

Pre-Analytical Variables In Coagulation Studies

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Abstract- Background: All coagulation tests critically depend on the quality of the plasma specimens obtained. Pre-analytical variability is a common source of errors in coagulation testing, as clotting assays are susceptible to poor standardization of the whole analytical process. Many misleading results in blood coagulation arise not from errors in testing but from carelessness in the pre-analytical phase. Pre-analytical factors influencing the reliability of laboratory testing are commonplace. Control is critical, since this has a direct influence on the quality of results and their clinical reliability. Internal Quality control should be maintained while performing the tests in the laboratory.

Aim: The aim of the study was to evaluate pre-analytical variables that influence the coagulation tests results.

Materials and Methods: A sample size of 100 were included in the study. The Pre-analytical variables during the sample collection and processing were analysed as per the proforma.

Results: It was found that the entire collection was done by vacutainer system. 83% of the collections were from non-fasting patients. Regarding tourniquet application, all the 100 were collected with tourniquet application. Of that 15 were applied for over one minute and 41% of the samples showed frothing. The tube order (2nd) of collection as per the NCCLS guidelines was followed for 96 samples. Mixing was adequate by inverting the tube (8-10) times for most (97%) of the collections. It was found that, the volume was inappropriate for 26 samples. And it was found that the samples were transported to the laboratory from the outpatient sample collection room within the stipulated time.

Ninety three out of hundred samples were centrifuged at a speed range of 4000-4500rpm. There was a yield of <10000/ul platelet in the PPP from 44 samples which is the acceptable limit, and the remaining 49 samples had a platelet count in the range 10,000-60,000/ul. Of the 49 samples, thirty six samples showed a platelet count in the range 10,000-20,000/ul, 9 samples were in between 21,000-40,000/ul and 3 samples in the range of 41000-60000/ul. Among the 49 samples, only 2 samples showed platelet count of 50,000- 60,000 and their PT and aPTT were affected. The occurrence of high platelet count may be due to the variation in the centrifugation speed. It was found that not even a single sample was lysed or clotted. Although, 83% of the collections were from non-fasting patients, the plasma was clear and there was no significant lipemia. It was also found that the three samples high HCT (> 55%) of the sample if not corrected, leads to false prolongation of the results. The time lapse between the sample collection and the report despatch was analysed and

found that for 92 out of 100 samples, the turnaround time was within the acceptable limits as defined by the laboratory.

Conclusion: Among the pre-analytical variables analysed in the study, tourniquet application, duration of application, order of draw, method of collection and mixing and centrifugation duration were found to be well under control. A better control is required in centrifugation speed to obtain PPP where the platelet count is less than 10000/ul.

Index Terms- Pre-analytical variables, Coagulation, Haemostasis, Icteric, Lipemic, Haemolysis

I. INTRODUCTION

Haemostasis is a host defence mechanism that protects the integrity of the vascular system after tissue injury. The mechanism has several important functions. It helps to maintain blood in a fluid state while it remains circulating within the vascular system, to arrest bleeding at the site of injury / bleeding loss by formation of a hemostatic plug and to ensure eventual removal of plug when healing is complete.[1]

There are five different components of haemostatic mechanism

- Blood Vessels
- Platelets
- Plasma coagulation factors
- Inhibitors
- Fibrinolytic system [2]

Any defect in the haemostatic mechanism can lead to excessive bleeding or thrombosis. Thrombosis occurs when the endothelial lining of blood vessels is damaged or removed. The processes include coagulation and platelet aggregation that cause formation and dissolution of platelet aggregation.[3]

There are four phases for haemostasis:

- (i) Constriction of the injured blood vessel to diminish blood flow.
 - (i) Formation of a loose and temporary platelet aggregate at the site of injury, platelets bind to collagen and are activated by thrombin. After the activation, platelets change their shape and in presence of fibrinogen, aggregate to form haemostatic plug in haemostasis or thrombus in thrombosis.

- (ii) Formation of a fibrin mesh that binds to the platelet aggregate, which gives to a more stable haemostatic plug or thrombus.
- (iii) Partial or complete dissolution of the haemostatic plug or thrombus by plasmin.

Stasis alone may not be a sufficient stimulus for generation of thrombin locally addition of activated clotting factors rapidly results in thrombosis. Blood coagulation takes place by the action of thrombin on soluble fibrinogen and numerous insoluble fibrin threads are formed.[4] Principal function of fibrinolytic system is to ensure that fibrin deposition in excess that required to prevent blood loss from damaged vessels is either prevented or rapidly removes.

