A multicentric verification of hereditary and acquired thrombophilia coagulation assays

ABSTRACT

Aims: With an ever-growing number of patients being admitted to our University Hospital Sveti Duh (Zagreb, Croatia), there was a need to positively respond to clinicians' demands about hereditary and acquired thrombophilia testing.

Study design: An extensive and multicentric verification of coagulation assays included in thrombophilia testing was performedon the BCS XP coagulation analyzer (Siemens Healthineers, Marburg, Germany): antithrombin activity (AT) (InnovanceAntithrombin), protein C activity (PC) (Berichrom Protein C), protein S activity (PS) (Protein S Ac), free protein S antigen (free PS:Ag) (Innovance Free PS Ag), activated protein C resistance (APCR) (ProC Ac R and ProC Global + Coagulation Factor V Deficient Plasma), lupus anticoagulant (LA) screening (LA1 and activated partial thromboplastin time by using Dade Actin FSL as reagent) and confirmation test (LA2), factor VIII activity (FVIII) (Dade Actin FS and coagulation FVIII Deficient Plasma).

Place and Duration of Study: Department of Medical Laboratory Diagnostics, University Hospital Sveti Duh, Zagreb, Croatia, between January 2020 and December 2021.

Methodology: Verification protocol included: the precision study (CLSI EP15-A3 protocol), trueness estimation by comparison of seventy remnant plasma sampleswith two large Croatianhospital laboratories with established thrombophilia testing, and verification of reference intervals and cut-off values(CLSI EP28-A3C and CLSI HA-60 guidelines).

Results: All of the obtained imprecision CVs were within the manufacturer's claims(<5/10/15%). While the observed bias for PC(+1.9%) was within the EFLM performance specifications (6.7%), the average bias for AT was higher than acceptance criteria(+10.8% vs 3.2%allowed). P&B regression revealed a significantpositiveproportional difference(slope=1.14)Comparison of PS activity yielded a high negative bias (-33.4%) that exceeded the acceptance criteria of 8.1%. Regarding LA testing, the diagnostic accuracy was 99% when compared to Sestremilosrdnice UHC (N=70) and 95% when compared to University Hospital Centre Zagreb (N=19). All manufacturer's reference intervals and cut-offs were verified.

Conclusion: The verification study confirmed all manufacturer's claims, except for PS which has been replaced with free PS:Ag determinations.

Keywords: thrombophilia, hereditary, acquired, verification, coagulation

1. INTRODUCTION

Thrombophilia testing has long been limited to specialized centers. However, due to the high annual incidence rate of venous thromboembolism of 1 to 2 cases per 1000 individuals, as wellas automation of the majority of assays, there is an ever-increasing demand for thrombophilia screening. It has been documented that ready availability of thrombophilia tests, particularly for heritable disorders, leads to their excessive and inappropriate use [1]. However, with expansion and

an ever-growing number of patients being admitted to the Hematology and Obstetrics and gynecology departments in our University Hospital Sveti Duh (Zagreb, Croatia), there was a need to positively respond to clinicians' demands about hereditary and acquired thrombophilia testing. It was particularly challenging to implement the non-standardized coagulation assays, never before assayed in our coagulation laboratory. To assure high-end quality, up-to-date assays, a literature search was performed [2-6].After the selection of assays, and prior to implementation of new assays into everyday laboratory routine, an extensive and multicentric verification of coagulation assays included in both hereditary and acquired thrombophilia testing was performed.

