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Original Article

Pathology Section

Effect of Pre-Analytic Variables on Prothrombin Time and Activated Partial Thromboplastin Time

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ABSTRACT

Introduction: Inadvertent inappropriate coagulation tests results are reported frequently inspite of using automated instruments and quality assurance measures, mostly due to pre analytical errors associated with sample collection and processing.

Aim: This study was undertaken to evaluate pre-analytical variables in coagulation tests by inducing variables through simple experiments. The study also aimed to evolve rejection/ sample acceptability criteria for coagulation tests to minimise rejection of sample at the level of pre analytical phase.

Materials and Methods: This cross sectional and observational study was carried out in the Pathology department of a tertiary care hospital from January 2014 to December 2014. Results obtained from reported blood samples with normal Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT) values were used to induce processing variables. Coagulation tests results were collected by running samples in ideal conditions. Same samples were rerun with introducing variation in parameters and coagulation test measurements were taken. Differences between first and second coagulation measurements were noted and compared by applying t-test using IBM SPSS software version 25. For observational study, data were based on results of samples received and rejected in Clinical Pathology laboratory.

Results: PT value was not significantly affected by haemolysis unlike aPTT which was altered due to haemolysis. By altering ratio of blood and anticoagulant, PT results were affected significantly when citrate concentration was increased by 0.4ml and aPTT results were affected more significantly when citrate concentration was increased by 0.1 ml. Centrifugation of samples for 10 minute at 1500xg or 3000xg did not alter the tests' results significantly. PT values were not significantly affected upto 24 hours at room temperature. But aPTT values were significantly affected when sample was stored for more than 12 hours even on freezing temperature. In observational study, the major reason for rejection in the pre-analytical phase was clotted samples, followed by samples rejected due to underfilling.

Conclusion: Samples should be properly identified and labeled. PT of haemolyzed samples giving results in normal range can be reported. A 67% of the optimum filled volume for PT and 86% for aPTT should be acceptable. A 10 minute centrifuge time at 1500xg might be suitable. Samples can be run for PT within 24 hours at room temperature and 48 hours in freezing condition and for aPTT within 12 hours.

Keywords: Coagulation Tests, Sample Collection, Sample Processing

INTRODUCTION

We use modern laboratory instrumentation with high levels of test reliability and appropriate quality assurance measures just to minimize the errors within coagulation testing. Nevertheless, inappropriate test results are still reported due to pre-analytical, analytical & post analytical errors, often due to pre analytical events which are associated with sample collection and processing.

Endothelium is a source of von Willebrand factor (vWF) which is the essential cofactor for platelet adhesion. Damaged endothelial cells are capable of encouraging platelet adhesion, activation, and aggregation. Hence, endothelial injury results in blood loss and sets the stage for thrombus formation [1,2].

This study was focused on pre-analytical phase in routine coagulation tests, highlighting contributory elements and providing suggestions on how errors can be minimised. Errors in reporting coagulation tests are most prone to occur in the preanalytical phase. The pre-analytical activities and sample rejection criteria are diverse throughout the world. The different strategies for managing, processing and reporting unacceptable samples are also non uniform worldwide [3]. A variety of standards related to blood sampling and sample trans-portation and handling are available, but compliance to those guidelines is low, especially if it is not supervised by laboratory staff and director. For some pre analytical procedures, internationally accepted guidelines, recommendations and related quality measures are not available.

The most frequently ordered tests in the clinical laboratory PT and aPTT have the greatest potential for pre-analytic variability and errors. Many standards set by the Clinical and Laboratory Standards Institute (CLSI) for testing in the coagulation laboratory has been developed in an effort to improve reproducibility, precision, and accuracy. Despite documentation, there is still a lack of standardisation among clinical laboratories regarding pre-analytic variables [4,5].

The aim and objectives of this study were to study pre-analytical variables in coagulation tests by carrying out simple experi-ments which involve induced variables and to analyse data of rejected/ erroneously reported samples for PT & aPTT tests. The study also aimed to evolve a system whereby rejection/ sample acceptability criteria for coagulation tests are defined in laboratory so as to minimize rejection of sample at the level of pre analytical phase.

MATERIALS AND METHODS

This was cross sectional and observational study ethically approved by Institutional Ethics Committee based on results of samples re-ceived and rejected due to pre analytical errors in Clinical Pathology laboratory of a Smt. NHL Municipal Medical College, Ahmedabad, from January 2014 to December 2014 over a period of twelve months. In present study, four major parameters were included as according to CLSI guidelines, these parameters are critical for Coagulation tests which includes- Haemolyzed samples, Altered blood Volume- overfilled or underfilled tube, Variable centrifugation time & speed and Variable storage time [4].

