sciendo

Original professional article

DOI:10.2478/rrlm-2019-0034

Hemolysis has no influence on routine coagulation tests in subjects without anticoagulant therapy - a referral Romanian emergency hospital laboratory experience

Elena Binzari[1], Mihaela Zaharia[2*], Stefan Barbu[1,2], Oana Roxana Oprea[1,2], Minodora Dobreanu[1,2]

1. George Emil Palade University of Medicine, Pharmacy, Science, and Technology of Targu Mures, Romania
2. Districtual Clinical Emergency Hospital Targu-Mures, Romania

Abstract

The aim of this study was to determine the rate of hemolyzed specimens sent to our laboratory for coagulation testing, assess the interference of hemolysis on coagulation for patients without anticoagulant therapy and to determine the reference intervals for PT, INR and aPTT for our laboratory in order to test our own limitations. ***Methods****: To determine the hemolysis rate, 1,689 specimens were evaluated on a visual scale and with the hemolysis icterus lipemia (HYL) test on Architect c4000 instrument. 125 blood samples collected from subjects without anticoagulant therapy were hemolyzed in vitro and the PT, INR and aPTT results were compared before and after hemolysis.To determine reference intervals (RI) for PT, INR and aPTT in our population, 125 apparently healthy human subjects (according to CLSI C28-A2) were enrolled and tests were performed on Sysmex CS 2000i, using Siemens reagents.* ***Results****: Out of 1,689 samples, 9.46% were assessed as hemolyzed by the visual scale, while HYL test showed a 6.63% hemolysis rate. We found a shortening of 0.1s for PT, a diminution with 0.01 units for INR and a prolongation with 0.9s for aPTT from in vitro hemolyzed compared to non-lyzed samples. As to the reference intervals, we obtained in our laboratory versus reagents producer: for PT 9.8-13.9 s vs 9.8-12.1 s, and for aPTT 19.1-31.5s vs 23-31.9 s respectively; 28.38% more PT results and 13.44% more aPTT results were within range when we used local laboratory RI, compared to the manufacturer's RI.* ***Conclusions****: The rate of hemolyzed coagulation samples in our laboratory is higher than the rate found in the literature. Nevertheless, for patients without anticoagulant therapy hemolyzed samples should be processed. Using our own reference interval leads to a significant reduced number of abnormal results.*

Keywords: *hemolysis, coagulation, reference intervals*

Received: 25th July 2019; Accepted: 10th September 2019; Published: 20th October 2019

***Corresponding author**: Mihaela Zaharia, Targu Mures Emergency Clinical Hospital, Romania.
E-mail: mihaela2006_cv@yahoo.com

Introduction

The pre-analytical phase is a very important step in any laboratory testing. Correct patient identification, sample collection, transportation, and storage are simple yet effective ways of avoiding misleading the test result, preventing misdiagnosis, treatment delay, and resources waste for the hospital.

Several studies state that pre-analytical errors represent 70% of all detected errors during the testing process, with spurious hemolysis representing 40% (1–4) of all unsuitable specimens in the coagulation laboratory (5). By far, the leading cause of sample rejection by the laboratory is due to pre-analytical errors (6).

Hemolysis represents the pathological process of red blood cell destruction resulting in red tinge in serum or plasma, visible after sample centrifugation. Hemolysis is typically detected when free hemoglobin exceeds 30mg/dL (7). The process of hemolyzation can occur in vivo, where a variety of medical conditions cause cell disruption. Depending on the mechanism, in vivo hemolysis can be intravascular or extravascular and can lead to hemolytic anemia. In vitro, or spurious hemolysis, is caused by incorrect blood collection, handling or transportation.

Routine coagulation testing PT (prothrombin time) and aPTT (activated partial thromboplastin time) are mainly performed to evaluate anticoagulant therapy (PT for oral anti vitamin K treatment and aPTT for non-fractioned heparin treatment), but also as pre-operatory screening of coagulation state. Two methods of coagulation measurement are currently available: the mechanical method, which uses a magnetic sensor to monitor the movement of a steel ball in the test solution, and the photo-optical method, which uses the change in optical density to detect clot formation (8,9). Primarily, laboratories use the photo-optical method to assess coagulation.

