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Evaluation of the detection of blasts by Sysmex hematology instruments, CellaVision DM96, and manual microscopy using flow cytometry as the confirmatory method

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Abstract

Introduction: The aim of the present study was to evaluate the diagnostic ability of blast flags generated by Sysmex instruments (XE/XN) by comparing with immunophenotyping by flow cytometry (IFCM). Additionally, the ability of manual microscopy and CellaVision DM96 (pre- and reclassification) to predict the presence of "true" blasts was investigated.

Methods: Blood samples (n = 240) with suspect pathology flags reported by the XE were collected from the daily workload and examined by the XN, by manual microscopy, by CellaVision DM96 and by IFCM (CytoDiff Panel).

Results: The ROC analysis for blasts showed an area under the curve of 0.64 ("Blasts?") (XE), 0.57 ("Blasts/Abn Lympho?") (XN), 0.75 (CellaVision preclassification procedure), 0.78 (CellaVision reclassification procedure), and 0.81 (manual microscopy). The sensitivity of blast detection varied between the methods from 0.41 (XE) to 0.90 (XN), and the specificity varied from 0.17 (XN) to 0.95 (CellaVision reclassification).

Conclusions: The CellaVision reclassification procedure has a diagnostic ability for predicting blasts close to that of manual microscopy. The blood smear methods show a notable number of false negative results. The Sysmex XN reported a higher rate of true positive blast flags than the XE. Taken together, the CytoDiff method could be a useful alternative to smear examination to correctly identify blasts.

KEYWORDS
blast flag, CellaVision, CytoDiff, flow cytometry, Sysmex hematology instruments

1 | INTRODUCTION

A complete blood cell count that includes a differential count of white blood cells (WBCs) is an important test to detect hematology abnormalities and to monitor patients' disease status. Most hematology analyzers identify samples with suspect abnormal cells and report the results by pathology flags. The diagnostic ability of a flag is a function of the underlying technology, and the computerized algorithms designed to evaluate the data. Concerning the presence of blasts, the Sysmex XE and XN analyzers report the flag "Blasts?" and "Blasts/Abn.Lympho?", respectively. On the XE analyzers, the flag "Blasts?" is derived by a cluster analysis of scatterplots from the differential (DIFF) and the immature myeloid information (IMI) channel. On the XN, the flag "Blasts/Abn Lympho?" is triggered based on information from the white blood cell differential channel (WDF channel). The flag "Blasts?" indicates the possible presence of myeloblasts, and the flag "Blasts/Abn Lympho?" indicates the possible presence of blasts not specified by lineage and/or abnormal lymphocytes.[1,2] Despite the continuous improvement of the hematology analyzers, the flags are subject to ambiguities that require further examination. Several

previous studies have evaluated the flagging performance of modern hematology instruments by comparing the data with the manual blood smear examination.[2-7] Microscopic counting and characterization of blood cells have been considered as the reference method despite the fact that the method is cumbersome, time-consuming, and also affected by inter-observer differences. In order to improve the efficiency of cell characterizations, the use of digital microscopy systems is increasing. CellaVision DM96 is an automated pattern recognition system where the morphology of the cells in the sample is classified by the use of an artificial network and a predefined database (preclassification). The preclassified cells are presented on a screen and thereafter reviewed by a competent technologist who verifies the correctly characterized cells and assigns the misclassified cells to the appropriate category using a drag and drop functionality (reclassification).[8] An alternative method to the blood smear examination is immunophenotyping by flow cytometry (IFCM). The use of specific monoclonal antibodies against various epitopes on the cell surface, in addition to the large number of cells that are counted, reduce the imprecision, and improve the ability to correctly characterize cells that are rare or difficult to characterize morphologically.[9] However, there are not specific recommendations on how to perform a WBC differential count by IFCM.[10] The CytoDiff IFCM method from Beckman Coulter uses a 5 colors/6 antibody reagent cocktail, and an auto-gating algorithm for differential counts of WBCs. Eighteen different leukocyte subsets, that include blasts and immature granulocytes (IGs), are visualized and reported.[11] The use of a premixed CytoDiff reagent and an auto-gating procedure simplifies the analysis and makes the method useful in regular routine laboratories.[12-14] The aim of the present work was to investigate the diagnostic ability and usefulness of the flag "Blasts?" reported by the Sysmex XE-5000, the flag "Blasts/Abn Lympho?" reported by the XN-9000, suspect blasts identified by manual microscopy, and CellaVision DM96 (pre- and reclassification procedure) by using the CytoDiff method (IFCM) as the confirmatory method for the detection of blasts.

