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Pre-Analaytical Factors Influencing IL 6 Levels in COVID Positive Patients

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ABSTRACT

BACKGROUND

IL-6 has been a useful marker for diseases which involve immune related reactions. It is a cytokine having both pro- and anti-inflammatory properties. Viral infections like SARS-CoV-2 often enhance the transcription or translation of IL-6 from cells such as fibroblast, mesenchymal, endothelial and other cells.IL 6 is also used in monitoring response to treatment using certain drugs which are being used against SARS-COV-2.As a normal IL 6 level indicated a good prognosis in COVID 19, monitoring of IL 6 levels in patients diagnosed with COVID 19 had plenty of clinical utility. However IL 6 being a cytokine was prone to blood sample handling and storage procedure errors. The study was designed with the intention of detecting pre-analytical factors influencing IL 6 levels in COVID positive patients.

METHOD

Two samples were collected from RT-PCR confirmed COVID-19 cases, one in red Tube & the other in a Heparin tube for 20 patients.IL-6 values were measured for both samples. The samples were further separated into two equal groups, wherein one group was stored at room temperature of 25 degrees centigrade & the other group was stored at 2 to 8 degrees centigrade. The samples stored in different temperatures were further divided into 2 groups to study the effect of centrifuged, i.e., separated plasma & un centrifuged plasma which was in contact with the RBC's.IL 6 was assayed for all the samples & the data was analysed.

RESULTS

Increase in IL-6 concentrations irrespective of whether the sample was serum or plasma, was noted with higher storage temperature and longer the storage time. The longer the plasma was in contact with the RBC's there was an increase in IL 6. We concluded that measurement of cytokine like IL-6 is highly sensitive and pre-analytical errors could lead to erroneous results. Separated serum or heparinized plasma kept at 2 to 8 degrees can keep IL-6 values stable up to 24 hours. If refrigeration is unavailable, separated serum or plasma is recommended for measuring IL-6 levels as soon as possible

KEYWORDS: *COVID-19; IL-6; T-cells; SARS-CoV-2*

ABBREVIATION: IL 6: Interleukin 6; ECLIA: Electrochemiluminescence Immunoassay; RT PCR: Reverse Transcriptase Polymerase Chain Reaction

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INTRODUCTION

IL-6 has been a useful marker for diseases which involve immune related reactions. It is a cytokine having both pro- and anti-inflammatory properties. Viral infections like SARS-CoV-2 often enhance the transcription or translation of IL-6 from cells such as fibroblast, mesenchymal, endothelial and many other cells [1]. This triggering in turn causes a fatal immune reaction to the hyper activated T-cells. Hence accurate IL-6 measurements become crucial in understanding, monitoring and treatment of SARS-CoV-2 infected patients. Though with the advent of newer detection methods like ECLIA the analytical errors might have fallen down drastically, IL-6 measurement is likely to get affected by several pre-analytical variables. This possibly may be attributed to the different changes in the cytokines that may occur during those pre-analytical periods of routine sample collection, transport and processing. With this in mind, we had intended to find the effect of blood sample handling and storage procedures on measurement of IL-6 levels among RT-PCR confirmed SARS CoV-2 infected patient samples.