2. MATERIAL AND METHODS

2.1 Materials and methods

The following assays were verified on the BCS XP coagulation analyzer (Siemens Healthineers, Marburg, Germany): antithrombin activity (AT) (InnovanceAntithrombin), protein C activity (PC) (Berichrom Protein C), protein S activity (PS) (Protein S Ac), free protein S antigen (free PS:Ag) (Innovance Free PS Ag), activated protein C resistance (APCR) (ProC Ac R and ProC Global + Coagulation Factor V Deficient Plasma), lupus anticoagulant (LA) screening (LA1 and activated partial thromboplastin time by using Dade Actin FSL as reagent) and confirmation test (LA2), factor VIII activity (FVIII) (Dade Actin FS and coagulation FVIII Deficient Plasma). All reagents were manufactured by Siemens Healthcare Diagnostics (Marburg, Germany). Verification protocol included the determination of within-run, between-run and total precision by analyzing Siemens Control plasma samples in the normal and pathological range (listed in Table 1.) for five consecutive days in quintuplicate, following the Clinical and Laboratory Institute (CLSI) EP15-A3 protocol [7], trueness estimation by comparison with two large hospital laboratories (Sestremilosrdnice University Hospital Center and University Hospital Centre Zagreb, Croatia) with established thrombophilia testing, as well as verification of reference intervals and cut-off values, as defined in the CLSI EP28-A3C and in the CLSI HA-60 guideline[8,9].The SestremilosrdniceUniversity Hospital Center, as well as University Hospital Centre Zagreb utilized the same reagents as our department. The coagulation analyzer in the Sestremilosrdnice University Hospital Centerwas the same as in our department (Siemens BCS XP), however, University Hospital Centre Zagreb analyzed their samples on Siemens Atellica COAG 360.Remnant plasma samples from patients for whom thrombophilia screening was ordered were separated into clean plastic tubes without additives and no additional blood draw was performed for the purposes of this study. Samples were frozen at -35 °C and transported to our department within two weeks from blood draw because of limited sample stability for LA samples. All samples were assayed in our institution immediately after delivery. Frozen plasma samples were thawed in a water bath at 37 °C, well stirred and assayed as a batch. Imprecision coefficients of variation (CVs, expressed in percentages) were compared to manufacturer's claims.

2.1 Statistical analysis

The comparison of methods was performed by Bland-Altman plot and Passing-Bablok regression. Biases, obtained from Bland-Altman analysis and expressed as percentages, were compared with minimum criteria available within the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) Biological Variation Database[10]. To assure independent evaluation of the assays under verification, during the verification period we have participated in the external quality control assessment scheme by the External quality Control of diagnostic Assays and Tests with a focus on Thrombosis and Haemostasis (ECAT) foundation. All data were stored in Microsoft Excel 2010 software. Statistical analysis was performed in MedCalc® v22.016 statistical software (MedCalc Software Ltd, Ostend; Belgium).

3. RESULTS AND DISCUSSION

3.1 Imprecision

Regarding imprecision study, all of the obtained imprecision CVs were within the manufacturer's claims, as shown in Table 1.

Table 1. Results of the total precision verification of a multicentric verification of hereditary and acquired thrombophilia coagulation assays

Assay (unit) (reagent)	Coefficients of variations (CVs) / %			
	Control Plasma N	Control Plasma P	Manufacturer's claim	Within manufacturer's claim (YES/NO)

	(lot No. 507767A, exp. 20/05/2021)	(lot No. 556714A, exp. 06/01/2021)		
Antithrombin (%) (INNOVANCE® Antithrombin) (lot No. 00325, exp. 06/07/2022)	3.0	7.8	<10.0	YES
Protein C (%) (Berichrom® Protein C) (lot No. 49884, exp. 25/07/2021)	2.2	2.0	<10.0	YES
Protein S (%) (Protein S Ac) (lot No. 50009, exp. 21/10/2021)	8.1	8.4	<10.0	YES
Free PS:Ag (%) (INNOVANCE® Free PS Ag) (lot No. 00477, exp. 16/06/2023)	2.5	2.3	<10.0	YES
FVIII (%) (Dade® Actin FS and Coagulation Factor FVIII Deficient Plasma) (lot No. 547693, exp. 04/09/2021)	7.1	9.0	<15.0	YES
	Control Plasma N (lot No. 507767A, exp. 20/05/2021)	ProC Control (lot No. 524479A, exp. 14/10/2023)	Manufacturer's claim	
APCR (ProC® Ac R) (lot No. 49840, exp. 24/10/2020)	6.5	4.0	<10.0	YES
APCR (ProC® Global + Coagulation Factor V Deficient Plasma) (lot No. 00468, exp. 16/11/2022)	4.8	3.4	<5.0	YES

