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Automated measurement of the erythrocyte sedimentation rate: method validation and comparison

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Abstract

Background: Development of automated analyzers for erythrocyte sedimentation rate (ESR) has imposed the need for extensive validation prior to their implementation in routine practice, to ensure comparability with the reference Westergren method. The aim of our study was to perform the analytical validation of two automated ESR analyzers, the Ves-Matic Cube 200 and the TEST1.

Methods: Validation was performed according to the recent International Council for Standardization in Hematology recommendations and included determination of intrarun and inter-run precision, assessment of sample carryover, hemolysis interference, sensitivity to fibrinogen, method comparison with the gold standard Westergren method and stability test.

Results: The highest intrarun imprecision was obtained for the low ESR range (33.5% for Ves-Matic Cube; 37.3% for TEST1) while inter-run coefficients of variation on three levels were much better for the TEST1 (0%, 2% and 1.2%) compared to the Ves-Matic Cube 200 on two levels (24.9% and 5.8%). Both Ves-Matic Cube 200 and TEST1 showed no statistically significant difference when compared with Westergren. Bland-Altman analysis yielded overall insignificant mean biases for all comparisons, but a wider dispersion of results and 95% limits of agreement for comparisons including the Ves-Matic Cube 200. Carryover was considered insignificant, while hemolysis had a negative effect on all assessed ESR methods. The highest sensitivity to fibrinogen was observed for the Ves-Matic Cube 200, followed by Westergren and the least sensitive was the TEST1.

Conclusions: The obtained results proved the analytical validity of the TEST1 and the Ves-Matic Cube 200, and high comparability with the gold standard Westergren method, showing obvious improvements in standardization of ESR methods.

Keywords: erythrocyte sedimentation rate; standardization; TEST1; validation; Ves-Matic Cube 200; Westergren method.

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Introduction

The erythrocyte sedimentation rate (ESR) is a traditional laboratory test used for assessment of inflammation that dates back to the early 1900s [1–3]. Its usefulness is nowadays being largely debated, mainly because ESR lacks analyte and disease specificity and is affected by a variety of physiological and pathophysiological conditions [4–6]. Nevertheless, it is still considered a valuable laboratory test in selected clinical conditions. Specifically, ESR is incorporated in the diagnostic criteria for rheumatoid diseases (rheumatoid arthritis, polymyalgia rheumatica and giant cell arteritis) [7–9], as a screening test for orthopedic infections [10] and presents prognostic significance in Hodgkin's lymphoma [11].

The original ESR method proposed by Westergren in 1921 is performed in diluted whole blood samples and remains the gold standard, as defined in the International Council for Standardization in Hematology (ICSH) review from 2011 [12]. The recent classification of ESR methods proposed by the ICSH defines, beside the gold standard, two further ESR method categories: modified Westergren methods that are based on the Westergren methodology with acceptable modifications including shorter assay time, use of undiluted blood or different diluents, and alternate methods that incorporate novel technologies for ESR measurement [13]. The introduction of these new methodologies on semi-automated and automated analyzers started about two decades ago and opened a new era of ESR analysis [3, 13–15]. The many advantages of these automated systems include the use of ethylenediaminetetraacetic acid (EDTA) undiluted blood that increases

sample stability and allows using a single sample for both ESR and other hematologic tests, thus enhancing patient safety, workflow optimization, increasing personnel safety by using closed systems and shortening turnaround time [13, 14, 16]. In the paradoxal era of increased workloads with the continuous need for reducing costs while enhancing patient and employee safety, automation of ESR measurement was considered attractive and soon a lot of laboratories transitioned to the use of these novel methods [13]. Clearly, method standardization and comparability of ESR results emerged as a problem. The continuous development of modified and alternate methods for the measurement of ESR and their ongoing implementation in laboratory routine imposed the need for universal recommendations that will serve as a framework for laboratories prior to the introduction of ESR methods in routine practice. The ICSH and the Clinical Laboratory Standards Institute (CLSI) documents from 2010 and 2011 define that all new analyzers and/or methodologies should be evaluated against the reference Westergren method and only systems providing results as the Westergren method with diluted blood in 60 min or normalized to 60 min can be used in clinical practice [12, 17]. In 2017, a group of experts on behalf of the ICSH, published recommendations for establishing a unique validation protocol for ESR methods and prerequisites for quality control and results reporting, in order to ensure comparability with the reference method [13].

The aim of this study was to perform the analytical validation of two automated ESR analyzers, the Ves-Matic Cube 200 (Diesse Diagnostica Senese Spa, Siena, Italy) and the TEST1 (Alifax Spa, Padua, Italy). The ICSH recommendations were followed for method validation and the results were compared with the gold standard Westergren method.

Materials and methods

Validation protocol

Validation of two automated ESR analyzers, the Ves-Matic Cube 200 and the TEST1 was performed at the Department of Laboratory Medicine, University-Hospital of Padova according to the ICSH recommendations [13] and included precision study (intrarun and interrun), method comparison, hemolysis interference study, sensitivity to fibrinogen, carryover and sample stability assessment.

