



Effect of Time and Temperature Variables on Prothrombin Time and International Normalized Ratio of Plasma in Patients in Gampaha, Sri Lanka

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Abstract: Generating accurate results for Prothrombin time (PT) and International Normalized Ratio (INR), PT/INR test is paramount in treatment monitoring. The diagnosis process measures how long it takes blood to clot. The main objective of this study was to determine the effect of storage time and temperature variables on PT and INR results in patients attending Hematologic clinics in Sri Lanka. This cross-sectional study was conducted during June and July 2021. PT was measured in the plasma samples, stored at different temperatures at baseline (0 hours), and then after 12 hours, 18 hours, and 24 hours after specimen collection. INR was calculated from each result, and the deviation percentage of PT and INR was calculated compared to the baseline result (0 hours). Then PT and INR percentage deviation is grouped as <10% and >10% to find biologically significant variations while paired t-test was performed to find statistical significance using GraphPad prism 8.4.3. At refrigerated temperature, both PT and INR results showed >10% variation once kept 24 hours, and this variation was statistically significant ($p < 0.01$, Wilcoxon matched paired sign ranked test). The plasma specimens kept at laboratory temperature and specimen collection room temperature for 18 hours indicated >10% variation of PT and INR values, while these variations were significant at the same criteria above. These results suggest that the separated plasma could only be recommended to test up to 12 hours. Therefore, the laboratories that operate in the same climatic conditions can now use these results to plan the specimen collection accordingly.

Keywords: International normalized ratio; prothrombin time; storage time.

INTRODUCTION

Prothrombin time (PT) and International Normalized Ratio (INR) are used to identify patients with bleeding and coagulation disorders (Shikdar and Bhattacharya, 2018). PT is used to evaluate the coagulation process, especially tissue factor (TF) activities and common coagulation pathways (Smith et al., 2006). INR, which is calculated using patient prothrombin time and ISI (International Sensitivity Index) provided by the manufacturer of tissue thromboplastin reagent also used in treatment monitoring. Specifically, PT/INR values are used to monitor the treatment of patients on oral anticoagulants. For example, patients on warfarin treatment minimize the risk of thrombosis due to deep vein thrombosis (Yamashita et al., 2019; Loeliger et al., 1985) and cardiovascular diseases, including ischemic heart disease congestive heart failure (Shikdar and Bhattacharya, 2018). PT/INR could also provide different

indications for diagnosis. For instance, it evaluates the extrinsic clotting pathway (Smith et al., 2006), and thereby clotting factor deficiency could result in abnormal PT/INR values (Dorgalaleh et al., 2021; Ciavarella et al., 1987). In addition, patients with liver diseases possess higher PT values as clotting factors are synthesized in the liver (Ozougwu, 2017).

PT/INR values may be affected by different factors. The physiological factors that affect PT/INR results are age and gender. Sivrikaya et al. (2013) identified that PT and INR values of males are significantly higher than female patients. Irrespective of the gender, PT/INR values of the (0-14) age group are significantly higher than in patients with age more than 50 years (Sivrikaya et al., 2013). In addition, factors related to the pre-analytical and analytical phases of the laboratory testing process were identified to affect the PT/INR results (Geest-Daalderop et al., 2005). Among the pre-analytical variables, using proper anticoagulant, that is, 3.2% trisodium citrate (Dufour et al., 2000) and maintaining correct plasma: anticoagulant ratio of 9:1 is crucial in obtaining accurate PT/INR results (Huang et al., 2019; Dufour et al., 2000). Additionally, plasma should be separated from the whole blood within one hour of collection by centrifuging at 1500g for 15 minutes (Boissier et al., 2017). It is essential to have a speed monitor in the sample separation centrifuge, while centrifugation time should be monitored manually when a timer is unavailable (Geest-Daalderop et al., 2005). This standard centrifugation speed and time results in platelet-poor plasma (10,000 platelets/ μ L), and proper centrifugation parameters ensure obtaining the recommended plasma level for PT/INR (Sultan, 2010). Despite the variables mentioned above, storage time and temperature between specimen collection and analysis were essential factors that affect PT/INR assay results (Toulon et al., 2017). It is identified that, in less than 25% of the patients, the INR values could be changed >10% by 6 hours post blood collection when the sample is stored at room temperature or 4-6°C. Further delays of more than 24 hours could change INR by >10% in more than 25% of patients' samples (Valiuddin et al., 2020; Geest-Daalderop et al., 2005).