A total of 510 tests were performed on 34 samples received. Reported blood samples with normal PT and aPTT values were used to induce processing variables. The normal range of PT was defined as 9-16 seconds and normal range of aPTT was defined as 24-40 seconds. The entire data was obtained by creating variation in four parameters (15 variables are introduced for each sample). Samples were first run in ideal condition which is recommended by CLSI and then again run after creating variations in parameters which affect PT & aPTT values. ACL Elite Pro which is a fully automated coagulometer was used for running samples for PT and a PTT.

STATISTICAL ANALYSIS

Results were compared by applying t-test using IBM SPSS software version 25, to reach an inference regarding each parameter and significance level of each variable. Statistical analysis was done using paired t-test with significance level at 0.05. Differences between co-agulation measurements on samples run in ideal condition and samples run with introducing variation in parameters were measured.

RESULTS

Induced processing Variations in Parameters:

1) Induced haemolysis: (Closed to venipuncture induced haemolysis)

A 23 G needle and syringe were used for rapidly aspiring and strongly expulsing the blood into the test tube. This was repeated 5 times for inducing haemolysis in the sample. Thereafter samples were kept at room temperature for three hours. As shown in [Table/Fig-1,2], it was concluded after analysis of results for induced haemolysis, PT value was not significantly affected but aPTT value was affected significantly in induced processing variables due to haemolysis.

Parameter for PT of samples received for coagulation tests	Mean	SD	No. of Observations on each sample	t-test values	P(T<=t) two-tail values	Level of Significance
In Normal condition*	11.44	1.16	20			
Induced haemolysis	11.85	0.72	20	-1.558253	0.135674	Not Significant
Citrate conc. increased by 0.1mL	11.66	0.86	20	-0.249947	0.805310	Not significant
Citrate conc. increased by 0.2 mL	12.16	1.10	20	-1.612389	0.123364	Not Significant
Citrate conc. increased by 0.3 mL	12.59	1.88	20	-3.202437	0.004688	Not significant
Citrate conc. increased by 0.4 mL	13.77	1.21	20	-2.218433	0.038905	Significant
Citrate conc. increased by 0.5 mL	27.94	1.52	20	-5.897603	1.11666	Significant
Citrate conc. increased by 0.6 mL	35.79	1.41	20	-6.724562	1.99527	Significant
Centrifuge for 5 min at 1500xg	12.05	1.34	20	-3.281704	0.003923	Significant
Centrifuge for 10 min at 1500xg	11.31	0.76	20	1.057556	0.303519	Not significant
Centrifuge for 5min at 3000xg	11.67	0.92	20	-1.407854	0.175325	Not significant
Centrifuge for 10min at 3000xg	11.33	1.81	20	0.937226	0.360403	Not significant
Storage for 12 hrs at room temp	11.52	0.78	20	-0.095807	0.924676	Not significant
Storage for 24 hrs at room temp	11.61	1.84	20	-0.188435	0.852533	Not significant
Storage in (-40oC) for 48 hrs	11.69	1.65	20	-0.276822	0.784905	Not significant
Storage in (-40oC) for 48 hrs	32.81	1.77	20	-2.439098	0.024706	Significant

[Table/Fig-1]: Comparison of PT in samples with induced processing variables.
*At Normal condition – PT value (blood to citrate ratio 9:1 & proper mixing with 20 min at 1500 centrifugation at room temperature). All samples for variable parameters were compared with samples run in ideal condition.

Parameter for aPTT of samples received for coagulation tests	Mean	SD	No. of Observations on each sample	t-test values	P(T<=t) two-tail values	Level of Significance
In Normal condition*	25.25	1.42	20			
Induced haemolysis	29.21	1.56	20	-2.791964	0.011625	Significant
Citrate conc. increased by0.05ml	29.05	1.23	20	-1.336230	0.197258	Not significant
Citrate conc. increased by 0.1ml	32.55	1.89	20	-6.588252	2.6339	Significant
Citrate conc. increased by 0.2ml	33.67	1.32	20	-4.624850	0.000184	Significant
Citrate conc. increased by 0.3ml	37.08	1.46	20	-3.500018	0.002395	Significant
Citrate conc. increased by 0.4ml	48.98	1.67	20	-8.813327	3.86475	Significant
Citrate conc. increased by 0.5ml	52.84	1.82	20	-8.933777	3.13234	Significant
Centrifuge for 5min at 1500xg	28.04	1.34	20	-3.279916	0.003939	Significant
Centrifuge for 10min at 1500xg	25.40	1.54	20	-1.738160	0.098363	Not significant
Centrifuge for 5min at 3000xg	25.75	1.61	20	-2.191536	0.041075	Not significant
Centrifuge for 10min at 3000xg	25.23	1.76	20	0.200210	0.843443	Not significant
Storage for 12 hrs at room temp	25.50	1.81	20	-0.090130	0.929122	Not significant
Storage for 24 hrs at room temp	33.37	1.86	20	-2.689269	0.014521	Significant
Storage in (-40oC) for 48 hrs	32.81	1.77	20	-2.439098	0.024706	Significant