Patient samples

All patient samples used in the study were leftovers selected from daily routine samples, including both hospitalized and ambulatory patients from the University-Hospital of Padova. They were collected in 3.0 mL tripotassium EDTA (K_3-EDTA) vacutainers (Becton Dickinson, UK), processed according to manufacturers' specifications and analyzed within 4 h from venipuncture. The study was performed following the requirements of the Declaration of Helsinki.

Description of the evaluated analyzers

The Ves-Matic Cube 200 is a closed automated ESR analyzer that performs analysis from primary EDTA tubes. The method is based on a modified Westergren sedimentation technique. ESR is determined by optical recording of the difference of the red blood cell (RBC) column height before and after sedimentation in a determined period of time, with the first result obtained after 20 min and the following ones every 18 s. Results are corrected for variations of temperature and the obtained results are converted to Westergren values [15, 18, 19].

The TEST1 is an automated ESR analyzer that equally uses EDTA anticoagulated blood whose measurement principle is based on the capillary photometric-kinetic technology. Specifically, 175 µL of anticoagulated blood is aspirated in a capillary tube and centrifuged, causing RBC sedimentation which is eventually measured photometrically. The obtained result is extrapolated to be comparable with the conventional Westergren method. The TEST1 analyzer actually calculates a mathematically derived ESR based on the Rouleaux formation. The first ESR results are available within 5 min from analysis start, with the release of subsequent results every 20 s [13, 20, 21]. The new generation of the TEST1 analyzer differs from the previous one inasmuch it has a new aggregation sensor that is declared to enhance measurement precision as well as an automatic washing cycle after three consecutive analysis errors and a predefined timeout period, preventing capillary clogging and minimizing the need for manual maintenance.

The Westergren method

The manual ESR measurement using the Westergren method was performed according to the ICSH's recommendations. The samples were analyzed within 4 h from blood sampling. The EDTA-anticoagulated blood was thoroughly mixed by complete inversion of the tube 20 times and diluted 4:1 using a trisodium citrate dihydrate solution (3.8%). The diluted sample was mixed by inversion of the tube 20 times to obtain homogenosity and was immediately mechanically aspirated to a colorless, circular glass Westergren tube with an inner diameter of 2.55 mm. The Westergren tube was allowed to stand for 60 min in an appropriate supporting rack in a stable, vertical position in a fume hood, at constant temperature (18–25 °C) and free from external influences such as vibrations, heat and direct sunlight. ESR was read by visual determination after 60 min as the distance from the top of the plasma level to the top of the RBC layer and recorded in mm. All Westergren analyses were performed by a single analyst to minimize pipetting and reading variations.

Method comparison

Method comparison was performed as a consecutive study and included 245 patient samples (149 male and 96 female; median age 64,

ranging from 0 to 96 years) with ESR values spanning the whole analytical range of the respective analyzers (from 2 to 120 mm), accommodating the ICSH recommendation's requirement that each third of the analytical range, i.e. the low (<40 mm), middle (40–80 mm) and high (>80 mm) third, was covered by at least 20 samples. All samples were analyzed on the Ves-Matic Cube 200, the TEST1 analyzer and the Westergren method within 4 h from venipuncture.

Precision study

Intrarun precision was estimated using five patient samples with ESR values covering the low (<40 mm), middle (40–80 mm) and high (>80 mm) third of the analytical range. All samples were analyzed 10 times on the Ves-Matic Cube 200 and 5 times using the TEST1 analyzer. As the TEST1 consumes a definite amount of sample for every analysis, intrarun precision was performed in five consecutive measurements. Inter-run precision was performed using commercial quality control (QC) samples in the normal and abnormal range; specifically, on the Ves-Matic Cube 200 using commercially available control ESR Control Cubes (Diesse Diagnostica Senese SpA, Siena, Italy) composed of stabilized human blood on two levels while for the TEST1 commercial latex control samples (Alifax Spa, Padua, Italy) it was on three levels. QC samples were analyzed three times a day for 5 consecutive days.

Hemolysis interference

Hemolysis interference was assessed in 20 randomly selected routine patient samples covering the whole ESR range. Two aliquotes of 3 mL of whole blood for each patient were prepared. Hemolysis was induced *in vitro* by addition of 25 µL of lysing solution composed of 1.3% glycerol (Osmored, Eurospital Spa, Trieste, Italy) in one aliquot, while 25 µL of 0.9% saline was added to the other aliquot to eliminate the dilution effect. Samples were analyzed on all assessed analyzers and the Westergren method. The hemolysis index (HI) was determined on a Cobas 8000 modular analyzer series (Roche Diagnostics, Mannheim, Germany). The analyzer dilutes an aliquot of patient plasma with 0.9% saline and the HI is calculated from the absorbance measurement at 600/570 nm wavelengths, with corrections made for absorption due to lipemia in the sample. HI corresponds to the concentration of free hemoglobin expressed in g/L.