The routes by which thrombin forms in cell-poor plasma has been called intrinsic pathway, since it does not require the participation of substance extrinsic to the blood. Most of the clinical investigations are on the intrinsic clotting system.[5] Many of the reactions have been demonstrated with plasma obtained from patients with congenital deficiencies and with partially purified preparations of clotting factors. The laboratory screening tests of coagulation include Prothrombin Time, activated Partial Thromboplastin Time, & Thrombin Time. All coagulation testing is critically dependent upon the quality of the plasma specimen obtained. So, quality control should be maintained while performing the coagulation tests.

Q.C Pre analytical variables
Analytical Variables
Post – analytical variables
In which the analysis part is done by the automated machines and the pre-analytical variables should be monitored and controlled to minimize errors in test results.

Hence, an analysis of pre-analytical variables in coagulation tests is very important because many misleading results in blood coagulation arise not from errors in testing but from carelessness in the pre-analytical phase.

Control of pre-analytical phase is critical, since this has a direct influence on the quality of results and on their clinical reliability. The aim of this study is to evaluate pre-analytical variables that may affect the coagulation tests in our laboratory

II. AIM AND OBJECTIVE

To study the pre-analytical variables that influence the coagulation tests results.

III. MATERIALS AND METHODS

100 outpatient samples received in the central diagnostic laboratory of St. Johns Medical College Hospital for coagulation studies are analysed for pre analytical variables. The following are the pre-analytical variables assessed.

1. Patient Identification
2. Sample Collection
 - Open/Closed, Single/Double puncture
 - Sample volume
 - Type of sample – anticoagulant
 - Method of sample mixing after collection.
3. Transportation

- Timing
- 4. Processing
 - Clot in the sample
 - Centrifugation speed, time and Temperature
 - Clear/turbid/lysed/icteric/lipemic plasma after centrifugation.
- 5. Hematocrit volume
- 6. Measurement of platelet in Platelet Poor Plasma
- 7. PT, aPTT analysis

METHODS:

3.1 Platelet count and hematocrit percentage

Platelet count and HCT% were performed on automated haematology analyser Sysmex XT – 1800 I using anticoagulated fresh venous blood sample based on DC detection method.

3.2 Analysis of PT and aPTT

PT and aPTT were measured by ACL ELITE PRO. The mechanisms used for the measurement of the coagulation end point are conductivity, mechanical resistance, and turbidity.

IV. 4. RESULTS

the study was conducted on 100 outpatient samples received in central diagnostic laboratory of SJMCH, for the coagulation parameters. Present study was performed to analyse the preanalytical variables which may affect the reliability of test reports. All the age groups both males and females were included in the study. The preanalytical variables of coagulation tests were analysed as per the proforma

- Patient status(fasting/non-fasting)
- Tourniquet application
- Order of collection
- Collection method(opened/closed)
- 1/>1 puncture
- Presence of frothing
- Appropriate mixing (inverted mixing for 8-10 times)
- Volume of sample
- Proper centrifugation criteria for PPP
- Packed cell volume of the sample
- Platelet count of PPP
- Plasma status(clear/icteric)& Volume