[Table/Fig-2]: Comparison of aPTT in samples with induced processing variables.
*At Normal condition – aPTT value (blood to citrate ratio 9:1 & proper mixing with 20 min at 1500 centrifugation at room temperature). All samples for variable parameters were compared with samples run in ideal condition.

2) Changing blood to anticoagulant ratio:

To know the variations in results, citrate was added & run in sequential order like: increasing citrate concentration by 0.1 mL, 0.2 mL, 0.3 mL, 0.4 mL, 0.5 mL and 0.6mL. Each vacutte had already fixed quantity of anticoagulant. As shown in [Table/Fig-1,2], PT results

were affected significantly when citrate concentration was increased by 0.4ml and aPTT results were affected more significantly when citrate concentration was increased by 0.1 mL.

3) Centrifugation:

To know the effect of centrifugation time on results of PT & aPTT tests, received samples were centrifuged for 5 min and 10 min respectively. Variations in centrifugation speed were also introduced at 1500xg and 3000xg for each. PT & aPTT value were significantly af-fected when samples were centrifuged for 5 minute at 1500xg only. As concluded from [Table/Fig-1,2], centrifugation of samples for 10 min at 1500xg or 3000xg did not alter the tests' results significantly.

4) Storage Condition:

Samples were stored for 12 and 24 hours at room temperature and for 48 hours at (-400C) to know the effect of storage time on PT and aPTT results. From [Table/Fig-1,2], we see that PT values were not significantly affected for received samples upto 24 hours at room temperature. But aPTT value was significantly affected when sample is stored for more than 12 hours even on freezing temperature.

Observational Study of Various Reasons for Rejection of PT & aPTT Requests (Samples/Forms) in Pre-Analytical Phase:

In observational study [Table/Fig-3,4], show percentage distribution of various causes of rejection of samples during the pre-analytical phase in the year 2014. The total number of samples analysed were 52680 and the total number of rejections were 5370. The data shows that the major reason for rejection in the pre-analytical phase were clotted samples (52.44%), followed by samples rejected due to underfilling (22.85%). Considerable number of cases of rejection due to overfilling of samples, haemolyzed samples and mismatch between label of sample and request form were also observed.

DISCUSSION

The pre-analytical phase includes many manual activities which are more prone to induce errors. Inappropriate collection or processing of specimens can lead to most of the erroneous results.

A. Sample Collection:

Patient identification and sample labeling

Sample volume & sample collection tube

Phlebotomy Technique

B. Sample Processing:

Storage, Agitation, centrifugation & transportation

Delays in transport to the laboratory for processing

SAMPLE COLLECTION:

Minimizing the errors in accurate patient identification is the highest priority for patient safety [6]. Inappropriate procedure for venous blood collection accounts for 60% of the pre-analytical errors [7]. Coagulation tests play a major role in management of patients. Conventional quality control measures focus only on assessing the analytical phase of coagulation testing. Further quality improvement policies should be targeted to monitor the preanalytic variables such as phlebotomy which cannot always be supervised by laboratory staff. Due to high personnel turnover rates, inadequate training, lack of understanding of the importance of good laboratory practices, many errors occur during phlebotomy. These mainly include misidentification of patients, collection of unsuitable specimens for testing due to inappropriate collection site and procedure, inappropriate collection vaccuttees and containers. There has to be internationally accepted standardised guidelines for proper phlebotomy techniques, continuous medical education and training of health care professionals and technicians. The CLSI, in its guidelines for PT and aPTT testing, states that samples with visible haemolysis should not be used due to possible clotting factor activation and interference with end point measurement [8].

The specimen is collected directly into the appropriate anticoagulant evacuated tube collection systems and immediately mixed by gentle inversion thereby minimizing the possibility of inadvertent sample clotting, haemolysis and tube under-filling. Tubes should have a minimum fill volume of 80% of the demarcated level. Proper filling is essential for ensuring the proper blood to anticoagulant ratio. A 3.2% sodium citrate concentration tube is preferred over 3.8% citrate tube [7].