To assess the hemolysis effect on ESR determination, bias was calculated using the following formula:

$$\text{Bias (\%)} = \left(\frac{\text{ESR in hemolyzed sample} - \text{ESR in nonhemolyzed sample}}{\text{ESR in nonhemolyzed sample}}\right) \times 100$$

Sensitivity to fibrinogen

A total of four healthy volunteers (three females and one male) recruited from laboratory staff participated in the study. Concentrated fibrinogen solution (expected concentration 20 g/L) was made by dissolving lyophilized human fibrinogen (Sigma-Aldrich, St Louis, MO, USA) in warm (37 °C) 0.9% saline and sterile-filtering using a 0.22 µm filter (Merck Millipore Ltd., Carrigtwohill, Co. Cork, Ireland), as recommended by the manufacturer. Fibrinogen concentration in the stock solution was measured using the automated Clauss method on a coagulation analyzer Sysmex CS-5100 (Siemens Healthineers, Erlangen, Germany). Aliquots of 2 mL of normal blood were prepared, and 400 µL of saline alone, saline spiked with stock fibrinogen (in ratios 3:1, 1:1 and 1:3) or original stock solution was added to each aliquot. ESR was determined in the prepared mixtures on analyzers subject to validation (Ves-Matic Cube 200 and TEST1) as well as using the Westergren method.

Carryover

Carryover assessment was conducted according to the CLSI H26-A2 protocol [22], i.e. a sample with a high target value (HTV) was analyzed in triplicate, followed by three measurements of a sample with a low target value (LTV). Carryover was calculated using the following formula:

$$\text{Carryover (\%)} = \left(\frac{\text{LTV1} - \text{LTV3}}{\text{HTV3} - \text{LTV3}}\right) \times 100$$

Carryover was assessed only for the TEST1 analyzer because it aspirates a definite amount of blood in a single capillary that is used for the analysis of all samples, with a washing cycle between each analysis. This measurement principle is, however, prone to sample carryover if washing is not complete. The Ves-Matic Cube 200, on the other hand, allows the samples to settle and simply measures the settling distance, without any sample consumption, thus making sample contamination impossible.

Sample stability

Stability was tested for 20 randomly chosen patients that had two EDTA tubes drawn for routine laboratory diagnostics. ESR was determined in fresh samples, as well as 4, 8 and 24 h after sample collection. One paired sample was stored at room temperature (RT) while the other was stored at 4 °C, and ESR was measured on the Ves-Matic Cube 200 and TEST1 analyzers at all the assessed timepoints. Samples stored at 4 °C were allowed to return to RT before retesting. The Westergren method was performed only in fresh samples and after 24 h storage, because of scarce sample volume.

Statistical analysis

For precision study, mean values, standard deviations (SD) and coefficients of variation (CVs) were calculated. The D'Agostino-Pearson test was used to assess data distribution normality. Spearman's rank correlation coefficient (ρ) was calculated to assess the strength and direction of the association between the compared data. A paired sample t-test and Wilcoxon signed-rank test were used for comparison of groups, $p < 0.05$ was considered statistically significant. Linear regression was carried out using Passing-Bablok regression. Differences between methods were evaluated by calculating bias and

limits of agreement using Bland-Altman analysis. Statistical analysis was performed with the MedCalc statistical software, version 14.12.0 (MedCalc, Ostend, Belgium).

Results

Precision study

The results of intrarun and inter-run precision study are presented in Tables 1 and 2 as mean ± SD, and CV (%), respectively.

Method comparison

A total of 245 patient samples were analyzed from original EDTA vacutainers on two automated analyzers (Ves-Matic Cube 200 and TEST1) and in diluted EDTA blood using the gold standard Westergren method. Mean ESR values were 28 mm (95% CI: 24–32 mm) for both the Ves-Matic Cube 200 and the TEST1 analyzers while for the Westergren method a mean of 27 mm (95% CI: 23–31 mm) was obtained. The ESR results obtained by the Ves-Matic Cube 200 and the TEST1 analyzers in comparison to the Westergren method showed no statistically significant difference ($p = 0.218$ and $p = 0.165$, respectively). High correlation was demonstrated for the comparison of the TEST1 analyzer with the reference Westergren method ($\rho = 0.99$, $p < 0.001$) while comparison of the Ves-Matic Cube 200 analyzer yielded lower and almost equal coefficients of correlation; i.e. 0.82 for the comparison with Westergren, and 0.81 with TEST1 analyzer, with $p < 0.001$ for both comparisons.