Fig 1. Preanalytical variables during sample collection

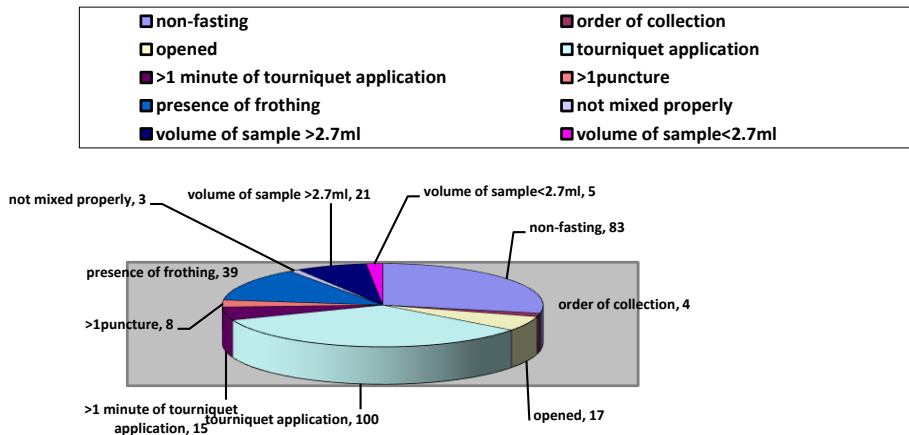


Fig 2. Preanalytical variables of sample processing

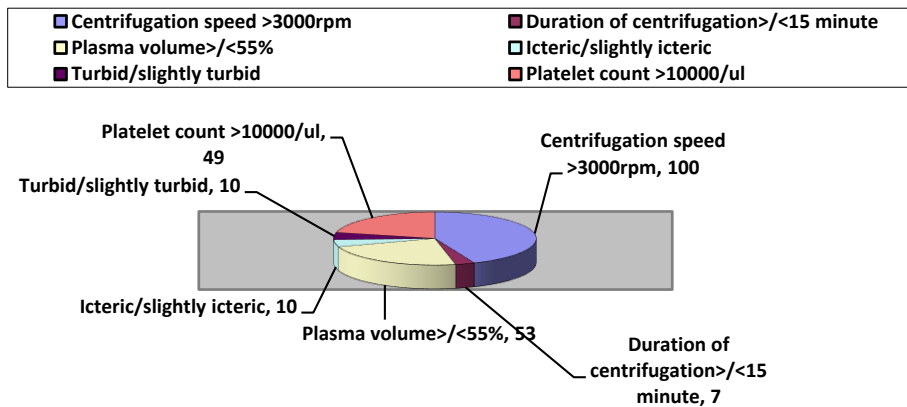


Fig 3. Correlation of variables during sample collection

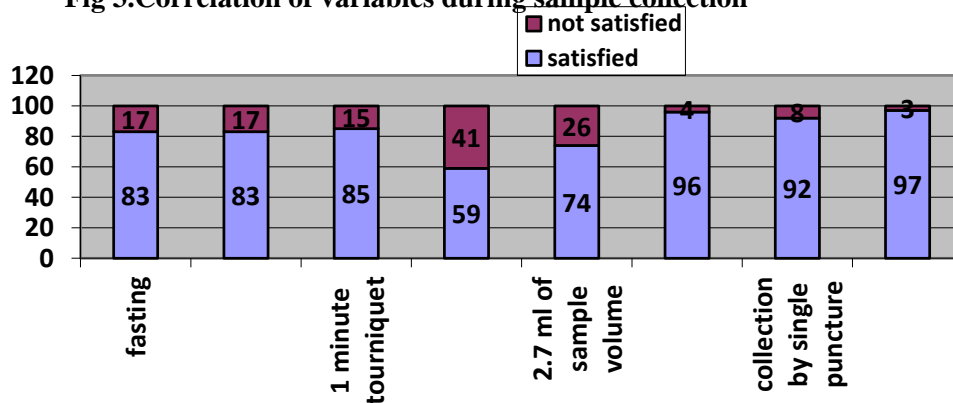
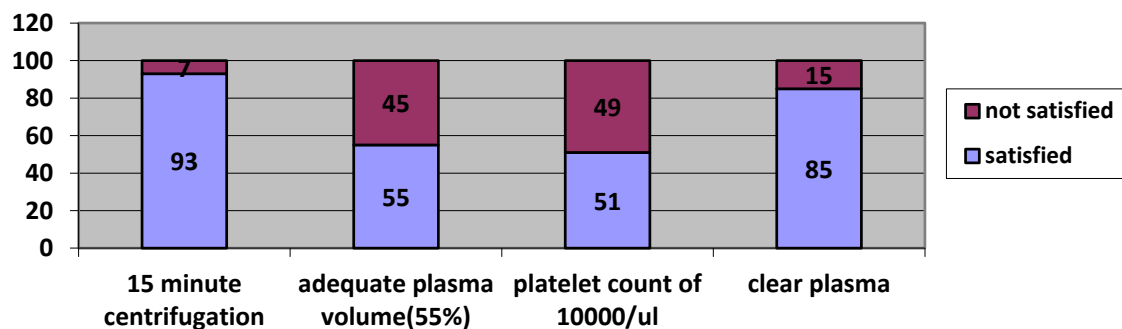


Fig 4. Correlation of variables during sample processing



The pre-analytical variables analyzed as per the proforma are:

Table I Pre analytical variables Phase 1

Patient status	Fasting	17
	Non fasting	83
Method of collection	Closed collection	83
	Opened collection	17
Tourniquet application	+	100
	-	0
Duration of tourniquet application	Duration >1 minute	15
	1 minute	85
Order of draw as per NCCLS guidelines	+	96
	-	4