2 | MATERIALS AND METHODS

A total of 240 K_2EDTA- anticoagulated blood samples (Greiner Bio-One, Frickenhausen, Germany) flagged by the Sysmex XE-5000 (software version 00-14) were randomly selected from the routine workload. The selected flags included "Blasts?", "ImmGran?", "AbnLy/L-blast?", and/or "Atypical Lymph?". The background for including samples with these flags was to have a representative sample of samples that needed a confirmation by smear review. The thresholds for trigging flags were factory settings (ie, Q value 100).[6,15] The results of the WBC counts performed on the XE varied from leukopenia to severe leukocytosis. The median leukocyte count was 10.2 × 10^9/L (ranged from 0.3-252.4 × 10^9/L)

Two blood smears were immediately prepared from each sample and stained with May-Grünwald Giemsa using a Sysmex SP-1000 automated stainer. The evaluation of the smears was performed on both CellaVision DM96 (pre- and reclassification procedure) and by manual microscopy. The Norwegian Accreditation board (NA) has granted accreditation for the analyses according to the NS-EN ISO15189 standard.

Using manual microscopy, pair of smears was counted by two highly trained technologists each counting 100 WBCs per slide (ie, 2 × 100 cells). The reviews included a WBC differential count and a morphological examination of WBC abnormalities.

The same smears were examined by the preclassification feature of CellaVision DM96, reviewing 120 WBCs per slide. By processing one slide each, two highly trained technologists reviewed the images of the preclassified cells to verify the preclassification or reclassify incorrectly classified cells.

For the smear analyses, the percentage of blasts was calculated as the average percentage of pairs. To define a sample as blast-containing or not, blast counts ≥0.5% were used as a criterion for a positive result. The criterion was used in order to compare the results with prior studies from our group.[6,15]

Within 6 hours after blood collection, the samples were analyzed on a Sysmex XN-9000 (XN) using the WDF channel and the software version 00-18, and by IFCM using the premixed CytoDiff Panel and analysis software CytoDiff CXP version 2.0 from Beckman Coulter.[13,16] The XN-9000 analyzer was not equipped with the extended white precursor cell channel (WPC channel).[2]

For IFCM, cells were stained using FITC conjugated anti-CD36, PE-conjugated anti-CD2 and anti-CD294 (CRTH2), ECD-conjugated anti-CD19, PC5-conjugated anti-CD16 and PC7-conjugated anti-CD45 monoclonal antibodies in accordance with the manufacturer's instruction before analysis on a FC500 flow cytometer. After installing CytoDiff CXP, auto-setup was performed periodically during the study to ensure the correct setup and compensation values. Adequate instrument performance was ensured prior to the running of samples by using Flow-Check, Flow-Set, and PC7 Setup Kit reagents (all Beckman Coulter). The CytoDiff Panel has been CE-IVD approved in the European market for the following hematological parameters: B-lymphocytes, total T and NK-lymphocytes, total lymphocytes, total monocytes, immature granulocytes (IGs), eosinophils, mature neutrophils, basophils, and total blasts, where the total number of blasts is the sum of the Xb (B-lymphoblast), Xt (T-lymphoblast), Xn (myeloblast) and Xm (monoblast) subpopulations. To date, the CytoDiff Panel has not been submitted to FDA for approval.[11,17] Cell population counts were collected directly based on auto-gating without evaluation of populations or adjustment of gates by the operator.

The present study is a method comparison for detection of blast cells in peripheral blood using the CytoDiff method as the confirmatory method, and the result from manual microscopy, CellaVision (pre- and reclassification procedure), and two different Sysmex instruments as the variable to analyze. The demographic data, the clinical background, or the results from previous blood count analyses were not possible to retrieve and were therefore not included in this study. Because the CellaVision DM96 and the manual microscopy do not specify blasts by linage, the total blasts from the IFCM measurements were used in the comparison between the methods.

TABLE 1 The classification of 240 patient samples into blast-containing (Positive (Pos)) or not (Negative (Neg)) by comparing IFCM (confirmatory method, cutoff ≥0,5% blasts) with the CellaVision preclassification and reclassification methods, manual microscopy, the Sysmex XE flag "Blasts?", and the Sysmex XN flag "Blasts/Abn Lympho?"