Regression analysis revealed a minor constant and proportional difference between the ESR results obtained with the TEST1 analyzer and the Westergren method with a regression equation $y = -0.28 + 1.04x$ (Figure 1A) and the mean bias of –1.1 (95% CI: –1.8 to –0.4) obtained by Bland-Altman analysis, with the value of 9.8 for the upper limit and –11.9 for the lower limit (Figure 1B). Neither a constant nor a proportional difference was obtained for the comparison between the Ves-Matic Cube 200 and the reference Westergren method, yielding a regression equation $y = 1.42 + 0.92x$ (Figure 1C), and a mean bias of 0.4 (95% CI: –2.2 to 2.9). The upper and the lower limits of bias were 39.9 and –39.1, respectively (Figure 1D). Constant and proportional difference was evidenced for the comparison of the Ves-Matic Cube 200 with the TEST1, yielding regression equation $y = 1.86 + 0.89x$ (Figure 1E). The mean bias was 1.4 (95% CI: –1.4 to 4.3) with a value of 45.2 for the upper and –42.3 for the lower limit (Figure 1F). The obtained biases for all evaluated comparisons were not significant.

The results were further divided into subgroups of low (<40 mm), medium (40–80 mm) and high (>80 mm) ESR values obtained with Westergren. Passing-Bablok regression analysis, Bland-Altman analysis and Spearman's rank correlation coefficient results are presented in Table 3.

Table 1: Intrarun precision of the Ves-Matic Cube 200 and TEST1 analyzers.

	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5	
	Ves-Matic Cube 200 (n = 10)	TEST1 (n = 5)	Ves-Matic Cube 200 (n = 10)	TEST1 (n = 5)	Ves-Matic Cube 200 (n = 10)	TEST1 (n = 5)	Ves-Matic Cube 200 (n = 10)	TEST1 (n = 5)	Ves-Matic Cube 200 (n = 10)	TEST1 (n = 5)
Mean ± SD	5 ± 2	2 ± 1	19 ± 2	19 ± 1	26 ± 4	50 ± 9	86 ± 4	58 ± 1	76 ± 4	119 ± 1
CV, %	33.5	37.3	9.2	4.4	14.6	18.3	4.6	2.0	5.3	1.1

SD, standard deviation; CV, coefficient of variation.

Table 2: Inter-run precision of the Ves-Matic Cube 200 and TEST1 analyzers obtained by analysis of commercial control samples in the normal and abnormal range, i.e. on the Ves-Matic Cube 200 using ESR Control Cube on two levels while on the TEST1 latex control samples on three levels.

	Ves-Matic Cube 200		TEST1		
	ESR Control Cube normal (lot 413)	ESR Control Cube abnormal (lot 413)	LATEX CONTROL 2 (lot 1943B)	LATEX CONTROL 3 (lot 1943B)	LATEX CONTROL 4 (lot 1943B)
Mean ± SD	5.8 ± 1.4	55.6 ± 3.2	8.0 ± 0	17.9 ± 0.4	61.7 ± 0.7
CV, %	24.9	5.8	0	2.0	1.2

The control samples were analyzed in triplicate on 5 consecutive days. SD, standard deviation; CV, coefficient of variation.