Samples should be processed as shown in [Table/Fig-5] [9], failing which samples should be spun down, the plasma portion removed, and snap frozen to a minimum of -20°C. Only one freeze thaw cycle is permissible [4].

Specimen Processing in Laboratory:

If clots or haemolysis are observed, the specimen must be discarded and recollected. Specimens must be centrifuged within one hour of collection at 1500xg for atleast 15 minutes at room temperature that consistently produces platelet-poor plasma.

1. Effect of Haemolyzed Samples on Result of PT & aPTT:

The relative prevalence of haemolyzed specimens is as high as 3.3% of all samples afferent to a clinical laboratory [10]. In the present study, it was found that PT values were not significantly affected however; aPTT values were significantly affected due to haemolysis. It was found that there aPTT had prolonged value in haemolyzed samples and 6.63% samples were rejected due to haemolysis.

Year-2014 (Month wise Record)	Total requests received for PT & APTT	Forms re-ceived with-out Sample	More than mark (overfill-ing)	Clotted samples	Quantity insufficient (underfilling)	Haemolysed samples	High hemat-ocrit	Mismatch: Sam-ple received and requested in the form	Total rejected samples
Jan	4931	20	24	112	54	38	2	6	256
Feb	4647	30	30	102	82	28	2	10	284
Mar	4304	12	38	136	60	44	0	8	298
Apr	4360	40	30	254	112	44	0	4	484
May	4389	28	46	224	134	46	0	0	480
Jun	4036	44	36	230	256	24	0	4	594
Jul	4290	74	24	304	88	14	0	0	506
Aug	4088	60	40	410	90	0	0	6	606
Sep	4216	56	34	280	78	30	2	0	480
Oct	4284	34	60	250	116	18	0	8	486
Nov	4327	42	48	278	84	34	0	4	490
Dec	4808	30	32	234	72	36	2	0	406
Total	52680	470	442	2814	1226	356	8	50	5370

[Table/Fig-3]: Rejection of samples/forms for PT & APTT due to various reasons in 2014

Various reasons for samples rejection	Samples rejected	Percentage of samples rejections due to various reasons
Forms received without Sample	471	8.76%
More than mark (overfilling)	442	8.24%
Clotted samples	2815	52.44%
Quantity insufficient (underfilling)	1226	22.85%
Haemolyzed samples	357	6.63%
Results withheld due to high hematocrit	9	0.15%
Mismatch: Sample received and requested in the form	50	0.93%
Total samples rejected	5370	

[Table/Fig-4]: In 2014, frequency of rejection of samples due to various reasons (Total 52680 received samples for PT & aPTT)

Test	Whole blood specimen (unopened)		Processed and aliquoted plasma (capped)	
	Room temperature	Refrigerated	Room temperature	Refrigerated
Prothrombin time (PT)	Up to 24 hours	Unacceptable	Up to 24 hours	Unacceptable
Activated partial thromboplastin time (APTT) (non-heparin)	Up to 4 hours	Unknown	4 hours	4 hours
Activated partial thromboplastin time (heparinised)	1 hour	Unknown	4 hours	4 hours
Special coagulation testing	4 hours	Unacceptable	4 hours	4 hours

[Table/Fig-5]: Acceptable time delay and short-term storage for coagulation test [9].

According to Arora et al., differences in the results of coagulation tests PT and aPTT performed on haemolyzed and non-haemolyzed pairs of samples from normal individuals were not statistically significant. But on the other hand, the difference between the results of PT and aPTT between haemolyzed and non haemolyzed pairs of samples from patients was statistically significant. This might be due to heparin therapy. Lysis of the platelets along with the lysis of red blood cell could release platelet factor 4 which would have shortened the clotting time by partially neutralizing the heparin [11].

According to present study, the samples sent for PT which are haemolyzed; should not be rejected. It is concluded that issuing results of PT of samples sent for routine screening, which are giving results in normal range, can be reported.

2. Effect of Blood Volume to Anticoagulant Ratio:

Adequate blood volume to anticoagulant ratio is another parameter which affects PT & aPTT results. According to CLSI guidelines, blood & anticoagulant should be taken in 9:1 ratio [4]. PT value was affected significantly when citrate concentration was increased by 0.4ml from baseline (>67%) & aPTT value was affected more significantly when even we increased citrate concentration by 0.1 ml from baseline (>86%). Result of aPTT was more affected in patient population due to depletion of blood to anticoagulant ratio.

According to Chuang J et al., in order to maintain a ratio of 9 parts blood to 1 part anticoagulant, the collection tube must be filled to at least 90% fill volume. Under filling or overfilling results in an imbalance in the blood to anticoagulant ratio which can lead to artificially prolonged or shortened clotting times, respectively [12].