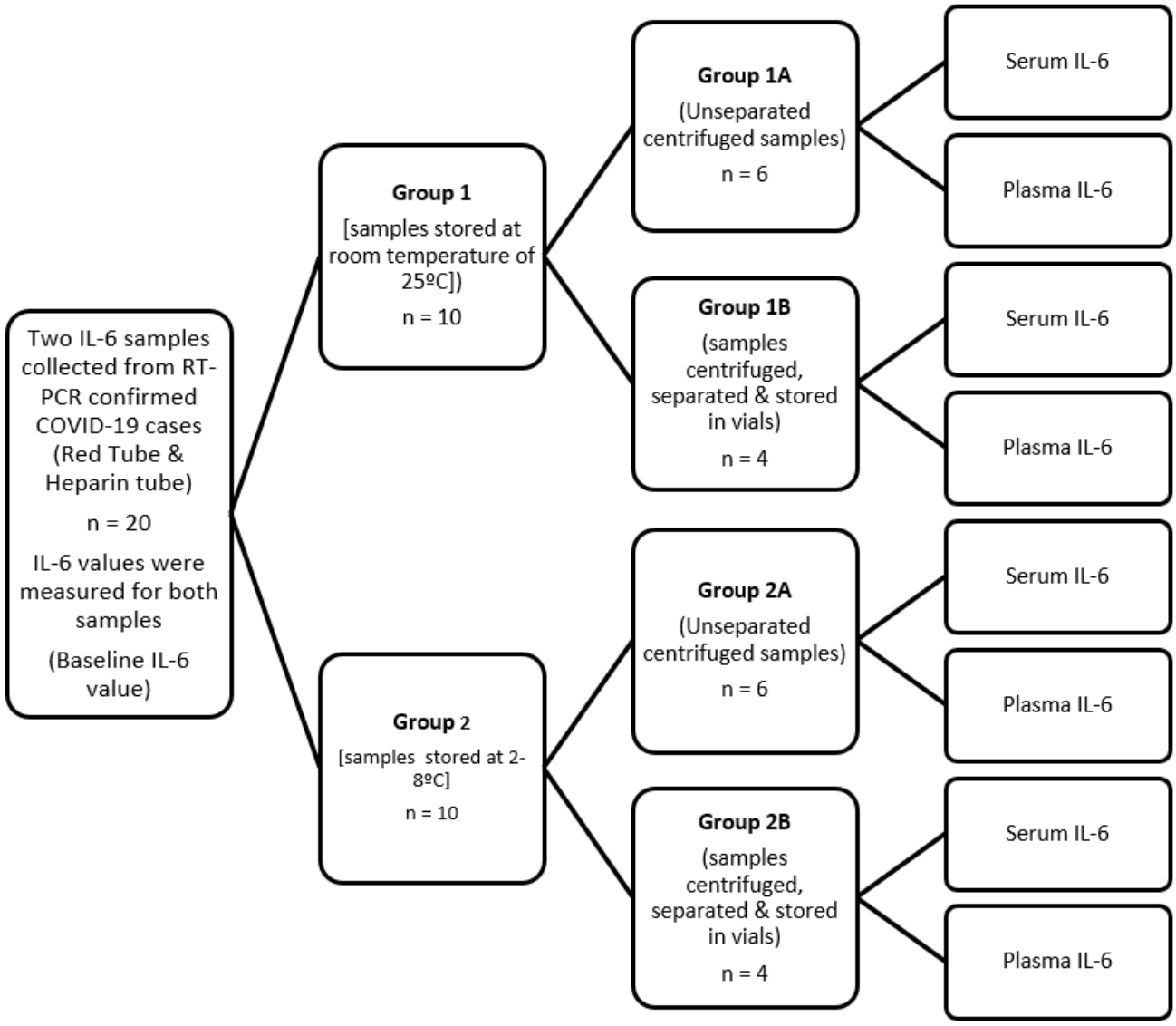

Figure 1: Study design.

MATERIALS AND METHOD

Collection & processing of sample

This study was carried out at Apollo Diagnostics, Regional Research Laboratory (RRL), Chennai for a period of 2 months from September to October 2020. Two whole blood samples from 28 RT-PCR confirmed COVID-19 patients were collected in vacutainer tubes (BD vacutainer clot activator tubes - red top) and plasma tubes (BD Vacutainer green top tubes containing Lithium heparin). After collecting, tubes were gently inverted 10 times for proper mixture of additives. All study participants were divided into four groups with reference to Figure 1 and different handling procedures were performed accordingly. All samples were centrifuged at 1760 g for 15 minutes and one aliquot from each sample that had been centrifuged and tested immediately were considered as baseline serum/ plasma IL-6 values.

Group 1 samples were stored at room temperature (25°C) while Group 2 samples were stored at 2°C - 8°C. Each group is further divided into respective A and B groups where 'A' group comprised of samples which remained unseparated post centrifugation and 'B' group samples were aliquoted after centrifugation. IL-6 readings were taken for each subgroup samples after 4 hours, 8 hours, 12 hours and 24 hours post sample collection. It was ensured that each sample in subgroup had IL-6 readings measured from serum and plasma simultaneously. Random samples were selected for the above process.

Statistical analysis

Statistical analyses were performed using IBM SPSS statistical software, version 20, of Windows. Results were expressed as median and interquartile ranges of 25^{th} and 75^{th} percentiles as the values obtained were found to be non-parametric. Median baseline IL-6 values in serum and plasma were compared using Wilcoxon signed-rank test and Spearman's rank correlation. The Friedman test was used to evaluate the changes in serum or plasma IL-6 values overtime. Probability value (p value) of <0.05 was considered statistically significant.