In the present study, out of 100 patients, percentage of fasting patients is 17 and non-fasting is 83%. Of the total 100 samples except 17, all were collected by closed collection system in the study. Among the 100 samples in the study 96 were collected according to the NCCLS guideline order. (96%)

Table II. Pre analytical variables Phase 2

Collected by (no. of punctures)	>1 puncture	8
	1 puncture	92
Presence of frothing	+	41
	-	59
Proper inverted mixing	+	97
	-	3

Sample volume	Appropriate (2.7 ml)	74
	Inappropriate(>2.7ml)	21
	(<2.7 ml)	5

Ninety-seven of the total samples followed the correct method of mixing. (97 of the total). Out of 100, it was found that the 41 samples showed the presence of frothing in the study. The tube should be adequately filled to 2.7 ml to meet the sample anticoagulant ratio(9:1).In the present study, It was found that 5 were underfilled,21 were overfilled and 74 were adequately filled.

Table III. Pre analytical variables Phase 3

Proper duration of centrifugation	+	93
	-	7
Centrifugation speed	3000 rpm	0
	3500-3700 rpm	7
	4000-4500 rpm	93

Of 100 patients, 93 were found to be centrifuged for appropriate time. Of 100 samples included in the study, not even a single fulfilled the requirement of centrifugation at 3000 rpm. 5 out of the 7 which were centrifuged at a speed range of 3500-3700rpm, yielded a platelet count <10000/µl. Among the 93 samples centrifuged at 4000-4500 rpm, 44 yielded <10000/µl of platelet count.

Table IV. Pre analytical variables Phase 4

Platelet count in PPP	<10000/ul	51
	10000-15000/ul	26
	16000-20000/ul	10
	21000-30000/ul	7
	31000-40000/ul	3
	41000-50000/ul	1
	51000-60000/ul	2
Plasma volume in the whole blood	55%	47
	>55%	42
	<55%	11

Requirement of PPP is mandatory in coagulation tests. Among the 100 total samples, the percentage of samples which met the requirement were 51. And in the rest of 49% platelet count was found to be more than 10000/ul. It was found that, 47% of the total sample, yield 55% plasma volume(1.48ml) of the total volume(2.7ml).

Table V. Correlation of HCT and PT results

HCT (%)	PT (seconds)
56	44.1
55.6	27.4
57	32.1

In the present study, except 3 of the 100, the HCT % was found to be in the range of 15-55%.

Table VI. Plasma status of the centrifuged samples

Status	Percentage
Clear	85%
Icteric	9%
Turbid	5%
Lipemic	1%
Lysed	0%

Although the samples were collected in non-fasting, the number of lipemic sample in the present is 1. And not even a single sample was lysed. The time laps between the sample collection and the report dispatch for 92 out of 100 total samples were found to be in the limit of the turnaround time as defined by the laboratory and it was found that the samples were transported to the laboratory from the outpatient sample collection room within the stipulated time.

In the present study, PT was done on 63 samples. aPTT on 37 samples and out of that both PT & aPTT done on 34 samples. Out of the 63 samples of PT analysis, 36 were prolonged and 13 out of 37 samples of aPTT analysis showed abnormal results. Both PT and aPTT were abnormal in 6 samples. PT prolongation of the 15 samples among the 36 were due to the patient's clinical condition and rest were of some of the preanalytical errors.

V. DISCUSSION

In haemostasis, even more than in other disciplines of biology, quality is determined by a pre-analytical step that encompasses all procedures, starting with the formulation of the medical question, and includes patient preparation, sample collection, handling, transportation, processing, and storage until time of analysis. (6) A sample size of 100 was included in the study which was conducted to analyze the preanalytical variables in coagulation tests. The errors that can occur during the sample collection and processing were analyzed.

5.1 Fasting status of the patient

Non-fasting samples may cause lipemic plasma, which interfere the assay by optical detection method (light scattering). Rajesh kapur and et al in 1996 conducted a study on Postprandial Elevation of Activated Factor VII in Young Adults and they hypothesized that postprandial elevation of FVIIa would produce intermittent activation of factor X to Xa and, subsequently, prothrombin to thrombin. (7)

In the present study, of 100 samples, 83 were collected in non-fasting and only 17 samples were collected in fasting. Among that only 1 sample was found to be lipemic and the PT, aPTT values of that samples were not affected. It was found that non fasting samples do not influence the PT, aPTT values. And the post prandial activation of FVII is not clear from the study.