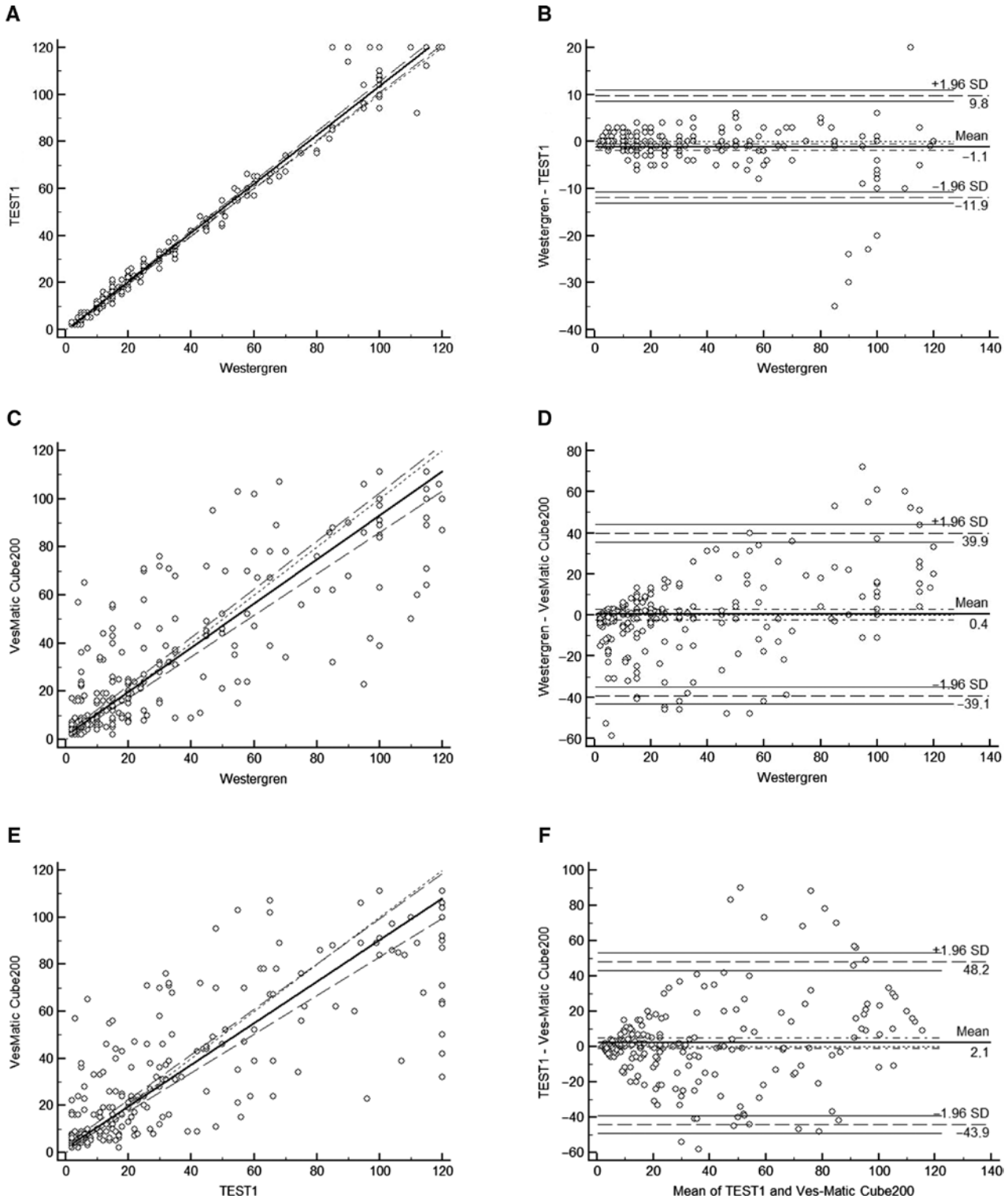

Figure 1: Regression and bias plots for all assessed comparisons.
Passing-Bablok regression analysis for comparisons between: (A) TEST1 analyzer vs. Westergren method (y = – 0.28 + 1.04x, intercept A –0.28 [95% CI: –1.17 to –0.10], slope B 1.04 [95% CI: 1.02 to 1.06]); (C) Ves-Matic Cube 200 vs. Westergren method (y = – 1.42 + 0.92x, intercept A –1.42 [95% CI: 0–2.46], slope B 0.92 [95% CI: 0.86–1.0]); (E) TEST1 analyzer vs. Ves-Matic Cube 200 (y = 1.86 + 0.89x, intercept A 1.86 [95% CI: 1.11–3.14], slope B 0.89 [95% CI: 0.82–0.96]). Corresponding scatter diagrams obtained by Bland-Altman analysis with mean biases are shown as B, D and F, respectively.

Table 3: Results of Passing-Bablok regression analysis, Bland-Altman analysis and Spearman's rank correlation coefficient for the comparison of the Ves-Matic Cube 200 and the TEST1 with the original Westergren method, divided per low, medium and high ESR subgroups.

	Ves-Matic Cube 200				TEST1			
	Intercept (95% CI)	Slope (95% CI)	ρ	Mean bias (95% CI)	Intercept (95% CI)	Slope (95% CI)	ρ	Mean bias (95% CI)
Low (n = 169)	−1.5 (−3.7 to −0.3)	1.5 (1.3–1.9)	0.65	−5.6 (−7.7 to −3.4)	−0.7 (−1.1 to 0)	1.1 (1.0–1.1)	0.97	0 (−0.3 to 0.3)
Medium (n = 35)	−157.1 (−365 to −83)	9.2 (−18 to 18)	0.48	2.1 (−6.3 to 10.5)	1.0 (−8.6 to 4.7)	1.0 (0.9–1.2)	0.94	−0.8 (−1.9 to 0.3)
High (n = 41)	−170.8 (−416 to −58)	2.5 (1.4–5.0)	0.39	23.3 (16.5–30.1)	15 (−13.6 to 30.7)	0.8 (0.7–1.1)	0.56	−5.7 (−9.3 to −2.0)

Hemolysis interference

Table 4 summarizes the results of the assessment of hemolysis effect on ESR determination, and outlines a constant negative bias throughout the assessed hemolysis range for all methods, and a statistically significant difference for the Ves-Matic Cube 200 (p = 0.032) and the Westergren method (p = 0.008). The median HI value was 2.14 g/L (ranging from 0.22 to 6.77 g/L).

Sensitivity to fibrinogen

The sensitivity of ESR to added fibrinogen is presented in Figure 2. The final concentration of the fibrinogen stock solution was 9 g/L due to loss of fibrinogen during the filtering process.