The study location of this project, the District general hospital (DGH) Gampaha, has more than 600 registered patients at the end of June-July 2021, while there are 30 new patients registered for the treatments every month. All the registered patients attend the clinics that monitor treatments once a month and most of the patients (about 75%) tested for PT/INR (Clinic observations). Therefore, about 20 patients attend the clinic each day (Haematology clinic registers, DGH, Gampaha) (Katulanda et al., 2010). As the storage temperature is the primary factor that decides the accuracy of PT/INR testing results, it is essential to consider the room temperature of the study area. The maximum temperature of the Gampaha district in June-July 2021 was 31°C. The minimum temperature within the year 2019 was 22°C (Annual weather report, 2019), while the temperature of the Hematology laboratory is reported to be maintained at 25°C.

All of the above studies considered the effects when samples were kept either at refrigerated conditions or room temperature, primarily 25°C. The recommendations provided by those studies also indicate a controversy as some studies recommend only 6 hours of storage duration while others recommend 24 hours for both temperatures (2-6°C and 25°C). Moreover, in the hospital setting of Sri Lanka, sample collection is usually performed at room temperature, which is nearly 30°C. Neither the above studies were conducted in Sri Lanka nor evaluated the effects of storage temperatures >25°C. Therefore, it is essential to investigate the impacts of storage conditions, both time and temperature, during the preanalytical phase to optimize the accuracy of the PT and INR results in the Sri Lanka setting. The findings obtained here

will not only be necessary to DGH, Gampaha but also to any hospital that operates within the same climatic conditions throughout the country. Moreover, as shown before, an increased number of specimens due to an increased number of cases for routine testing could cause delays in testing due to the deficiencies of human and physical resources. Therefore, it is paramount to evaluate the effect of storage time and the temperature under local conditions to maintain the integrity of the test results.

MATERIALS AND METHODS

Population, Participants, Study Design, and Setting

Patients attending the HEC, hematology clinic District General Hospital, Gampaha, Sri Lanka, have been considered the population of this study. Participants for the study will be recruited during June and July 2021. According to the cross-sectional study in the hematology clinic (HEC), District General Hospital, Gampaha, Sri Lanka, this study was done.

After considering a 20% non-response rate, 156 patients will be recruited for the study. Participants will be recruited using the convenience sampling method due to the limited time availability for a research project. A comparable number of males and females will be compelled to make the study gender-balanced.

Inclusion and Exclusion Criteria

Inclusion criteria as patients registered in hematology clinic, District General Hospital, Gampaha and age group considered as >19 years. Exclusion criteria as vulnerable participants such as pregnant women and critically ill patients will be excluded from the study.

PT assays and INR calculation

Samples were collected, maintaining proper blood to anticoagulant ratio. 3.6 ml of blood was collected into a sample container with 0.4 ml of anticoagulant (INR tubes containing 3.2% trisodium citrate.). Temperatures are measured by a thermometer at the sample collection area and testing area and recorded. Platelet poor plasma is obtained by centrifugation (Thermo Fisher Scientific, US) of the sample at 2500 r.p.m. for 15min. Collected plasma is then aliquoted into 12 containers (one aliquot > 200µl). Label aliquots as 0 hours, 12 hours, 18 hours, and 24 hours and the temperature regime administered; room temperature or refrigerated (4°C). Then store two sample sets at the refrigerator (4°C) and room temperature in the laboratory (25°C) and room temperature in the specimen collection area (28-30°C).

Screening Tests in the Haemostasis method are used as, each plasma sample, an aliquot of 100µl plasma is incubated for 5 min at 37°C. Add 200µl of tissue thromboplastin (Manchester Reagent, UK) and start the stop-watch. Measure the time for visible clot formation. The procedure will be repeated for all the other aliquots of different storage times (0, 12, 18, and 24 hours) at different temperatures (room and refrigerator temperatures). A control sample will be tested along with the test samples. A Normal control is made using human plasma (pooled human plasma from a healthy person is aliquoted and frozen at -4°C) with normal (9 – 12 seconds) PT values. Then using patient test value and Control PT value, INR is calculated using the following formula.

$$INR = \left(\frac{PT \text{ value of the test sample}}{PT \text{ value of the control}} \right)^{ISI}$$

International Sensitivity Index (ISI) value is provided by the manufacturer who makes prothrombin reagent.