Although no mechanism of interference of hemolysis on coagulation testing has been fully explained, two theories are possible. One theory speculates that cell-free hemoglobin present in samples after centrifugation has a high absorbance at the wavelengths conventionally used by the optical instrumentation for coagulation testing. At the same time, the assumption that hemolysis is due to incorrect blood collection allows to consider concomitant endothelium injury with release of thromboplastin-like pro-coagulation activity and exposure of the subendothelial surface, thus compromising the sample (3).

The other theory presumes that red blood cell lysis results in cytoplasmatic and plasma membrane molecules, such as phospholipids, tissue factor, proteases, etc., which can produce spurious activation of coagulation cascade or consumption of clotting factors leading to prolongation of test results (3).

The aim of this study was to determine whether in vitro hemolysis interferes in the coagulation process and influences test results in patients not undergoing anticoagulation treatment. A secondary aim was to determine reference intervals (RI) for our laboratory in comparison to reference intervals provided by reagent manufacturer.

Materials and methods

Our study was divided into two parts. In part one, we determined the hemolysis rate in coagulation tests requested within the Emergency County Hospital Targu-Mures, Romania.

We received a total of 1,689 samples for coagulation testing from December 2018 to February 2019. Samples were collected in clinical wards by nursing staff unrelated to laboratory. We first evaluated them on a visual scale and then on Architect c4000 (Abbott Diagnostics, IL, USA) instrument using HYL (hemolysis icterus lipemia) assay to determine the degree of hemolysis, after

the completion of any diagnostic tests, otherwise the residual blood samples were discarded.
In practice, samples can be classified into 4 categories using the visual scale:

- "yellow" corresponding to no hemolysis,
- "pale red" corresponding to mildly hemolyzed,
- "red" corresponding to moderately hemolyzed,
- "dark red" corresponding to severely hemolyzed (10).

HYL is a spectrophotometric method performed on an automated instrument and results in a semi-quantitative expression of hemoglobin concentration:

- < 30 mg/dL corresponding to "+/-",
- 30-99 mg/dL (1+),
- 100-199 mg/dL (2+),
- 200-499 mg/dL (3+),
- >500 mg/dL (4+) (11).

We evaluated residual blood samples that would otherwise be discarded, after the completion of any diagnostic tests. Therefore, in accordance with the Declaration of Helsinki and under the terms of the local laws, no informed consent was needed from the patients. Samples that were lipemic or had high bilirubin levels were excluded.
For the second part of the study, approved by the Ethics Comity of our hospital, we obtained a written consent from those who agreed to participate and we completed a survey concerning medical history, treatment and lifestyle. We collected the samples following the hospital sample collection standard operating procedure. Venous blood was collected by laboratory nursing staff in tubes containing Sodium citrate 3.2% as anticoagulant in 9:1 proportion.
For RI determination and for the study regarding the interference of hemolysis on coagulation test result we enrolled healthy subjects using the following exclusion criteria: patients with anticoagulant treatment, high levels of AST (aspartate transaminase), ALT (alanine transaminase) or GGT (gamma-glutamyl transferase) or other diseases that may influence coagulation tests.

Determining the reference interval of values for PT and aPTT for our laboratory

Following CLSI C28-A2 (Clinical Laboratory Standards Institute) document (12), we colected 162 specimens from apparently healthy subjects. After centrifugation for 20 minutes at 2,150 RCF (relative centrifugal force), PT and aPTT tests were performed on Sysmex CS 2000i (Sysmex Corporation, Kobe, Japan), using Siemens reagents: Thromborel and Actin FS (Siemens Healthcare, GmbH, Erlangen, Germany), within 4 hours from blood collection. AST, ALT and GGT were also performed on Architect c-4000 instrument in order to rule out any liver disease that could bias the results; 125 specimens were included in RI study (37 samples were excluded: 18 samples were discarded due to anticoagulant treatment, 5 samples presented a clot, 8 were not handled correctly and 6 had high levels of AST, ALT or GGT).
Furthermore, we compared all results from the coagulation tests performed for the surgical wards from December 5th, 2018 to February 12th, 2019 to both manufacturer's reference interval and to our reference interval.