The most significant rise of ESR related to increasing fibrinogen concentrations was observed for the Ves-Matic Cube 200, while the TEST1 proved to be the least sensitive to fibrinogen influence. A steady increase of ESR with increasing added fibrinogen concentrations was observed for all methods, however, for the lower added fibrinogen

Table 4: The results of the assessment of hemolysis interference on ESR determination for the Ves-Matic Cube 200 and TEST1 analyzers and the Westergren method.

	Median (min–max)	p-Value	Mean bias, %
Ves-Matic Cube 200			
Non-hemolyzed	10.5 (4–110)	0.032	−13.4
Hemolyzed	8.5 (2–102)		
TEST1			
Non-hemolyzed	20.5 (4–120)	0.562	−18.9
Hemolyzed	21 (2–120)		
Westergren			
Non-hemolyzed	10 (3–105)	0.008	−24.5
Hemolyzed	6.5 (2–82)		

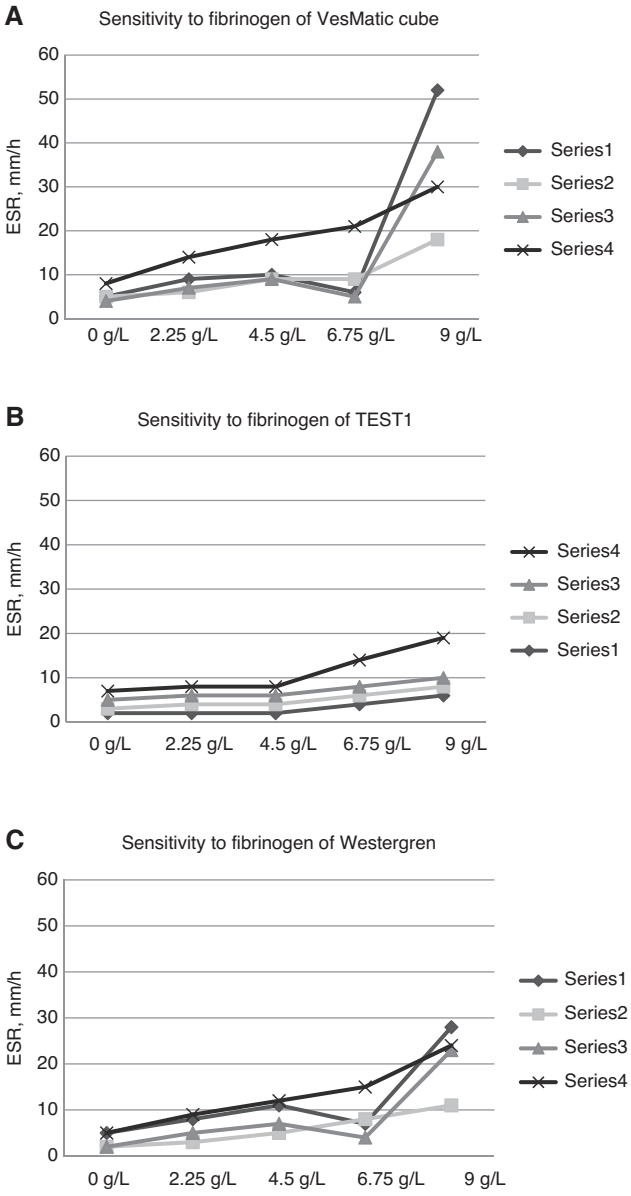

Figure 2: Sensitivity of ESR to added fibrinogen concentrations. Measured with (A) the VesMatic Cube analyzer, (B) the TEST1 analyzer and (C) the Westergren method.

concentrations were not clinically significant. A clinically meaningful increase was observed for the added concentration of 9 g/L, where the highest ESR elevation was 10.4-fold from baseline (from 5 mm to 52 mm) and was obtained for the Ves-Matic Cube 200. The Westergren method showed moderate sensitivity to fibrinogen, with an increase of up to 11.5-fold from the baseline (from 2 mm to 23 mm), while the TEST1 at tested fibrinogen concentrations did not show clinically significant changes in ESR values with the highest increase observed as 4.5-fold (from 2 mm to 9 mm).

Carryover

The evaluation of potential carryover for the TEST1 analyzer yielded a value of 4.2%. However, this results should not be interpreted solely but confronted with the intrarun CV obtained in the precision study. The intrarun CV for the low and middle analytical range was higher, thus implying that this result cannot be considered significant in terms of sample carryover but rather a consequence of analytical variation.

Sample stability

The results of sample stability assessment for the Ves-Matic Cube 200 and TEST1 analyzers are presented in Table 5. Overall, a decrease of ESR results was observed after storage at all timepoints and conditions, but a statistically significant difference was observed only for ESR results on the Ves-Matic Cube 200 after 24-h storage, both at RT and 4 °C. Moreover, the ESR results did not differ significantly after 24-h storage on RT and 4 °C when analyzed with the Westergren method ($p = 0.132$ and $p = 0.127$, respectively), yielding a mean difference of −4.2 (95% CI: −9.7 to 1.4) and 3.4 (95% CI: −1.1 to 7.9), respectively.