RESULTS

Out of 28 patient sample sets, eight serum samples showed IL-6 value less than the lower limit of detection (LOD) and were duly discarded. We hence included only 20 patient sample sets for further analysis. The median baseline concentrations of IL-6 values in serum and plasma were shown in Table 1. Baseline IL-6 values were found to be not significantly different between different types of sample among subgroups. Interestingly, baseline serum IL-6 levels were found to be slightly higher than corresponding IL-6 values measured in plasma up to 11 pg/mL, after which the plasma IL-6 values slightly overtakes corresponding serum measurements [serum IL-6 = 0.6088 X (plasma IL-6) + 4.3477] (Figure 2). In addition, significant correlation was found between serum and plasma measurements [$R = 0.976$; $R^2 = 0.953$] (Figure 2).

		n	Type	Median IL-6	Min - Max	Z	*p*
Group 1	**Group 1A**	6	Serum	18.4	13.1 - 104.1	-0.524	0.600
			Plasma	15.3	11.7 - 178.7		
	Group 1B	4	Serum	8.1	3.5 - 22.5	-0.365	0.715
			Plasma	11.0	7.8 - 12.9		
Group 2	**Group 2A**	6	Serum	6.5	2.9 - 9.9	-1.57	0.116
			Plasma	6.6	2.6 - 14.2		
	Group 2B	4	Serum	8.2	2.9 - 23.2	-0.73	0.465
			Plasma	8.8	2.0 - 22.3		

Z → Wilcoxon Signed Rank Test; p <0.05 Considered Statistically Significant

Table 1: Baseline IL-6 values of serum & plasma between groups.

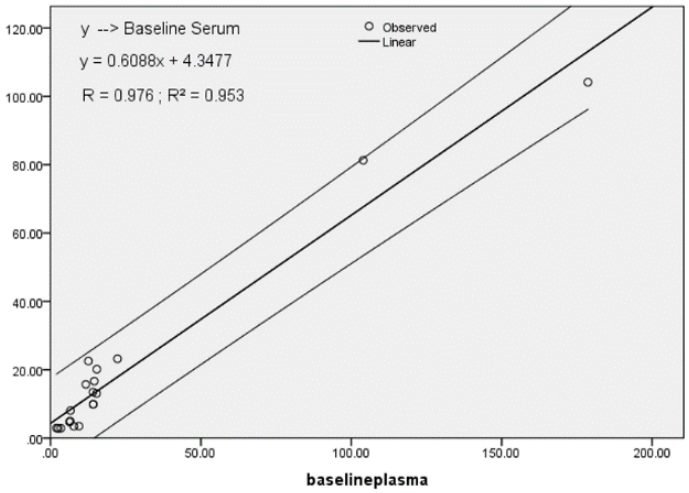

Figure 2: Correlation of baseline concentrations of IL-6 between serum & plasma with 95% confidence interval.

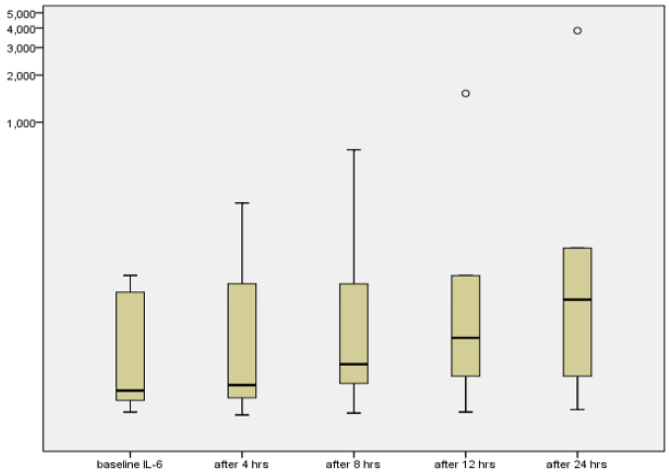

Figure 3: Median changes in IL-6 values at different time intervals.
Figure 3A: Group 1A (Serum) [$X^2 \rightarrow 20.74$; $p < 0.001$)].