5.2 Short term venous stasis and influence of frothing

Prolonged tourniquet times will induce increased vessel pressure, hypoxia, and lower pH below the tourniquet, thereby potentially masking mild deficiencies in VWF, FVIII, tPA, and other endothelial associated coagulation proteins. Application of the tourniquet for longer than 1 minute can result in hemoconcentration and endothelial cell release of proteins and elevate coagulation factors such as FVIII, VWF, and tPA, thereby affecting the accuracy of the diagnosis of coagulation defects. Prolonged tourniquet application may also create an acidic microenvironment, potentially leading to factitious prolongation of clotting assays. (8)

Among the 100 samples in the present study it was found that, tourniquet was applied in 85 patients for 1 min and 15 patients for more than 1 min and PT, aPTT values were not affected for

the samples consistent with reports of previous investigation. Of 100 samples 41 samples showed frothing and 59 samples did not showed the presence of frothing. It was found that frothing and tourniquet application does not affect the PT, aPTT values.

5.3 Sample volume and method of mixing

Khayati Siham et al in their study, the filling of the tubes, whatever its origin, is evaluated according to the latest recommendations of the GFHT: Recommended filling: to the mark noted on the tube, or more than 90%; Acceptable filling: tube filled to more than 80%; Under-filling: tube filled to less than 80%. (9) The tube should be adequately filled to 2.7 ml to ensure the sample anticoagulant ratio. Specimen tubes should be filled to at least 90% of capacity to avoid falsely elevated PT or APTT results, but values within the reference range may be acceptable even from underfilled tubes. (10)

Volume(ml)	PT(seconds)	aPTT(seconds)
2.4	74.5	-
2.5	22.6	-
1.62	24	54.5
1.89	11.1	-
2.65	14.1	97.9

In the present study among the 100 samples, five were under filled All the 5 were subjected to PT analysis. 3 gave prolonged PT results. The recommendation is to mix the tube properly by inverting the tube 8-10 times for the adequate mixing of the sample with the anticoagulant. (1) Proper mixing can avoid the incidence of clot formation. And the mixing should never be so vigorous as to cause frothing. (11)

5.4 Centrifugation duration

Lippi G and et al performed a study on the influence of the centrifuge time of primary plasma tubes on routine coagulation testing in 2007 to establish a minimal suitable Centrifuge time for primary samples collected for routine coagulation testing. Five sequential primary vacuum tubes containing 0.109 mol/l buffered trisodium citrate were collected from 10 volunteers and were immediately centrifuged on a conventional centrifuge at 1500 x g, at room temperature for 1, 2, 5, 10 and 15 min, respectively. Hematological and routine coagulation testing, including prothrombin time, activated partial thromboplastin time and fibrinogen, were performed. The centrifugation time was inversely associated with residual blood cell elements in plasma, especially platelets.

Statistically significant variations from the reference 15-min centrifuge specimens were observed for fibrinogen in samples centrifuged for 5 min at most and for the activated partial thromboplastin time in samples centrifuged for 2 min at most. Meaningful biases related to the desirable bias were observed for fibrinogen in samples centrifuged for 2 min at most, and for the activated partial thromboplastin time in samples centrifuged for 1 min at most. According to their experimental conditions, a 5-10 min centrifuge time at 1500 x g may be suitable for primary tubes collected for routine coagulation testing. (12)

Ninety-three samples were properly centrifuged for 15 minute in the present study which included a sample size of 100. Seven were centrifuged for less than 15 minute. The present study suggests that no significant differences were seen in the PT and aPTT reports of the samples centrifuged for less than 15 minutes.

5.5 Centrifugation speed

In 2006, Montagnana M and et al performed a study to investigate the influence of Centrifuge temperature on Routine coagulation Testing. They collected samples from patients, and mixed and finally divided into 3 identical 4-ml aliquots. The 3 aliquots were centrifuged at 1500g for 10 min at 4^oc, 12^oc, or room temperature (21^oc). After centrifugation, plasma was separated and immediately analysed. The study suggested that, centrifugation of whole-blood specimens at temperatures different from those currently recommended is not likely to generate significant analytical or clinical biases. (13) In the present study all the 100 samples included in the study was processed at RT.

The recommended speed of centrifugation to yield PPP for coagulation assay is 3000 rpm. In the present study, of hundred samples none of the samples were centrifuged at 3000 rpm. 7 samples were centrifuged at 3500-3700 rpm and out of the 7 samples, in 5 samples, there was a yield of <10000/ul platelet and in 2 samples the count was found to be 10000-11000 and the PT, aPTT values were not affected. 93 samples were centrifuged at a speed range of 4000-4500rpm and there was a yield of <10000/ul platelet from 44 samples and in 49 samples platelet count was >10000/ul. Of 49 only 2 samples showed platelet count of 50000-60000 and their PT and aPTT were affected.