	Preclassification		Reclassification		Microscopy		Sysmex XE		Sysmex XN[a]		
	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Total
IFCM Positive	122	42	87	77	109	55	67	97	146	16	164
IFCM Negative	31	45	4	72	6	70	14	62	62	13	76
Total	153	87	91	149	115	125	81	159	208	29	240

[a]Three samples disappeared in the diagnostic process.

For the IFCM, as for microscopy, a total blast count of 0.5% or more was defined as positive, whereas samples with fewer blasts were regarded as negative. Blood smears and instrument-generated flags were considered false positive if blast cells were not identified by the IFCM method and false negative if blast cells were recognized by the IFCM method. The samples were collected from November 2016 to June 2017 and analyzed at Akershus University Hospital, Lørenskog, Norway with the exception of the Sysmex XN-9000 analyses, which as a part of the study design were performed at Fürst Medisinsk Laboratorium, Oslo, Norway.

2.1 | Statistical analysis

Statistical analyses were performed using the SPSS software version 16 (SPSS, Chicago, IL) for Windows (Microsoft, Redmond, WA) and MedCalc Statistical Software version 17 (MedCalc, Ostend, Belgium). To describe the data, the cell counts were summarized as median and range. Bland-Altman analysis was used to assess agreement between the IFCM blast counts (%) and the blast counts (%) provided by the CellaVision and manual microscopy, respectively.[18] To determine the inter-method agreement for the classification of the sample as positive or negative for blasts, κ values were evaluated. The diagnostic ability of a method to correctly characterize samples as blast-containing or not was evaluated by receiver operating characteristic (ROC) curves analyses where the IFCM method was the confirmatory method. Sensitivity, specificity, predictive values, and likelihood ratios were calculated by the Galen and Gambino method.[19]

3 | RESULTS

When analyzed with the IFCM method, the blast counts ranged from 0.0% to 90.0%. The mean and the median counts were 7.2% and 1.1%, respectively. The mean difference (bias) between the smear methods and the IFCM: was −2.9 (95% CI: −4.3 to −1.6) for the manual blast counts, −3.4 (95% CI: −4.8 to −2.1) for the reclassified counts, and −2.4 (95% CI: −3.8 to −1.0) for the preclassified counts, and the IFCM reporting higher values on average than the smear methods. The cross tabulation on the categorical data is shown in Table 1. The IFCM method identified blasts (0.5% or more blasts) in 164 of the 240 samples (68%). Of these 164 samples, the manual microscopy method identified 109 as true positive (66%) and 55 samples as false negative (34%). In 41 of the 55 false negative samples, the IFCM reported a blast count below 2%. Thirty-five of these samples displayed irregularities when inspecting the IFCM dot plot. The irregularities were in some cases related to weak CD19 expression, and as a consequence CD19- and CD19+ B-cells were not separated. This had a direct effect on the identification and counting of subpopulations in the subsequent plots, including the blast subpopulations. A more prominent issue was the lack of the characteristic Side Scatterlow CD45low blast population, or that the population appeared diffuse in the Side Scatter CD45 dot plot, where the population resembled unspecific staining more than an actual population. Thus, the IFCM blast counts for these samples are questionable and would require further examination. The remaining six samples revealed no such issues making the blast count more trustworthy. Noteworthy, the IFCM blast counts for these samples were all above 1%. For the remaining 14 negative samples, the following was observed: two samples showed the presence of blasts when scanning the entire smear, four samples had reviewer comments on abnormal lymphocytes, two samples had comments on plasma cell-like cells, two samples had comments on damaged cells, and four samples had no comments, but two of them were leukopenic (WBC $<0.8 \times 10^9$/L).

The preclassification feature identified 122 samples as true positive (74%) and 42 samples as false negative (26%). Thus, the preclassification feature identified more true positive blasts and less false negative blasts than the manual microscopy method. On the other hand, the reclassification feature of the CellaVision reported fewer true positive samples (87 out of 164 IFCM-identified true blasts) (53%) and more false negative samples (77 out of 164 IFCM-identified true blasts) (47%) than the preclassification procedure and the manual microscopy method.

The Sysmex XN reported more true positive blast flag (90%) than the XE (41%). Consequently, the proportion of samples with false negative flags was higher for the XE (59%) than for the XN (10%).