Validity and Stability of the PT Assay Results

Stability and validity of assay results for PT test after freezing of samples at -20°C retained for up to two weeks (Zhao & Lv., 2013). The Clinical & Laboratory Standards Institute (CLSI) guidelines indicate storage at -20°C is acceptable for samples processed within two (2) weeks, given other collection and temperature monitoring standards are followed. Tested samples will be discarded according to the government guidelines (Annual health bulletin, 2018).

Percentage Deviation Analysis

PT and INR deviation were calculated by subtracting baseline values from stored specimen values from 12, 18, and 24 hours. The result is then divided by the baseline value and multiplied by 100 to calculate the % deviation. PT and INR percentage deviation were grouped as <10% and >10%. Then <10% and >10% groups were represented as a percentage out of 154 samples. Those calculations were performed for each temperature.

Statistical Analysis

Mean and standard deviations will be calculated for different storage times and temperatures. The 0-hour sample will be considered as the baseline. The results following storage for 12, 18, and 24 hours at different temperatures will be compared with the baseline results by using t-tests data are normal and continuous. The stability of the specimens will be evaluated using the percentage difference of PT values. According to the study conducted by Geest-Daalderop et al. (2005), the clinically significant difference is defined as a mean percentage difference of more than 10% if less than 25% of specimens show more than 10% of, considered as a moderate change. Suppose more than 25% of samples show more than 10% of the difference, thought a significant change. Statistical analysis will be done using Computer Applications.

Ethical approval was obtained from the ethics review committee, Faculty of Medicine, University of Kelaniya, Ragama, Sri Lanka P/22/05/2021. Written informed consent will be obtained before sample collection from all participants. Participants will be allowed to withdraw from the study without penalty ensuring voluntary participation.

RESULTS AND DISCUSSION

Demographic Data

One hundred and fifty-four patients out of 156 were agreed to participate in the study giving a response rate of 98.72%. Among these, 81 (52.6%) were males, and 73 (47.4%) were females. Participants were ranged from the age 27-94 years (Figure 1).

Clinical History of Participants

Sixty-five participants out of 154 were treated for heart-related diseases, all of which are on warfarin treatment (Figure 2). Among 65 patients, there were patients with ischemic heart diseases (n=21), patients with prosthetic heart valves and irregular heartbeat (n=18), and patients with previous myocardial infarction (n=26). The second most common was liver disease (n=22). Within the reported liver diseases, there were 12 patients with alcoholic cirrhosis, four (4) of non-alcoholic cirrhosis, and six (6) patients with factor deficiencies. In addition, there were 19 patients awaiting bone marrow investigation. To avoid bleeding risk, all patients were tested for PT/INR together with routine hematological investigations. There were fourteen patients with immune thrombocytopenic purpura, 14 patients with deep vein thrombosis, and 13 leukemic patients. Moreover, there were five (5) patients with post-splenectomy and two (2) patients with hemophilia.