	LA Control Low (lot No. 546097A, exp. 05/06/2021)	LA Control High (lot No. 545967A, exp. 02/06/2021)	Manufacturer's claim	YES
LA1 Screning Reagent (s) (lot No. 567211A, exp. 31/12/2020)	2.6	3.2	<5.0	YES
LA2 Confirmation Reagent (s) (lot No. 548881A, exp. 11/01/2021)	2.2	2.1	<5.0	YES
	Control Plasma N (lot No. 507767A, exp. 20/05/2021)	Citrol 2 (lot No. 548502, exp. 19/04/2023)	Manufacturer's claim	
Dade® Actin FSL Activated PTT Reagent (lot No. 556996. exp. 11/01/2021)	4.7	2.5	<5.0	YES
APCR – activated protein C resistance				

Although similar findings were obtained by Flieder and Hörber [11,12], their verification studies were performed on different coagulation analyzers (Sysmex CS-5100 and CS-2000i vs Siemens AtellicaCoag 360), thus disabling the direct comparison with our study performed on Siemens BCS XP analyzer. However, it is worth emphasizing the surprisingly high CVs for Protein C on the Sysmex CS-2000i analyzer in the Flieder study [11]: 7.92% for Control N and 10.02% for Control P, which even exceeds the manufacturer's claim of 10% allowed in the imprecision study. Protein C chromogenic assay was the assay that excelled in its performance during the verification study, thus the observed differences could be attributed to different coagulation platforms used. Surprisingly, the CVs for Protein S observed in the study by the Hörber et al. [12] were significantly lower than numbers obtained in our study. Possibly we can employ the same explanation as for the Protein C assay, incomparability of the coagulation analyzers.

3.2Accuracy (method comparison study)

Seventy remnant plasma samples were included in the inter-laboratory comparison of PC and AT activities, spanning the whole linearity range (PC: 49 – 149%, AT: 34 – 150%) (Table 2.). While the observed bias for PC was within the EFLM analytical performance specifications for minimum allowed bias, the average bias for AT was higher than the EFLM acceptance criteria. Passing and Bablok regression also revealed a significantpositiveproportional difference (Table 2).

Table 2. Results of method comparison study of a multicentric verification of thrombophilia coagulation assays

Assay (unit) (reagent)	N	Comparison range	Bland-Altman analysis			Passing and Bablok regression			
			Average bias% (95%	EFLM APS %	Within APS (YES/NO)	Intrecept (95% CI)	Significant YES/NO	Slope	Significant YES/NO

			CI)						
Antithrombin (%) (INNOVANCE® Antithrombin)	70	34 – 150	+10.8 (8.5 – 13.0)	3.2	NO	-2.28 (-13.8 – 5.25)	NO	1.14 (1.05 – 1.27)	YES
Protein C (%) (Berichrom® Protein C)	70	49 – 149	+1.9 (0.9 – 2.8)	6.7	YES	-1.02 (-4.90 – 3.11)	NO	1.02 (0.98 – 1.07)	NO
Protein S (%) (Protein S Ac)	30	32 – 130	-33.4 (-29.0 – (-37.8))	8.1	NO	-0.37 (-26.65 – 18.86)	NO	0.62 (0.44 – 0.87)	YES
95% CI – Confidence Interval EFLM APS – analytical performance specifications for minimum allowed bias, according to EFLM Biological Variation Database									