Interference of hemolysis on coagulation tests in patients without anticoagulant treatment

A total of 125 samples were included for analysis in this part of the study (74 women and 51 men). Subsequently, each sample was mechanically hemolyzed using a 20 mL syringe with 18G needle by rapid aspiration of blood and strong expulsion back into the test tube for 20 times. The samples were centrifuged again for 20 minutes at 2,150 RCF. PT, aPTT, and HYL tests were performed again.
All results and patients' data were tabulated on Excel spreadsheets. The results obtained for

each patient immediately after blood collection were compared to the results obtained after in vitro hemolysis.

Statistical analysis

For the determination of the reference intervals, statistical analysis was performed in Medcalc Software (Version 14.8.1, 64 bit for Windows). Continuous variables with normal distribution were expressed as mean ± standard deviation (SD) and those non-normally distributed were presented as median (min-max). According to CLSI protocol (12), we used the non-parametric percentile method. The normality of data was checked with the Kolmogorov-Smirnov goodness-of-fit test. The comparison between means or medians was performed by the Student t-test, Mann-Whitney or Wilcoxon tests as appropriate to identify significant differences between groups, while using the Tukey post test to identify possible outliers. Statistical significance was set at p value<0.05.

Results

Determining the hemolysis rate in coagulation test samples

Out of 1,689 samples, 9.46% (n=160) samples were assessed as hemolyzed by the visual scale (table 1 A.) while HYL test showed that 6.63% (n=112) were hemolyzed at different degrees. These results are shown in Table 1 B. Some differences appeared between the visual scale and HYL test: 48 more samples were assessed as hemolyzed with the visual scale than the HYL test. 48% of the samples analyzed with HYL test (n=54) were collected in the emergency department, 18% (n=20) in the surgical department, 14% (n=16) in the cardiology department, 10% (n=11) in internal medicine, and the other 10% (n=11) came from neurology, gastroenterology, and hematology departments.

Determining the reference interval of values for PT and aPTT for our laboratory

The reference intervals for PT, INR (international normalized ratio), and aPTT determined in our laboratory are slightly different from those provided by reagent manufacturer. These results are presented in Table 2.

For surgical wards 2,545 tests were performed from December 5th, 2018 to February 12th, 2019. In PT tests 28.38% more results were within range when we used local laboratory RI. In aPTT tests 13.44% more tests were within local laboratory RI than in manufacturer's RI. The results are presented in Fig.1.

Table 1 A, B. The rate of hemolyzed specimens in 1,689 coagulation tubes. 1A with visual scale and 1B with HYL test

1A. Visual scale

Hemolysis degree	No. of samples	%
Yellow (no haemolysys)	1,529	90.52
Pale red (mildly hemolyzed)	57	3.37
Red (moderately hemolyzed)	62	3.67
Dark red(severely hemolyzed)	41	2.42
Total haemolyzed	160	9.46

1B. HYL with Architect c4000 (Abbott,USA)

Hemolysis degree	No. of samples	%
(+/-)	1,577	93.36
(1+)	86	5.09
(2+)	17	1.01
(3+)	7	0.41
(4+)	2	0.12
Total haemolyzed	112	6.63

Table 2. Manufacturer's and local laboratory reference intervals, for routine coagulation tests, determined according to CLSI C28-A2

	Siemens reference interval for tests on Sysmex CS 2000i	The reference interval determined by local laboratory	CV (coefficient of variation for) local laboratory determination
PT	9.0-12.1s	9.8-13.9s	6.11%
INR	0.85-1.19	0.74-1.2	7.95%
aPTT	23.0-31.9s	19.1-31.5s	8.67%