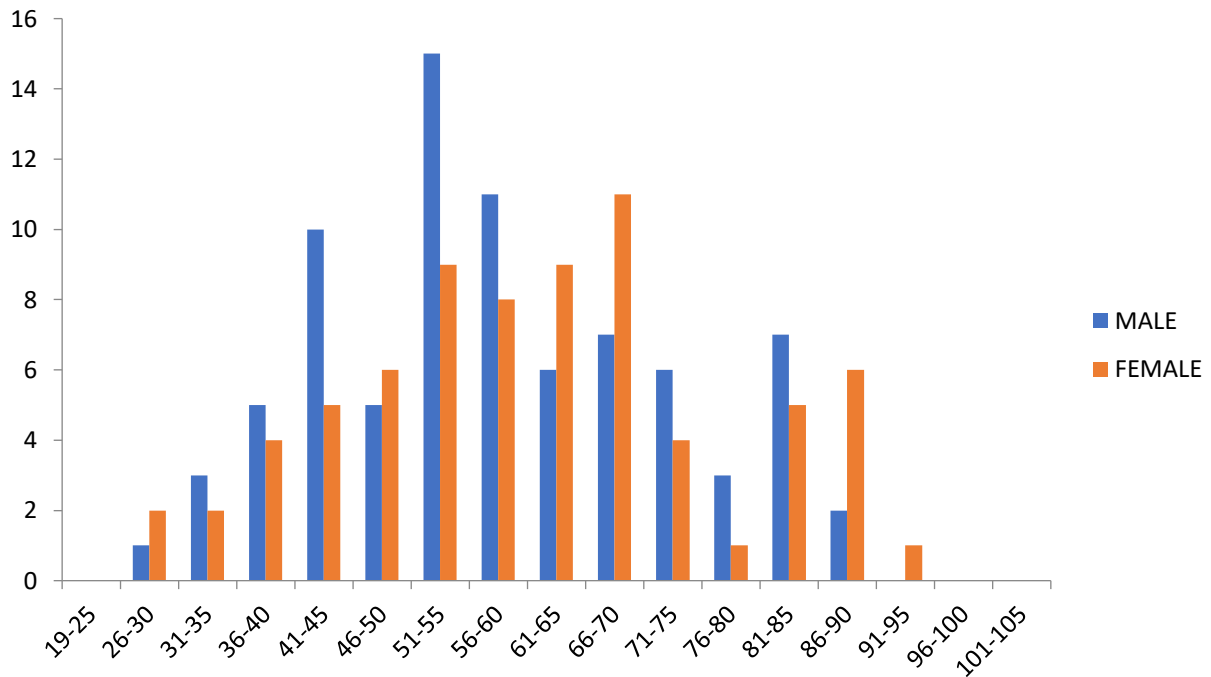


Figure 1 . Age Distribution According to Age and Gender

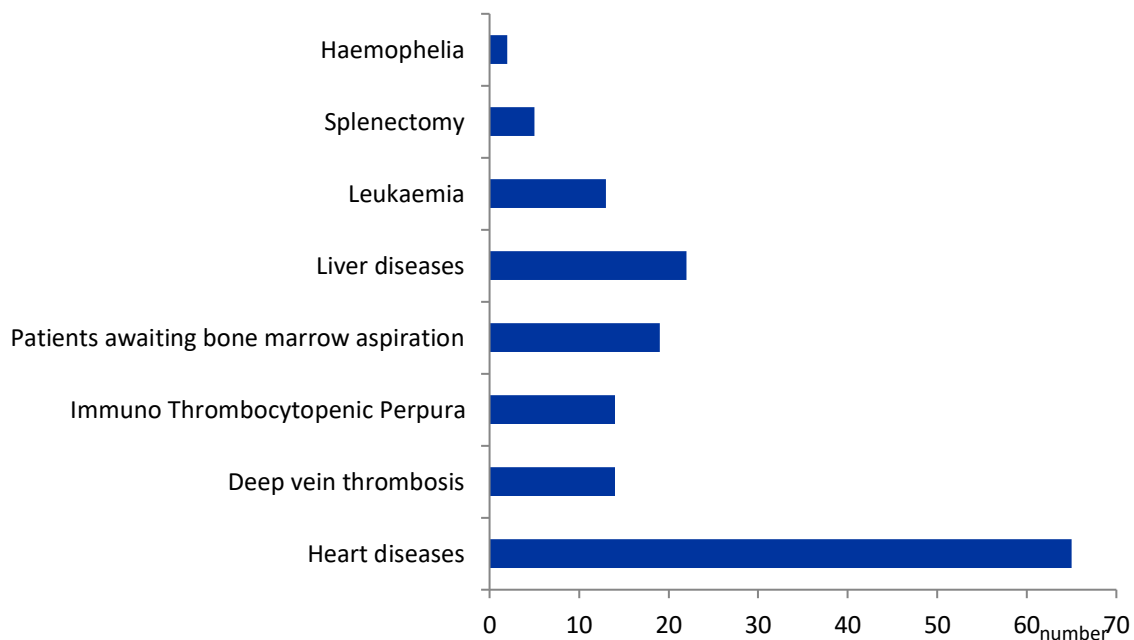


Figure 2. Clinical History of Participants Recruited for the Study

Temperature Measurements

We monitored the temperatures of the specimen collection room, laboratory room, and refrigerator during the analysis period (Table 1). Specimen room temperature was ranged $28.95 \pm 0.95^{\circ}\text{C}$; laboratory temperature fluctuated at $25^{\circ}\text{C} \pm 0.9$ and refrigerator temperature was ranged from $4 \pm 0.1^{\circ}\text{C}$.

Table 1. Temperature Measurements.