Although, similar results were observed in a comparison study of four automated coagulation analyzers by Scherer-Burić et al. [13], with a significant positive slope for AT (1.05), and in the Hörber study [12], with a positive antithrombin bias of 11.2%, the first received ECAT report revealed unsatisfactory result for AT activity with a positive bias (+9.8%) observed in comparison to other participants that used the same method.Although it is worth emphasizing that the biases of the commercial quality controls during the whole verification period were within the allowed manufacturer's claims (±20%), the received results were in accordance with the positive bias observed in the comparison study (irrespective of the centers involved in the comparison study), andcould not be ignored, nor explained by the incomparability of the coagulation analyzers, as the observed bias was consistent in all studied samples. With the second round of ECAT samples we recalibrated our assay, and re-analyzed the samples from the first round. The results were satisfactory, and we have received a certificate for AT activity with a negligible bias. Comparison of 30 samples for PS activity (range 32 – 130%) yielded a high negative bias (-33.4%) that exceeded the acceptance criteria of 8.1 %, which was also confirmed with a significant negative proportional error from Passing and Bablok regression (Table 2). During the verification period an instability of the reagent used was observed, with a need for recalibration with every batch. The on-board stability of the reagent is declared to be 2 hours, which put a lot of pressure on timely measurement of thawed samples. As the assay for free PS:Ag determination showed to be stable and reproducible, and the current recommendations [4] suggested that the free PS:Ag assay should be used as the initial assay of choice in the clinical coagulation laboratory, we have decided to dismiss the PS activity assay, and to implement the free PS:Ag assay, only.

Regarding LA testing, the two diagnosticcenters from the comparison study report LA results in a different manner. Sestremilosrdnice University Hospital Center reports the non-standardized ratio as the final result of the screen-mix-confirm protocol, whereas University Hospital Centre Zagreb reports results dichotomously as positive/negative. We preferably decided to compare the final classification of all received samples, then to assess the comparability of the individual measurements of the screen-mix-confirm protocol, as it was previously confirmed that some minor differences between reagents and instruments are to be expected and are also observed in EQA data [14]. The diagnostic accuracy was 99% when compared to Sestremilosrdnice UHC (N=70) and 95% when compared to University Hospital Centre Zagreb (N=19) (Table 3).

Table 3. Diagnostic accuracy for lupus anticoagulant comparison of a multicentric verification of thrombophilia coagulation assays

N=19	University Hospital CentreZagreb		
University Hospital Sveti Duh	LA positive	LA negative	Total
LA positive	9	0	9

LA negative	1	9	10
Total	10	9	19
Diagnostic accuracy = TP + TN/TP + FP +TN + FN = 18 / 19 = 0.95x 100% = 95%			
N = 70	Sestre milosrdnice University Hospital Center		
University Hospital Sveti Duh	LA positive	LA negative	Total
LA positive	4	0	4
LA negative	1	65	66
Total	5	65	70
Diagnostic accuracy = TP + TN/TP + FP +TN + FN = 69 / 70 = 0.99 x100% = 99%			

Two of the discrepant lupus anticoagulant results when compared to University Hospital Centre Zagreb and Sestre milosrdnice University Hospital Center were characterized as positive in those two centers with established thrombophilia testing, but they were falsely reported negative in our department. It is well documented that all tests examined could detect patients with strong anticoagulants, none was able to detect all patients, especially those patients with weaker anticoagulants could be missed [15], which could be the possible explanation for the observed misclassification. Additionally, as the verification protocol included frozen remnant plasma samples, perhaps residual platelets in plasma samples could result in shortening of DRVVT assay which may result in a false-negative LAC test result [16].

3.3Verification of reference intervals

Finally, we performed a verification of reference intervals and diagnostic cut-offs in plasma samples of 20 healthy volunteers without any coagulation abnormality (male/female ratio = 8/12). The health status was checked with complete blood count (CBC) and C-reactive protein (CRP) measurements. Coagulation samples were drawn into BD Vacutainer® 9NC 0.105M 3.2% Buffered Sodium Citrate Glass 4.5 mL Blood Collection Tubes (BD, Plymouth, UK). All manufacturer's reference intervals and cut-offs were verified.

4. CONCLUSION

The verification study confirmed all manufacturer's claims, except for PS which has been replaced with free PS:Ag determinations. Therefore, the assays subject to verification could be safely introduced into routine practice. Indeed, starting in December 2021, we have successfully started with coagulation testing of hereditary and acquired thrombophilia in our department.

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