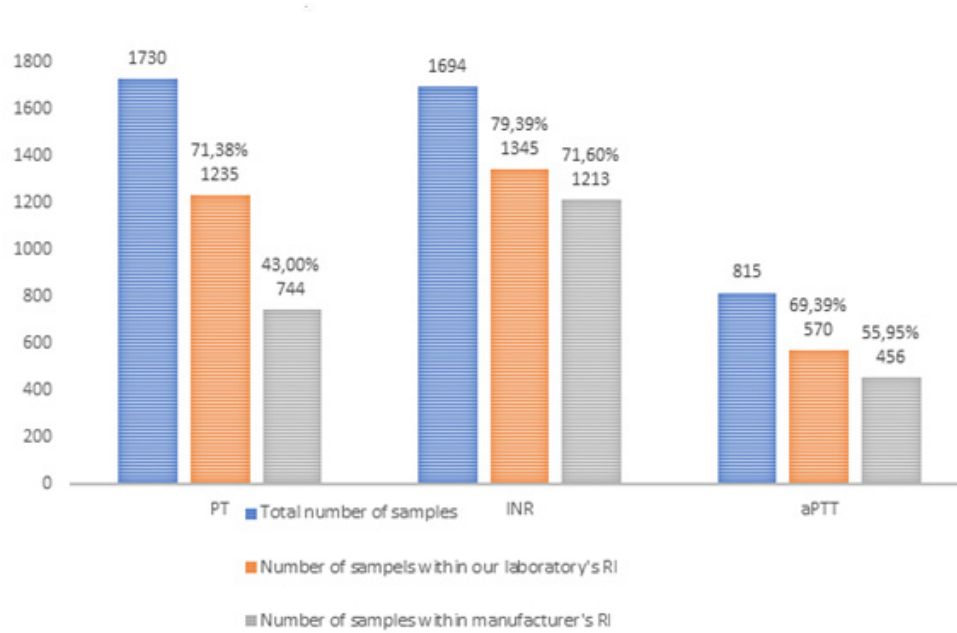

Fig. 1. Comparison of the numbers of tests performed (in emergency laboratory from December 5th, 2018 to February 12th, 2019) for surgical wards with results in the two different RI; left column represents total number of tests performed, middle column represents number of results within laboratory RI and right column represents number of results within manufacturer's RI.

Interference of in vitro hemolysis on coagulation tests in patients without anticoagulant treatment

Out of 125 included samples, before in vitro hemolysis three PT (2.4%) according to manufacturer's reference interval and one (0.8%) according to our laboratory's, and only one INR value (0.8%) according to both, were outside reference intervals.

After in vitro hemolysis, the degree of hemolysis of the specimens were as follows:

- No significant hemolysis: 3 samples (2.4%),
- (1+): 44 samples (35.2%),
- (2+): 55 samples (44%),
- (3+): 18 samples (14.4%),
- (4+): 5 samples (4%).

The coagulation test results after in vitro hemolysis, changed: nineteen (15.2%) PT values according to manufacturer's RI and only two (1.6%) according to ours, one INR value (0.8%) and six (4.8%) aPTT values according to both

were out of the RI. The change in results after in vitro hemolysis are shown in Table 3.

Discussions

Spurious hemolysis is the leading cause of sample rejection in clinical laboratories (3). However, the lack of a clear mechanism of interference does not allow us to decide whether those samples should be processed. What if in patients without anticoagulant treatment the difference in coagulation test results has no clinical significance nor patient safety?

Studies analyzing hemolysis interference with coagulation tests are not numerous nor comparable between each other due to different methodologies and sample patient size (3). Mainly, hemolysis technics used in these studies are: use of deionized water with or without detergents for whole anticoagulated samples lysis, freezing and thawing whole anticoagulated blood (13), spiking plasma with hemolysate products, and mechanical lysis by stirring with a metallic bar, sonication, application of the blade of a tissue homogenizer (7,14) and aspiration using a different diameter needle than the recommended one (3,5,14–17).

Table 3. PT, INR and aPTT test results before and after mechanical hemolysis induced in vitro (Wilcoxon statistical analysis was used)

	PT	
	Before	After
Median	11.20s	11.10s
95%CI	11.10- 11.50s	10.80- 11.39s
p	0.0012	
	INR	
	Before	After
Median	0.89	0.88
95% CI	0.87- 0.91	0.86- 0.90
p	0.0124	
	aPTT	
	Before	After
Median	25.20s	26.10s
95% CI	24.60- 25.69s	25.60- 26.60s
p	<0.001	

We have chosen to use the latter in order to reproduce as close as possible the physical damage produced by inappropriate traumatic blood collection to the plasma. Although spiking samples with hemolysate or pure hemoglobin solution might result in a more accurate hemoglobin concentration, during mechanical lysis not only erythrocytes are destroyed, but also platelets and leukocytes, and this leads to potential biological interferences independent of hemolysis degree (3). As for freezing whole anticoagulated blood, the challenge is to use a highly standardized technique, as temperature and duration of freezing are crucial to obtain a homogenous osmotically induced injury to cells (3).