Discussion

Erythrocyte sedimentation is still a partly understood physicochemical phenomenon of the susceptibility of RBCs to settle down, dependent on the influence of a variety of physiological and pathophysiological factors. The process is characterized by three distinct phases: the aggregation phase where negatively charged RBCs couple in Rouleaux formations, followed by a settling stage and finally a packing stage where RBCs are sedimented at the bottom of the tube. Rouleaux formation is crucial for the overall ESR and is largely influenced by the presence of positively charged plasma proteins that induce RBC aggregation, in particular fibrinogen and globulins [16]. Understanding this mechanism was important for the development of novel ESR technologies. While the Westergren method and its modifications simply measure the overall sedimentation phenomenon, alternate methods incorporate measurement principles that assess the erythrocyte sedimentation kinetics mainly in the initial phase and apply measurements at different time intervals [13, 15, 16].

In this study, we aimed to assess the analytical performance of two automated ESR analyzers based on different determination techniques. Similar intrarun CVs were obtained for both evaluated analyzers, with expectedly highest CVs in the lowest range, which is in concordance with the results from other studies that have also observed that precision substantially decreases at low ESR levels

Table 5: Evaluation of sample stability for the Ves-Matic Cube 200 and TEST1 analyzers after storage at room temperature and 4 °C for 4, 8 and 24 h.

Samples (n = 20)	Ves-Matic Cube 200			TEST1		
	Mean, mm	Mean difference, mm (95% CI)	p-Value	Mean, mm	Mean difference, mm (95% CI)	p-Value
Fresh	26			32.2		
Stored at RT						
4 h	25	−1.0 (−5.8 to 3.9)	0.685	32.1	−0.1 (−2.6 to 2.5)	0.967
8 h	20.6	−5.4 (−13.3 to 2.5)	0.166	30.5	−1.7 (−5.5 to 2.2)	0.384
24 h	10.2	−15.8 (−25.5 to −6.1)	0.003	27.5	−4.7 (−10.5 to 1.1)	0.106
Stored at 4 °C						
4 h	25.4	−0.6 (−4.7 to 3.5)	0.764	37.1	5.0 (−0.7 to 10.6)	0.082
8 h	24.7	−1.4 (−4.9 to 2.2)	0.436	34.5	2.3 (−2.8 to 7.4)	0.359
24 h	18.8	−7.1 (−11.8 to −2.4)	0.005	31.7	−0.5 (−6.1 to 5.1)	0.855

RT, room temperature.

[15, 18, 19, 21, 23]. These variations result from small numbers rather than impaired precision and as already reported, are not considered clinically relevant [24]. Interestingly, CVs for inter-run precision showed a significantly better performance of TEST1 in comparison to the Ves-Matic Cube 200. This can be explained by the different constitution of control samples used, namely the TEST1 uses latex controls while commercially provided stabilized human blood serve as control samples on the Ves-Matic Cube 200. The issue of stabilized human samples that serve for quality control was addressed by Plebani and Piva [14], emphasizing that their kinetic properties differ from fresh blood erythrocytes, thus cannot replace fresh human blood and are not suitable for methods that measure ESR kinetics.

Overall, linear regression and Bland-Altman analysis showed good agreement between the modified (Ves-Matic Cube 200) and alternate (TEST1) ESR method with the gold standard. This clearly demonstrates that newly developed ESR analyzers based on different methodological principles can serve as a valid substitute to the gold standard method, thus contributing to better harmonization of ESR determination. However, some differences have been observed that can be explained by different methods.

A larger dispersion of results in comparisons where the Ves-Matic Cube 200 was involved as well as a more random variation of ESR results at higher ESR levels was observed, as already reported in previously published studies regardless of the type of ESR analyzer [23, 25, 26]. This, however, can be affected by a smaller number of samples evaluated in the upper analytical range. Although no evidence of systematic bias was observed for all comparisons when all samples are included, it is important to emphasize that 95% CIs for the comparison of the Ves-Matic Cube 200 with Westergren were wider than the TEST1 analyzer. When samples were subdivided into groups according to respective ESR values, a much higher bias with rising ESR values was observed, which was probably affected by individual samples causing significant deviations.

Interestingly, lower correlation coefficients (0.81 and 0.82) were obtained for all comparisons involving the Ves-Matic Cube 200, indicating deviations in ESR results when compared to the Westergren method and the TEST1 analyzer that should not be overlooked. Similar correlation of the Ves-Matic Cube 200 with the Westergren method has already been reported in two studies [18, 19] while Perovic et al. [15] obtained a much higher correlation coefficient. This observation is even more obvious when evaluating the correlation coefficients per each ESR level group that are even lower, clearly indicating result discrepancies and implicating the need to reassess the interchangeable use of ESR methods.

