive samples for PC). However, it does not seem that an ET of LMR might be a professional or operative suggestion that can be adopted extensively in laboratories with high workflow and/or reduced resources.

In this scenario, a more extensive use of the technological resources could be a new opportunity to improve the clinical information provided by the laboratory. In recent years, the

availability of automated digital morphology and information systems to pre-classify circulating cells in peripheral blood changed some criteria for microscopic review for at least two reasons. The former is that the digitized microscopy systems can be the terminal step of the haematological workflow systems in which all peripheral blood smears are made, stained and read in real time along with the sample processing. The latter is that the automatic systems never get tired and they can withstand high workloads without of increasing professional resources because these can be dedicated to the usual tasks during the automatic scanning. Consequentially, less restrictive rules for microscopic review can be used to reduce the false negative incidence by increasing the percentage of slide review in respect to the total sample amount [3].

By using this strategy, we demonstrated that, in cases of samples with low amount of nRBC in PB, a decrease of the false negative from 39 (with 200LP) and 25% (with 400ST) to 0% can be achieved with an extended count up to 1000 cells by using an automatic system for cellular pre-classification and that an ET of up to 20 min is comparable. This last mode is proved more efficient in the underpopulated cells as demonstrated by the discovery of 17 PC respect to 11 PC with 1000VH (delta ratio equal to 1.54).

Conclusion

Both automated and manual systems by increasing the timing for microscopic review demonstrated that an improvement of the quality of diagnostic information in haematology is a reliable goal that can be achieved without additional resources (with 1000VH) or with a reasonable gain of these (with ET). This last option should be reserved to samples with higher pretest probability such as those with cytopenia or detected dysplasia, with or without associated morphological flags. Despite the clinical significance of underpopulated cells cannot always be established, their relevance particularly for the blasts cells was affirmed even when these are present in small number in peripheral blood [25]. So, for these samples, the total cost must be assessed mainly in relation to higher diagnostic benefit coming from information that otherwise would be lost with the use of normal and standard procedures.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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