False positive blasts were detected in 6 (out of 76 negative) samples (8%) by manual microscopy, 31 samples (40%) by the preclassification method, and in 4 samples (5%) by the reclassification method, 14 samples (18%) by the XE, and 62 samples (82%) by the XN (Table 1). Table 2 shows the kappa values for the agreement on the classification into blast-containing (positive) or not blast-containing (negative) samples comparing the test method with the

TABLE 2 Inter-method agreement for the classification of 240 patient samples into blast-containing (positive) or not (negative)

Method	κ	95% CI
IFCM versus Preclassification	0.323	0.195 to 0.451
IFCM versus Reclassification	0.380	0.286 to 0.474
IFCM versus Microscopy	0.499	0.397 to 0.601
IFCM versus Sysmex XE	0.173	0.077 to 0.269
IFCM versus Sysmex XN	0.089	-0.029 to 0.207

CI, confidence interval

IFCM method. The highest agreement was found between the IFCM method and the manual microscopy method (κ = 0.499), while the agreement between the IFCM method and the "Blasts/Abn Lympho?" flag generated by the Sysmex XN was found to be no better than chance (κ = 0.089).

The diagnostic ability of different methods to correctly identify samples as blast-containing or not blast-containing are shown by ROC curves in Figure 1. The area under the curves (AUCs) varied from 0.57 (the Sysmex XN "Blasts/Abn Lympho?" flag) to 0.81 (manual microscopy) using an IFCM finding of the presence of ≥0.5% blasts as the discriminant between positive and negative samples.

The sensitivity for detection of blasts varied between methods from 0.41 to 0.90 (Table 3). Correspondingly, the specificity varied from 0.17 to 0.95. The highest sensitivity and the very lowest specificity were obtained by the XN flagging procedure. It is interesting to note that the AUC for the Sysmex XN "Blasts/Abn Lympho?" flag did not differ significantly from 0.5, suggesting that this flag is not able to separate samples without blasts from samples where blasts are present.

4 | DISCUSSION

It is well known that an examination of a test's diagnostic ability depends on a well-defined definition of positive versus negative samples and the existence of an unambiguous diagnostic reference method. Microscopic examination of blood smears is still acknowledged as the current gold standard for performance evaluation of hematology analyzers.[20] Visual examination of the morphology of blood cells is important in situations where the hematology analyzer has reported a flag. Incorrect identification of cells, uneven distribution of cells on the smear, and the inherent statistical variability are well-known sources of error related to manual microscopy.[21] Errors associated with incorrect identification can be improved by training, and the uneven cell distribution may be reduced by the use of an automatic slide maker device.[22] However, the inherent variability of cell classification is due to the relatively low number of cells that are counted in total and it become increasingly problematic when the fraction of the cell type in question is low.[23,24] To address these concerns, enumeration of blasts by IFCM, which counts thousands of cells in each sample and the use of fluorescent labeled monoclonal antibodies for cell identification, has been highly recommended.[12,10] To our knowledge, the CytoDiff Panel together with the CPX auto-gating software is the only commercially available system for WBC differential count by IFCM. A simple "no-wash and lyse protocol" and differential count of WBC subpopulations from a single tube, using a 6 marker/5 color antibody cocktail, makes it an attractive approach to follow up flags by hematological analyzers in the clinical laboratory. It may also provide additional information compared to manual microscopy as it has been shown to classify cases of B-ALL to the Xb region.[25] However, because of the relative low number of monoclonal antibodies used, the system may separate subpopulations insufficiently