According to ICSH recommendations, it is also necessary to assess potential sample carryover. However, we assumed that is reasonable to assess it only for analyzers that use a pipetting system, as it is in our case the TEST1 analyzer, and observed no potential carryover that could cause spurious results.

Hemolysis in the studied range showed to have a negative effect on ESR values obtained by all methods, with a statistically significant decrease observed for the Ves-Matic Cube 200 and the Westergren method. Our approach for hemolysis interference assessment was similar to the ones reported in two previous studies [19, 23] that was based on spiking of native samples with a lysing solution that contains glycerol. Equally to our study, it was shown that hemolysis significantly decreases ESR levels when measured by the Ves-Matic Cube 200 [19, 23], while another ESR analyzer named iSED that utilizes photometric rheology principle for ESR determination did not show significative susceptibility to hemolysis [19]. The observed negative effect of hemolysis may result from RBCs lysis and a less packed RBC column that causes errors in reading, either optical as on the Ves-Matic Cube 200 or visual for the manual Westergren method. The TEST1 analyzer, on the other hand, showed to be less affected by hemolysis, most probably due to the kinetic principle of ESR measurement. However, as hemolysis was artificially induced using a 1.3% glycerol solution that could have increased sample viscosity, it is conceivable that the observed negative effect attributed to hemolysis could be, although minorly due to small added volume, caused by the lysing solution itself.

In this study, we also aimed to assess the sensitivity of response of ESR to increasing fibrinogen concentrations by adding fibrinogen *in vitro*. To the best of our knowledge, our study is the first one to have dealt with this topic. Fibrinogen is known to be the main protein responsible for aggregation and among the most important variables that affect the ESR value, along with hematocrit and variations in erythrocyte morphology [4–6, 27–30]. A kinetic study of the influence of fibrinogen on ESR demonstrated that its effect is most notable in the second phase of the erythrocyte sedimentation process where fibrinogen, due to its positive charge, mediates formation of large aggregates and enhances settling of RBCs [29]. This mechanistic principle and the methodology of the ESR methods could explain the results obtained in our study. Hereby, we show that the Ves-Matic Cube 200 analyzer is the most sensitive to increasing fibrinogen concentrations, the Westergren method being moderately affected while the TEST1

analyzer did not show clinically significant increases of ESR following fibrinogen addition. The TEST1 measures only the first phase of sedimentation that is considered to be only marginally affected by fibrinogen levels [29], while a more notable response obtained by the Westergren method and especially the Ves-Matic Cube 200 analyzer can be attributed to the fact that here, ESR is a result of RBC settling from the first two phases of the sedimentation process. However, as sensitivity to fibrinogen was studied only in healthy volunteers, the observed effects cannot be extrapolated to patients who have increased ESR values due to a disease-related imbalance between fibrinogen and globulins.

Additionally, sample stability for ESR determination was studied and we observed that delayed analysis causes a decrease in ESR values on both assessed analyzers, as already reported in previously published works [15, 19, 21, 23, 26]. Still, a significant difference was observed only for ESR results measured by the Ves-Matic Cube 200 when analyzed after 24-h storage both on RT and refrigerated at 4 °C, while other studies dealing with sample stability for the Ves-Matic Cube 200 observed the statistically significant difference of ESR results only when stored at RT [15, 19, 23]. Interestingly, although the results obtained by the Westergren method did not show statistically significant difference after 24-h storage, a decrease of ESR values was observed when samples were stored at RT, while an overall increase was evidenced for storage at 4 °C. Nevertheless, we believe that laboratories should perform ESR analysis within the same working day whenever possible, to avoid reporting of potentially altered ESR results.

ESR is not a measure of a single analyte but rather a result of a complex biophysical phenomenon and the ESR results can be affected in some particular cases by different methodological principles [15, 16]. In fact, the Ves-Matic Cube 200 simply measures the settling distance of RBCs, while the TEST1 utilizes a kinetic method that estimates ESR based on Rouleaux formation. The Ves-Matic Cube 200, similar to the Westergren method, can be affected by hematocrit, while the TEST1 with its capillary photometric kinetic method is less susceptible to variations in erythrocyte morphology or hematocrit levels [13, 16]. These automated technologies undoubtedly provide a faster and more reproducible determination of ESR, but results can differ because of different time intervals used for measurement [15, 16].

Our study proved the analytical validity of the TEST1 and Ves-Matic Cube 200 analyzers and their high comparability with the gold standard method. According to our findings, some differences can be observed, thus making their interchangeable use questionable. During two decades of use of automated ESR analyzers, efforts have been made in terms of standardization of ESR measurement. As evidenced in our study, improvements are obvious and the novel technologies show high comparability with the gold standard. Still, the interchangeable use of modified and alternate ESR methods should be applied with caution. It is, therefore, a requirement for laboratories to perform an extensive validation of ESR analyzers following the ICSH recommendations prior to their implementation in routine practice in order to assure clinically valid results that serve laboratories' own patient population and the imposed clinical needs.

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