From this study, 22.85% samples were rejected due to underfilling regardless of knowing percentage of underfilling volume to anticoagu-lant. Sample rejection due to underfilling was second most common cause next to clotted samples (52.44%).

3. Effect of Centrifugation:

According to CLSI guidelines, samples should be centrifuged for 15-20 minutes at 1500xg. PT & aPTT are emergency tests & in emergency condition because of necessity of centrifugation in coagulation tests, results get delayed. The centrifugation time is inversely associated with residual volume of blood cell elements, especially platelets [13].

By reducing the centrifugation time for coagulation testing and increasing the speed of centrifugation, blood cells are rapidly separated from plasma. The current laboratory practice is diverse based on local empirical observations [14].

In present study, results of centrifugation of samples for 5 & 10 minute both at 1500xg & 3000xg were analysed. PT & aPTT values were significantly affected when samples were centrifuged for 5 minute at 1500xg only. Centrifugation of samples for 10 minute at 1500xg or 3000xg did not alter the test results significantly. So for centrifugation, minimum 10 minute are required if we adjust centrifugation speed at 1500xg or 3000xg.

According to Kao C-H et al., the results achieved at 7000g for one minute centrifugation in platelet count were equivalent to that achieved by conventional centrifugation. They concluded that this high-speed technique for preparation of Platelet-Poor Plasma (PPP) is a reliable and useful option for minimizing turn-around time for PT and aPTT tests [15].

Lippi G et al., concluded that the centrifugation time was inversely associated with residual blood cell elements in plasma, especially platelets. Statistically significant variations from the reference 15-minute centrifuge specimens were observed for aPTT in samples centri-fuged for two minute at the most. According to them centrifugation for 5-10 minute at 1500xg is suitable for routine coagulation testing [16].

4. Effect of Storage Time & Temperature:

Samples were stored for 12 and 24 hours at room temperature (220c) and for 48 hours at -400C. From [Table/Fig-6] it was concluded that PT values were not significantly affected in received samples but aPTT values were significantly affected when samples were stored for more than 12 hours even at freezing temperature.

Test	Baseline	Sample size	12 h at 220c	24 h at 220c	48 h at -400c
	In received samples:				
PT (s)	11.44	20	11.525	11.61	11.695
p-value			0.92	0.85	0.78
APTT (s)	25.255	20	25.505	33.37	32.81
p-value			0.93	0.01	0.02

[Table/Fig-6]: Results and statistical significances of the coagulation tests of the plasma samples stored for 12 and 24 hours after collection at 22°C and 48 hours at -40°C, compared with the baseline results (According to present study)
*p-value < 0.05 is significant, compared with baseline results.

In other study, coagulation testing was done on seventy-two blood samples after storing them for different durations and at different temperatures. This included the results at 0, 2, 4, 6, 8, 12 and 24 hours at 25°C (room temperature) and 4°C. It was demonstrated that samples for PT could be safely stored for ≤ 24 hours both at 4°C and 25°C; and aPTT for ≤12 hours at 4°C and ≤8 hours at 25°C. The acceptable time interval for aPTT determination was longer than recommended in the CLSI H21-A5 guidelines [9].

Kemkes-Matthes B et al., observed that coagulation tests including PT and aPTT can be tested and reliably reported after storage for 8 hours at room temperature. This time interval can be extended to 24 hours for PT without altering the results [17]. Oddoze C et al., re-ported that acceptable time interval for aPTT determination is 6 hour at 4°C and 25°C [18].

In contrast, Feng L et al., in his studies showed that the acceptable time intervals for PT and aPTT determination were shorter than those recommended in the guidelines [9].

LIMITATION

This study has not taken into account the history of dose of anticoagulant therapy if any administered to the patients. The relationship between degree of haemolysis and the changes in coagulation readings have not been analysed.

CONCLUSION

We hereby conclude that quality maintenance of pre-analytic variables can significantly reduce the errors in PT and aPTT test results. Mislabeled samples or non-labeled samples, and samples without test orders are one of the important causes for sample rejection. To prevent this, labelling of test tubes should always be performed immediately before sample collection. PT of haemolyzed samples sent for routine screening, which are giving results in normal range, should not be rejected. A 67% of the optimum filled volume for PT and 86% of the optimum filled volume for APTT should be acceptable. 10 minute centrifuge time at 1500xg might be suitable for primary tubes collected for routine coagulation testing. For PT, samples can be run within 24 hours at room temperature and in freezing condition it can be stored for 48 hours. But for aPTT, samples should be run within 12 hours of receiving in laboratory.

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