5.6 Effect of haemolysis

A study was conducted by Laga and colleagues in 2006 on the effect of specimen hemolysis on coagulation test results. "Specimens from healthy human subjects were subjected to mechanically induced hemolysis, and PT and aPTT results compared with concurrently drawn non hemolyzed control samples. In 50 paired patient specimens, there were statistically significant differences in PT (15.8 ±8.4 vs. 16.3 ±8.7 seconds, p<.01) and aPTT (31.6 ±18 vs 32.5 ±19 seconds, p<.01) between hemolyzed and nonhemolyzed specimens, respectively. Specimens from healthy subjects showed no difference in PT and a minor difference in aPTT." (14)

In 2006 another study was conducted on Interference of blood cell lysis on routine coagulation testing, in which they found that a significant increase occurs in prothrombin time and dimerized plasmin fragment d were observed in samples containing final lysate concentrations of 0.5% and 2.7% respectively, whereas significant decreases were observed in aPTT and fibrinogen in samples containing final lysate concentration of 0.9%. And they conclude as that, although slightly hemolysed specimens might still be analyzable, a moderate blood cell lysis, as low as 0.9% influences the reliability of routine coagulation testing.

In the present study, on 100 samples, vacutainer system was used, and the percentage of lysis was zero. This confirms that, blood collection by vacutainer system by well-trained staffs can avoid the incidence of cell lysis that can occur in a syringe collection.

5.7 Hematocrit percentage of patient sample

Samples with high HCT (>55%, like in severe dehydration, neonates, burn patients & patients of polycythemia vera etc) may lead to factitiously increased clotting times due to presence of excess citrate in the sample [13]. To address this issue of HCT, Labs may use vacuum tubes with a lesser volume of anticoagulant; for example, by using small gauged needle and removing 20% volume of anticoagulant (without eliminating the vacuum). (15) In a study conducted on the Effect on Routine and Special Coagulation Testing Values of Citrate Anticoagulant Adjustment in Patients with High Hematocrit Values, they compared the effect of adjusted and non-adjusted citrate concentrations on coagulation test results in samples from 28 patients with high hematocrit values (55%-72% [0.55-0.72]). Prothrombin time (PT) and activated partial thromboplastin time (aPTT) results from nonadjusted and adjusted samples were statistically different and exponentially increased with increasing hematocrit values. Results for fibrinogen, factor VIII, and protein C activity were statistically different and increased linearly with increasing hematocrit values; however, the difference was not as clinically significant. The protein C antigen value increased with increasing hematocrit values but was not significant. The effects on PT and aPTT are due to a dilutional effect of plasma and an interference effect of the higher final citrate concentration on the clotting test result. For patients with high hematocrit values, citrate concentrations must be adjusted for accurate results. (16)

In the present study, 3 out of 100 samples had high HCT. Since correction of anticoagulant volume was not done, the PT results were prolonged for those samples consistent with reports of previous studies.

HCT Vs PT

HCT(%)	PT(seconds)
56	44.1
55.6	27.4
57	32.1

5.8 Platelet count in PPP

Platelet count in the Platelet poor plasma should be less than 10000/ul. (1) The present study reveals that a platelet count in the range 10000-15000/ul is not significant to affect the PT results. Whereas, platelet count more than 50000/ul was found to correlate with shortened PT values. The platelet count in the PPP is related with the speed of centrifugation. In the present study 7 samples were centrifuged at 3500-3700 rpm and out of the 7 samples centrifuged at 3500-3700rpm, 5 samples, there was a yield of <10000/ul platelet and in 2 samples the count was found to be 10000-11000 and the PT, aPTT values were not affected. Ninety three out of hundred samples were centrifuged at a speed range of 4000-4500rpm. There was a yield of <10000/ul platelet from 44 samples and the rest 49 gave a platelet count in the range 10000-60000/ul. Of 49 only 2 samples showed platelet count of 50000-60000 and their PT and aPTT were affected. Hence it was found that PT was shortened in that two cases probably because of high platelet count in the PPP.

VI. CONCLUSION

Among the preanalytical variables analyzed in the study, tourniquet application, duration of application, order of draw method of collection and mixing, centrifugation duration were found to be well under control. A better control is required in centrifugation speed to obtain PPP where the platelet count is less than 10,000/ul.

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CONFLICT OF INTEREST

There is no conflict of interest

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