As an important indicator of overall quality of the pre-analytical phase (18), it is essential for every laboratory to determine its in vitro hemolysis rate. Studies estimate this rate to be around 3.3% (13) whereas our study found a higher rate for coagulation samples, of 6.57% according to HYL assay and 9.53% according to the evaluation with our laboratory visual scale. This significant difference actually shows the high level of subjectivity when there are no strict boundaries of a test and humans have to decide what intensity to assign to a certain process.

In 2018, our laboratory assessed 47,248 samples for coagulation tests. Considering that 6.57% of all samples were hemolyzed (3,104 specimens), the hospital loss amounts to about 6,000 EUR, not to mention the waste of time, diagnosis delay, and unnecessary blood redraws. This short cost analysis puts hemolysis in the bigger picture – it is an expensive pre-analytical error.

Studies show that in most cases hemolyzed specimens are collected in emergency department (ED), pediatrics, and intensive care departments (5,18). However, in our case the top 3 depart-

ments sending hemolyzed samples to the laboratory are emergency, surgery, and cardiology departments. A higher rate of hemolyzed samples collected in the ED is said to be due either to the skills and experience of the staff or to the collection of blood on intravenous catheters instead of venipuncture (3). Further investigation is needed in order to see the whole problem and to design an intervention plan. Studies suggest that continuous education of health professionals concerning the best practice for blood collection, handling, and storage of blood samples can improve quality indicators in terms of hemolysis rate (2,19).

Regarding the interference of hemolysis on coagulation tests, this study shows a shortening of less than one second in PT test result and a prolongation of less than one second of aPTT test result after hemolysis. Although the difference is statistically significant, this still probably does not have any clinical significance. Arora et al (15) and Laga et al (14) stated the same findings as in our study: the PT and aPTT results' difference before and after hemolysis in healthy subjects is not clinically meaningful, although none of the studies performed coagulation factor assay, so the mechanism is not elucidated. As in Hernaningsih et al (8) and in Lippi et al (6) studies, we did not find any correlation between the hemolysis degree and the resulting variation in coagulation test results after hemolysis.

This study was performed only on patients with PT/INR and aPTT results within reference intervals before hemolysis. We cannot extrapolate our findings on patients using oral anticoagulant treatment or heparin.

Despite its limitations, one of the important strengths of our study is that we used a higher sample size than other studies and that we used the mechanical hemolysis technique reproducing the most closely hemolysis occurring during blood collection.

Conclusions

Although the reference intervals determined for our laboratory and on central Romania population are not critically different from those provided by reagent manufacturers, significantly more results obtained are within our RI than within RI recommended by the manufacturer. Our study suggests that for correct interpretation of test results it is mandatory for each laboratory to establish its own reference interval.

Our results confirm that in patients without anticoagulant treatment, there is no need to ask for a second sample of blood if the first sample is hemolyzed. Thus, good communication between the patient's treating team and laboratory staff is enough to solve this problem.

Abbreviations

aPTT - activated partial thromboplastin time
ED – emergency department
HYL - hemolysis icterus lipemia
INR – international normalized ratio
PT - prothrombin time
RI - reference intervals
SD - standard deviation

Conflict of interest

None to declare.

Authors'contribution

EB - design of methodology, investigation, data curation, original draft preparation; MZ - investigation, data curation, review and editing, SB - Software, formal analysis, data curation, visualization; ORO - design of methodology, resources, review and editing, visualization; MD - conceptualization, project administration, supervision, review and editing, validation