	Refrigerator temperature	Temperature inside laboratory	Temperatures inside sample collection room
Minimum	3.9	24	28
Maximum	4.1	25.8	29.9
Mean	3.993	25.03	28.95
Std. Deviation	0.07164	0.3991	0.6191

Changes in PT, Values at Refrigerated Temperature for Different Storage Times

At refrigerated temperature, there were statistically significant differences in PT values at 12, 18, and 24 hours when compared with that of 0 hours ($p < 0.0001$) (Table 2). However, there was no $>10\%$ deviation in the PT test results either at 12 hours or 18 hours compared to those values at 0 hours. Notably, at 24 hours, PT showed $<10\%$ deviation in 22.38% of samples and $>10\%$ deviation in 76.62% samples indicating the samples should only store up to 18 hours in the refrigerator (Table 2).

Table 2. Protrombin Time Values

(A)PT Values at Refrigerator Temperature (4°C)				
	0 hours	12 hours	18 hours	24 hours
Minimum	12.1	12.1	12.2	14.1
Maximum	85.5	85.9	86.2	94.2
Mean	29.54	29.65	29.81	34.19
Std. Deviation	11.91	11.98	12.02	12.83
Paired t-test vs 0 hours		< 0.0001	< 0.0001	< 0.0001
(B)PT Values at Laboratory Temperature				
Minimum	12.1	12.3	13.5	14.5
Maximum	85.4	85.8	91.1	96.9
Mean	29.55	29.64	32.86	35.74
Std. Deviation	11.91	11.98	12.68	13.15
Paired t-test vs 0 hours		0.0103	<0.0001	<0.0001
(C)PT Values at Specimen Collection Room Temperature				
Minimum	12.1	12.2	13.2	14.3
Maximum	85.7	85.8	90.5	96.1
Mean	29.71	29.7	33.12	36.18
Std. Deviation	11.86	11.97	12.54	13.03
Paired t-test vs 0 hours		0.9552	<0.0001	<0.0001

A) refrigerated specimens for different storage times, B) specimens kept at laboratory temperature for different storage, C) specimens kept at sample collection room temperature for different storage times.

Changes in PT, Values at Laboratory Temperature for Different Storage Times

At laboratory temperature, there were no statistically significant differences in PT values of 12 hours compared with 0 hours ($p = 0.01$), while there were statistically significant differences in PT values at 18 and 24 hours when compared with 0 hours

(Figure 3). There was no >10% deviation in the PT test results at 12 hours, whereas at 18 hours PT shows <10% deviation in 42.21% of specimens and >10% deviation in 57.79% samples when compared those values at 0 hours (Figure 3). At 24 hours, PT shows <10% deviation in 18.18% of samples and >10% deviation in 81.82% samples. These findings indicate that it is important to test specimens within 12 hours of storage at laboratory temperature.

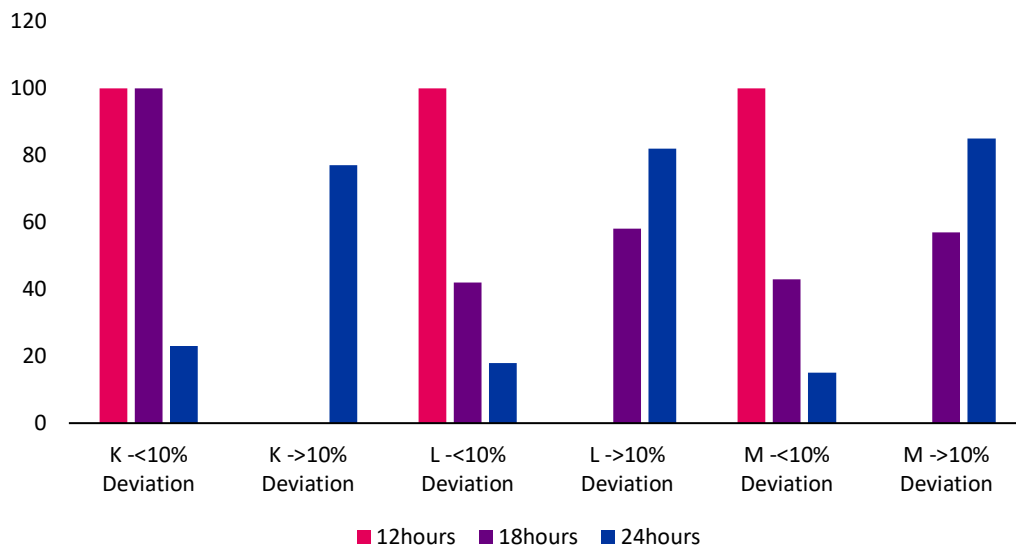


Figure 3. Percentage Deviation in PT Values at 12 hours, 18 hours and 24 hours
K) refrigerated temperature; L) laboratory temperature; M) specimen collection room temperature