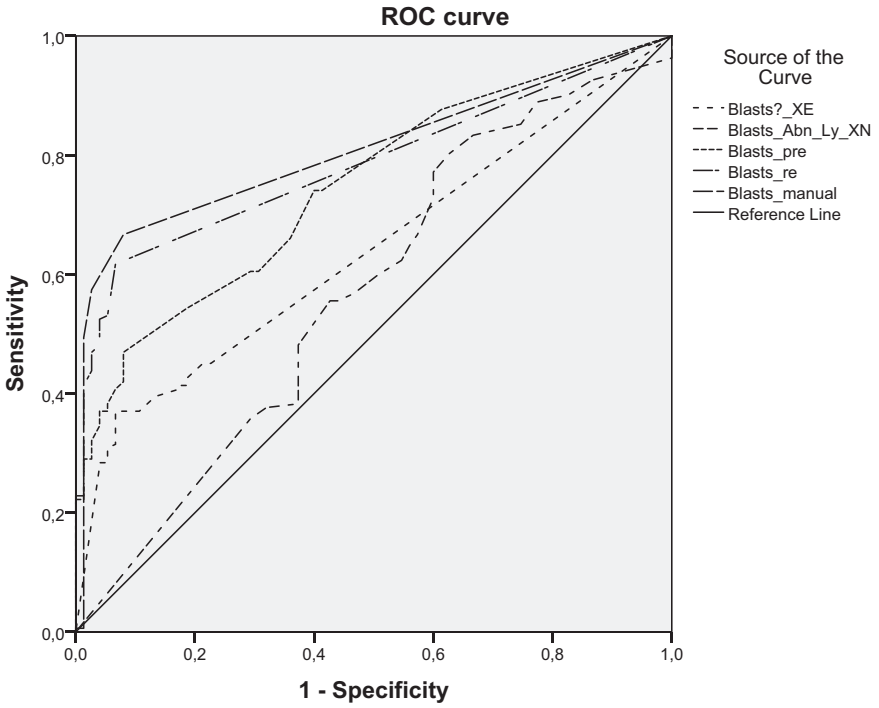

FIGURE 1 Receiver operator characteristic (ROC) curves for the overall performance of the blast flag on XE-5000 and XN-9000, the preclassified and reclassified blasts on CellaVision, and the blasts on manual microscopy, to predict the presence of blasts in blood samples. The area under the curves (AUCs) are presented in Table 3. The presence of blasts (total) ≥ 0.5% by IFCM was defined as a true positive finding

TABLE 3 Sensitivity, specificity, and area under the curves (AUCs) of the CellaVision preclassification and reclassification methods, the manual microscopy, the "Blast?" flag from XE, and the "Blast/Abn Lympho? flag from XN with reference to blast detection by IFCM (cutoff at ≥0,5% blasts)

	Preclassification	Reclassification	Manual microscopy	Blasts? XE	Blasts_Abn_Ly XN
Sensitivity (95% CI)	0.74 (0.67-0.81)	0.53 (0.45-0.61)	0.66 (0.59-0.74)	0.41 (0.33-0.49)	0.90 (0.84-0.94)
Specificity (95% CI)	0.59 (0.47-0.70)	0.95 (0.87-0.99)	0.92 (0.84-0.97)	0.82 (0.71-0.90)	0.17 (0.10-0.28)
AUC (95% CI)	0.75 (0.69-0.81)	0.78 (0.73-0.84)	0.81 (0.75-0.86)	0.64 (0.57-0.71)	0.57 (0.49-0.65)

or misclassify cells.[13,14,25,26] Accordingly, the International council for Standardization in Hematology (ICSH) has concluded that establishing an IFCM differential counting based on 5 or 6 colors as a reference method for differential counting is suboptimal. To meet the requirements, set forth by the ICSH, a novel method using 8 to 10 colors is being evaluated.[9,10]

However, it has been demonstrated that detection and counting of circulating blasts by IFCM with the "5 color single tube" mixture gave reliable results with good specificity and sensitivity when compared to smear analysis.[16,27] In this work, we have therefore used the CytoDiff method as the confirmatory method for determination of blasts.

4.1 | Evaluation of blast flag reports on Sysmex XE and XN instruments

We have in a previous study questioned whether the blast flags reported by automated hematology instruments are sufficient important criteria for performing a confirmative microscopic review. The study used microscopic reviews as the gold standard and concluded that the ability of the Sysmex XE analyzer to predict the presence of blasts was low.[6]

In the present study, this conclusion was confirmed and even strengthened, shown by a clearly lower sensitivity of the flag "Blasts?" (0.4 (present study) versus 0.7 (previous study)).[6] Comparing the diagnostic ability of Sysmex XE and Sysmex XN to flag samples with true circulating blasts (determined by IFCM), the XN analyzer provided less false negative samples, and the sensitivity increased from 0.41 (XE) to 0.90 (XN). The cost of the increased sensitivity was a lower specificity. The specificity decreased from 0.82 (XE) to 0.17 (XN) indicating a marked increase in false positive flagging of blasts in samples and thereby a subsequent increase in microscopic reviews that likely will result in a lower laboratory efficiency with increased turnaround time. However, the "Blasts?" flag generated by the XE identifies peripheral blasts, whereas the "Blasts/Abn Lympho?" flag generated by the XN identifies "blasts" and "atypical lymphocytes, suspect neoplastic".[28] Consequently, it is difficult to directly compare the "Blasts?" flag obtained by XE with the "Blasts/Abn Lympho?" flag obtained with XN. Considering the overall diagnostic ability of the XN, the AUCs were found to be closely to what would be expected by chance, indicating that the flag in fact is unable to separate between samples with or without blasts. Similarly, the agreement between the IFCM and XN on the categorization of the samples as blast-containing or not was equivalent to chance. This might be explained by the parallel identification of blast cells and atypical lymphocytes, suspect neoplastic. Only samples flagged by the Sysmex XE were selected for inclusion in the study, and the results may, therefore, to some extent reflect biases in the sampling procedure.