References

1. Lippi G, Blanckaert N, Bonini P, Green S, Kitchen S, Palicka V, et al. Haemolysis: An overview of the leading cause of unsuitable specimens in clinical laboratories. Clin Chem Lab Med. 2008;46(6):764-72. DOI: 10.1515/CCLM.2008.170
2. Söderberg J, Jonsson PA, Wallin O, Grankvist K, Hultdin J. Haemolysis index - An estimate of preanalytical quality in primary health care. Clin Chem Lab Med. 2009;47(8):940-4. DOI: 10.1515/CCLM.2009.227
3. Fraser Davidson D. A survey of some pre-analytical errors identified from the biochemistry department of a scottish hospital. Scott Med J. 2014;59(2):91-4. DOI: 10.1177/0036933014529056
4. Lippi G, Salvagno G, Montagnana M, Lima-Oliveira G, Guidi G, Favaloro EJ. Quality standards for sample collection in coagulation testing. Semin Thromb Hemost. 2012;38(6):565-75. DOI: 10.1055/s-0032-1315961
5. Lippi G, Plebani M, Favaloro EJ. Interference in coagulation testing: Focus on spurious hemolysis, icterus, and lipemia. Semin Thromb Hemost. 2013;39(3):258-66. DOI: 10.1055/s-0032-1328972
6. Lippi G, Cervellin G, Favaloro E, Plebani M. Management of hemolyzed specimens.Laboratornaya sluzhba. 2017;6:38-46. DOI: 10.17116/labs20176238-46
7. Lippi G, Plebani M, Di Somma S, Cervellin G. Hemolyzed specimens: A major challenge for emergency departments and clinical laboratories. Crit Rev Clin Lab Sci. 2011;48(3):143-53. DOI: 10.3109/10408363.2011.600228
8. Hernaningsih Y, Akualing JS. The effects of hemolysis on plasma prothrombin time and activated partial thromboplastin time tests using photo-optical method. Med (United States). 2017;96(38):1-5. DOI: 10.1097/MD.0000000000007976
9. Aggarwal S, Nayak DM, Manohar C. Discrepancy in optical & mechanical method in coagulation tests in a turbid sample. Indian J Hematol Blood Transfus [Internet]. 2014/08/17. 2014 Sep;30(Suppl 1):402-4. DOI: 10.1007/s12288-014-0438-5
10. Adiga U, Yogish S. Hemolytic index-A tool to measure hemolysis in vitro. IOSR J Biotechnol Biochem (IOSR-JBB [Internet]. 2016;2(2):49-52.
11. Luoma J, Seago J, Bates T, Campbell D. Reduced Tuarnaround Time for Haemolysis/Icterus/Lipaemia (HIL) Interferent Indices on Architect chemistry analyzers, AACC Annual Meeting, 2007
12. NCCLS, How to Define and Determine Reference Intervals in the Clinical Laboratory; Approved Guideline-Second Edition. NCCLS document C28-A2 [ISBN 1-56238-406-6]. NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pensylvania 19087-1898, USA 2000.
13. D'Angelo G, Villa C, Tamborini A, Villa S. Evaluation of the main coagulation tests in the presence of hemolysis in healthy subjects and patients on oral anticoagulant therapy. Int J Lab Hematol. 2015;37(6):819-33. DOI: 10.1111/ijlh.12417
14. Laga AC, Cheves TA, Sweeney JD. The effect of specimen hemolysis on coagulation test results. Am J Clin Pathol. 2006;126(5):748-55. DOI: 10.1309/03FK-3378YTRA1FRF
15. Arora S, Kolte S, Dhupia J. Hemolyzed Samples Should be Processed for Coagulation Studies: The Study of Hemolysis Effects on Coagulation Parameters. Ann Med Health Sci Res [Internet]. 2014;4(2):233-7. DOI: 10.4103/2141-9248.129049
16. Woolley A, Golmard JL, Kitchen S. Effects of haemolysis, icterus and lipaemia on coagulation tests as performed on Stago STA-Compact-Max analyser. Int J Lab Hematol. 2016;38(4):375-88. DOI: 10.1111/ijlh.12498
17. Anandani GM, Parikh SB. Effect of pre-analytic variables on prothrombin time and activated partial thromboplastin time. J Clin Diagnostic Res. 2018;12(7):EC01-5. DOI: 10.7860/JCDR/2018/32666.11719
18. Bölenius K, Söderberg J, Hultdin J, Lindkvist M, Brulin C, Grankvist K. Minor improvement of venous blood specimen collection practices in primary health care after a large-scale educational intervention. Clin Chem Lab Med. 2013;51(2):303-10. DOI: 10.1515/cclm-2012-0159
19. Lippi G, Salvagno G, Montagnana M, Franchini M, Guidi G. Phlebotomy Issues and Quality Improvement in Results of Laboratory Testing. Clinical laboratory 2006;52:217-230.