Changes in PT, Values at Specimen Collection Room Temperature for Different Storage Times

A specimen collection room temperature, there was no statistically significant difference in PT values of 12 hours compared with 0 hours ($p = 0.95$, Wilcoxon matched paired sign ranked test). In contrast, there was a statistically significant difference in PT values at 18 and 24 hours compared with 0 hours (Figure 3). There was no >10% deviation in the PT test results at 12 hours, whereas at 18 hours, PT shows <10% deviation in 42.86% of specimens and >10% deviation in 57.14% samples when compared those values at 0 hours (Figure 4M). At 24 hours, PT shows <10% deviation in 14.94% of samples and >10% deviation in 85.06% samples. These findings suggest that it is important to test specimens within 12 hours of storage at laboratory temperature.

Changes in INR and Values at Refrigerated Temperature for Different Storage Times

At refrigerated temperature, there were statistically significant differences in INR values at 12, 18, and 24 hours when compared with that of 0 hours ($p < 0.0001$) (Table 3). However, there was no >10% deviation in the INR test results either at 12 hours or 18 hours compared to those values at 0 hours. Notably, at 24 hours, INR showed <10% deviation in 22.73% samples and >10% deviation in 77.27% samples indicating the samples should only store up to 18 hours in the refrigerator (Table 3).

Changes in INR and Values at Laboratory Temperature for Different Storage Times

At laboratory temperature, there were statistically significant differences in INR values at 12, 18, and 24 hours when compared with that of 0 hours ($p < 0.0001$,

Wilcoxon matched paired sign ranked test) (Figure 4). There was no >10% deviation in the INR test results at 12 hours, whereas, at 18 hours, INR shows <10% deviation in 41.56% of specimens and >10% deviation in 58.44% samples when compared to those values at 0 hours. At 24 hours, INR shows <10% deviation in 17.53% of samples and >10% deviation in 82.47% samples. These findings indicate that it is important to test specimens within 12 hours of storage at laboratory temperature (Figure 4).

Table 3. INR Values of Specimens

(A)INR Values at Refrigerator Temperature (4°C)				
	0 hours	12 hours	18 hours	24 hours
Minimum	0.97	0.97	0.98	1.13
Maximum	6.97	7.01	7.03	7.69
Mean	2.385	2.394	2.408	2.764
Std. Deviation	0.9723	0.9781	0.9815	1.049
Paired t-test vs 0 hours		<0.0001	<0.0001	<0.0001
(B)INR Values at Laboratory Temperature				
Minimum	0.97	0.98	1.08	1.16
Maximum	6.96	7.00	7.43	7.91
Mean	2.386	2.393	2.656	2.891
Std. Deviation	0.9726	0.978	1.036	1.075
Paired t-test vs 0 hours		<0.0001	<0.0001	<0.0001
(C)INR Values at Specimen Collection Room Temperature				
Minimum	0.97	0.98	1.06	1.15
Maximum	6.99	7.00	7.38	7.85
Mean	2.399	2.404	2.676	2.926
Std. Deviation	0.969	0.974	1.025	1.066
Paired t-test vs 0 hours		<0.0001	<0.0001	<0.0001

A) refrigerator temperature for different storage times, B) I laboratory temperature for different storage times, C) sample collection room temperature for different storage times.