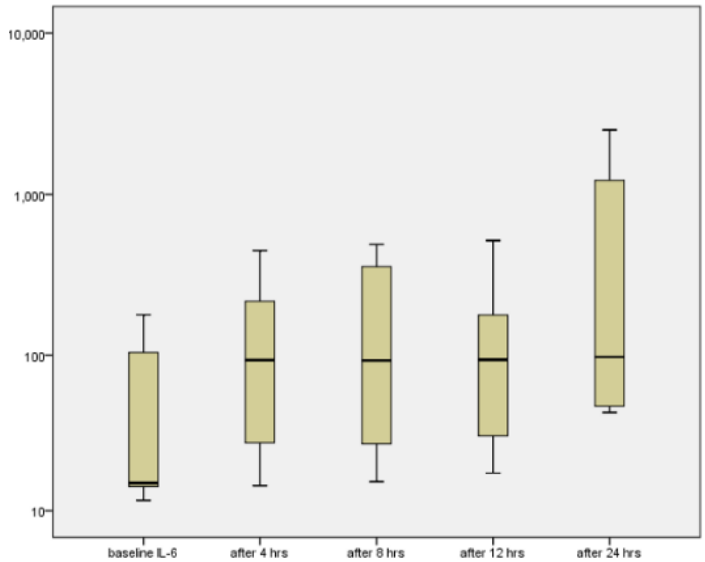

Figure 3B: Group 1A (Plasma) [$X^2 \rightarrow 17.46$; $p = 0.002$)].

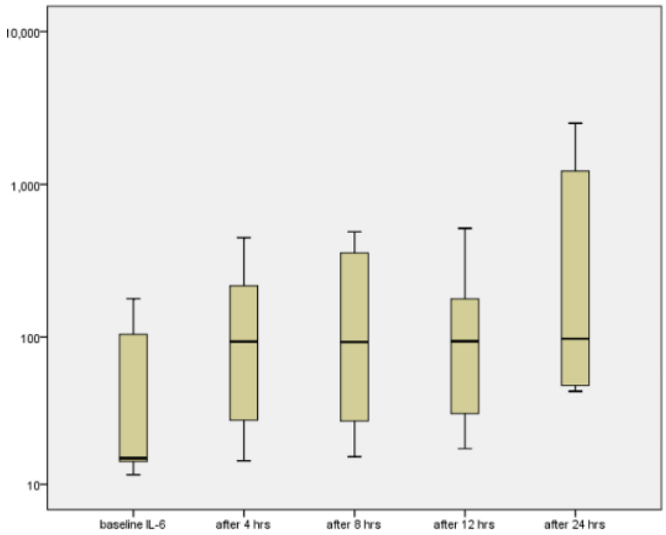

Figure 3C: Group 1B (Serum) [$X^2 \rightarrow 0.20$; p = 0.995)].

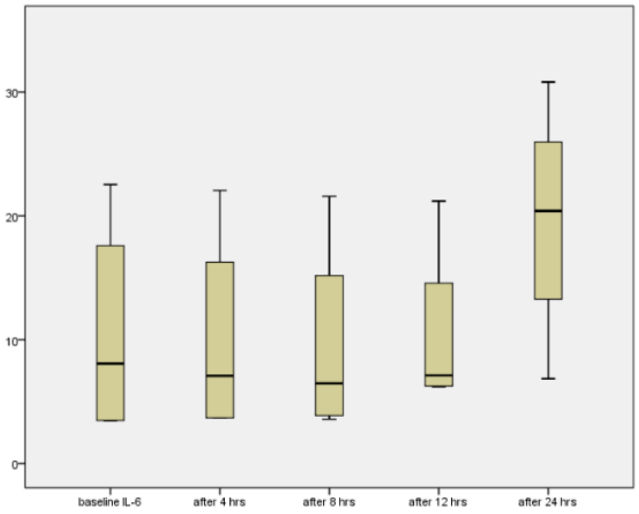

Figure 3D: Group 1B (Plasma) [$X^2 \rightarrow 11.80$; p = 0.019)].