4.2 | Evaluation of blast detection by manual microscopy in comparison with flow cytometry

In this study, we evaluated the diagnostic ability of manual microscopy to predict the presence of blasts using IFCM as the confirmatory method. Even though microscopic examination has acceptable AUC (0.81), the agreement between the two methods on classification of blasts was weak (κ = 0.499), implying considerable disagreement concerning the presence of blasts. The high specificity (0.92) and moderate sensitivity (0.66) of the manual microscopy method means that a substantial proportion of the samples are classified as false negative. In a substantial number of the false negative samples, the IFCM reported blasts counts below 2%. Accordingly, the apparent lack of sensitivity can be explained by the absence of detection of blasts due to the well-known limitations of manual microscopy associated with the inherent statistical variability.[16] In addition, the unspecific staining described in the Results section may also contribute to the number of false negative samples using microscopy.

The negative bias was -2.9% between the manual counts and the IFCM counts. With this bias, the low blast counts are expected to be more affected than the high blast counts, which may partly explain the low agreement between the methods on the bivariate classification of samples as blast-containing or not. These results are in accordance with Kahng et al who previously have shown that the CytoDiff IFCM method detects blasts in a higher number of samples than the manual microscopy method.[12] The relatively high AUC indicates that manual microscopy has a reasonable ability to distinguish between positive and negative samples determined by IFCM. The low sensitivity, however, reduces the usefulness of the test in diagnosis and treatment of hematological diseases.

4.3 | Evaluation of blast detection on CellaVision DM96

It has been shown that the performance of the WBC differential count by digital microscopes is similar to the manual microscopy.[29,30] We have in a recent study concluded that the preclassification feature alone may be sufficient to verify the absence of blasts in the sample. When blasts are detected by the preclassification procedure, the finding has to be verified by a reclassification procedure.[15] This conclusion was, however, based on a study using manual microscopy as the gold standard, a method that is affected by a number of confounding factors, as described above. The present study using IFCM as the confirmatory method does, however, confirm our previous findings, that is, that the sensitivity of detecting blasts is somewhat higher in the preclassification procedure compared to the reclassification procedure, while the specificity shows the opposite pattern. The preclassification procedure reports a higher number of samples as true positive compared to the reclassification procedure, which suggests that the technologists may have performed an incorrect classification of cells likely to be true blasts. Considering the low specificity, we assume that suboptimal learning algorithms can play a role for the potential overestimation of blasts, indicating that the finding has to be verified preferentially by reclassification or even better by IFCM. The ROC curve analysis showed that the manual microscopy method, and the reclassification procedure were similar with AUCs of 0.81 and 0.78, respectively; while the AUC for the preclassification procedure was slightly lower (0.75). Based on these AUC values, we conclude that the ability to discriminate samples with blasts from samples without blasts is almost identical for manual microscopy and the pre-and reclassification procedure.

In previous studies evaluating the performance of the CytoDiff method, the results were by definition always confirmed by visual inspection of the plots.[25,27] Furthermore, improved efficiency in laboratory validation practices has been demonstrated by applying rules and flagging systems as a supplement to the data generated by the auto-gating software.[14,26] In this study, the samples were selected on the basis of flags generated by the XE-5000, the cutoff value used for the presence of blasts was 0.5%, that is, a blast count that may be present in normal subjects, and the gating strategy was based solely on the auto-gating software. Important factors influencing the diagnostic performance of tests include technical variations as criteria for interpretation of positive samples, selection bias, and gating strategies. This must be taken into account when the results of this study are interpreted.

However, in conclusion, the CellaVision reclassification procedure has a diagnostic ability for predicting blasts close to that of manual microscopy. The blood smear methods show a notable number of false negative results. Compared with IFCM, the Sysmex XN reported a higher rate of true positive blast flags than the XE. Taken advantage of the automatic detection of blasts, the CytoDiff method could be a useful alternative to smear examination to correctly characterize a sample as blast-containing or not.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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