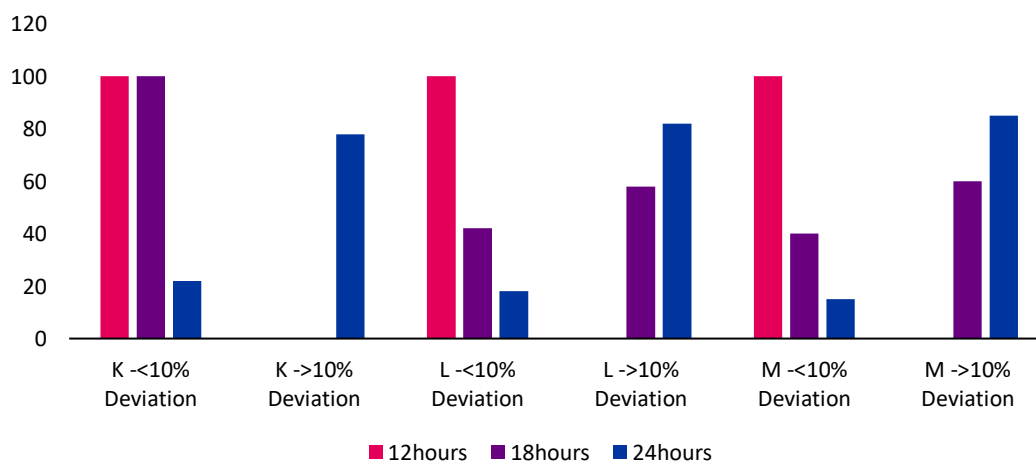


Figure 4. Percentage Deviation in INR Values at 12 hours, 18 hours, and 24 hours K) refrigerator temperature; L) laboratory temperature; M) specimen collection room temperature.

This study is the first study that assessed the effect of temperature and storage time on routine PT/INR testing conducted in a Sri Lankan hospital, as per the available literature. According to the results and calculations of our study, we can conclude that the samples should only store up to 18 hours in the refrigerator ($4 \pm 0.1^{\circ}\text{C}$) and up to 12 hours in laboratory temperature ($25^{\circ}\text{C} \pm 0.9$) and specimen collection room temperature ($28.95 \pm 0.95^{\circ}\text{C}$) for both PT and INR assays. In contrast to our results, Zhao & Lv (2013) study in China says that PT/INR specimens can store up to 24 hours without any changes at 2°C to 6°C and 20°C to 22°C . Geest-Daelderop et al., (2005) study at Netherland identified that the PT/INR results are stable only up to 6 hours at 4°C to 6°C , 25°C , and 37°C . However, similar to our findings, Rao et al., (2000) study in the United States of America says up to 12 hours in laboratory temperature. Then in the case of refrigerator storage, our study is not compatible with previous studies (Awad et al., 2004; Zhao and Lv 2013; Rao et al., 2000). However, our storage period lies mid-range of all previous studies.

Similar to our study, Rao et al. (2000) demonstrated that at laboratory temperature, specimens could store up to 12 hours for both PT and INR. But Zhao and Lv (2013) found that PT/INR specimens can store up to 24 hours, while Toulon et al. (2017) argued that it should only store up to 8 hours at 25°C . While testing, we needed to repeat PT tests for various reasons, including unacceptable results, extreme values of baseline, and incompatibility with previous results and clinical history. However, we didn't have enough volume to repeat tests as the total liquated volume was about 200 μl , and we had to use 100 μl for the first attempt. The remaining 100 μl for repeat test was not always exact due to wastage in pipetting/ air bubbles in the pipette tip, etc. Therefore, it is recommended to have adequate volume for repeat testing. However, it was noted that the participants were not willing to see inserting a 5cc syringe as it uses a high in whole blood for her bore size of the needle, and it is more painful than routine specimen collection. Therefore, future studies should carefully consider the number of storage times and temperatures in designing the study.

Our study's primary objective was to find the maximum delay before testing PT and INR at three different temperatures. However, because the coagulation factors are not stable in whole blood for longer times, we have performed this study after centrifuging and separating plasma and then storing it at the refrigerator for up to 18 hours, at laboratory temperature, and specimen collection room temperature up to 12 hours. Therefore, further studies are recommended to find the effect of temperature on unseparated specimens stored at different periods. According to the findings of this study, we can recommend that if there is a delay due to unavoidable reasons, it is possible to centrifuge the specimens at the sample collection room and process them within 12 hours.

CONCLUSION

We assessed the effects on PT/INR results after storage at sample collection area temperature (29°C), which none of the previous studies have investigated at such a range. Therefore, this study provides an essential insight into all the laboratories that operate under the same climatic conditions. We identified that PT/INR specimens could store up to 12 hours at sample collection area temperature before testing. However, it is essential to note that the samples should be centrifuged before storage in the specimen collection area to maintain integrity even after storing for 12 hours.

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CONFLICT OF INTEREST

The author(s) declare(s) that there is no conflict of interest.

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