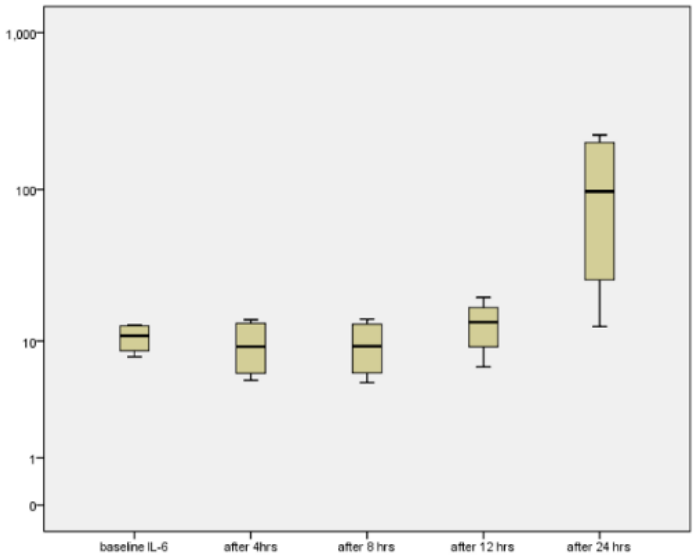

Figure 3E: Group 2A (Serum) [$X^2 \rightarrow 8.50$; p = 0.075)].

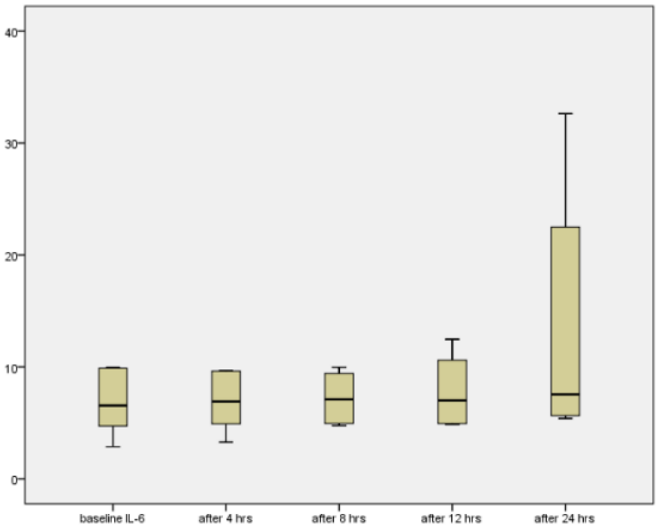

Figure 3F: Group 2A (Plasma) [$X^2 \rightarrow 6.15$; p = 0.188)].

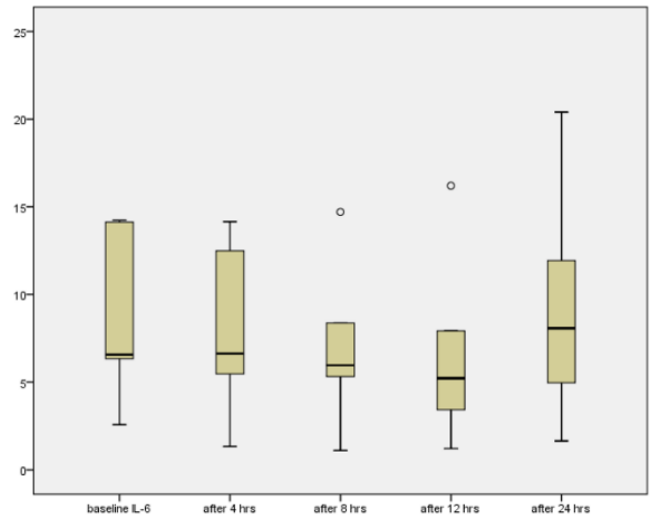

Figure 3G: Group 2B (Serum) [$X^2 \rightarrow 1.40$; p = 0.844)].

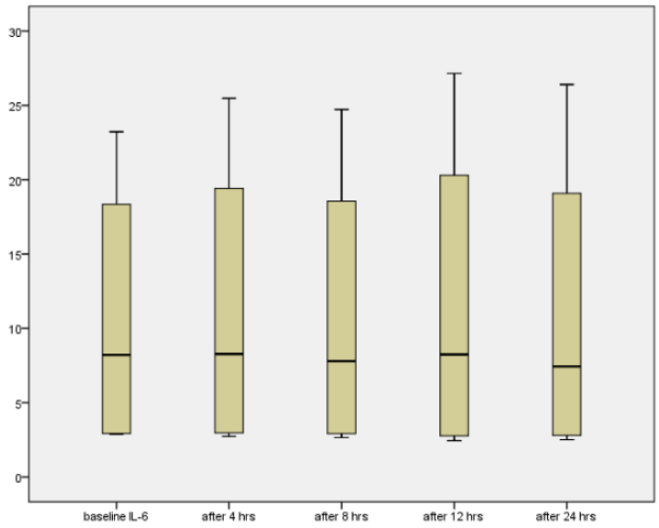

Figure 3H: Group 2B (Plasma) [$X^2 \rightarrow 5.80$; p = 0.215)].

Table 2 shows the changes in IL-6 values from baseline measurements at various storage conditions at different time intervals in serum and plasma. Samples which were centrifuged but not separated into aliquots and stored at room temperature (25°C) showed a statistically significant increase in median IL-6 concentrations in both serum [$X^2 \rightarrow 20.74$; $p < 0.001$] and plasma [$X^2 \rightarrow 17.46$; $p = 0.002$] with increasing storage time. Aliquoted samples stored at room temperature did not show much of a variation from median baseline concentrations up to 12 hours for serum and plasma. The trends with serum and plasma IL-6 values stored at 2°C - 8°C remained unchanged in both unseparated and separately aliquoted samples (Table 2 and Figure 3A - Figure 3H).

In short, higher the temperature and longer the storage time without separation, the more obvious the increase in IL-6 concentrations irrespective of serum or plasma although the increase in plasma is much more rapid and prominent. This could be prominently seen in (Figure 4A – Figure 4D) where the IL-6 values measured at various time intervals at different storage conditions has been represented as multiples of baseline concentration in both serum & plasma. Unseparated samples after centrifugation and kept at room temperature shows an increase of more than five times the baseline value in serum and plasma.

			Median IL-6 values (Serum) [IQR]	X^2	Median IL-6 values (Plasma) [IQR]	X^2
Group 1	**Group 1A**	**Baseline**	18.4 [15.1,87.0]	20.74* (<0.001)	15.3 [13.8,122.7]	17.46* (0.002)
		After 4 hours	20.0 [15.4,145.2]		94.9 [24.6,275.0]	
		After 8 hours	27.6 [18.6,235.7]		94.3 [24.6,389.4]	
		After 12 hours	41.3 [20.4,460.0]		95.5 [27.7,262.7]	
		After 24 hours	84.7 [20.6,1079.0]		100.1 [46.8,1544.8]	
	Group 1B	**Baseline**	8.1 [3.5,20.1]	0.20 (0.995)	11.0 [8.2,12.9]	11.80* (0.019)
		After 4 hours	7.1 [3.7,19.2]		9.6 [5.6,13.7]	
		After 8 hours	6.5 [3.7,18.4]		9.5 [5.5,13.7]	
		After 12 hours	7.1 [6.2,17.9]		13.6 [8.1,18.5]	
		After 24 hours	20.4 [10.1,28.4]		115.4 [22.6,212.2]	
Group 2	**Group 2A**	**Baseline**	6.5 [4.3,9.9]	8.50 (0.075)	6.5 [5.4,14.2]	6.15 (0.188)
		After 4 hours	6.9 [4.5,9.6]		6.6 [4.4,12.9]	
		After 8 hours	7.1 [4.9,9.6]		6.0 [4.3,10.0]	
		After 12 hours	7.0 [4.9,11.1]		5.2 [2.9,10.0]	
		After 24 hours	7.5 [5.6,25.0]		8.1 [4.1,14.1]	
	Group 2B	**Baseline**	8.2 [2.9,20.8]	1.40 (0.844)	8.8 [2.4,20.2]	5.800 (0.215)
		After 4 hours	8.3 [2.9,22.4]		8.7 [2.5,19.8]	
		After 8 hours	7.8 [2.8,21.6]		8.3 [2.2,22.3]	
		After 12 hours	8.2 [2.6,23.7]		7.8 [1.9,21.3]	
		After 24 hours	7.4 [2.7,22.7]		7.5 [2.1,20.7]	

Table 2: Changes in IL-6 values at various storage conditions at different time intervals. **Note:** *p <0.05 considered statistically significant. $X^2 \rightarrow$ Friedman Test (with Chi-square) was used to find the significance of differences in IL-6 values at different time intervals. IQR → Interquartile ranges at 25th & 75th percentile.

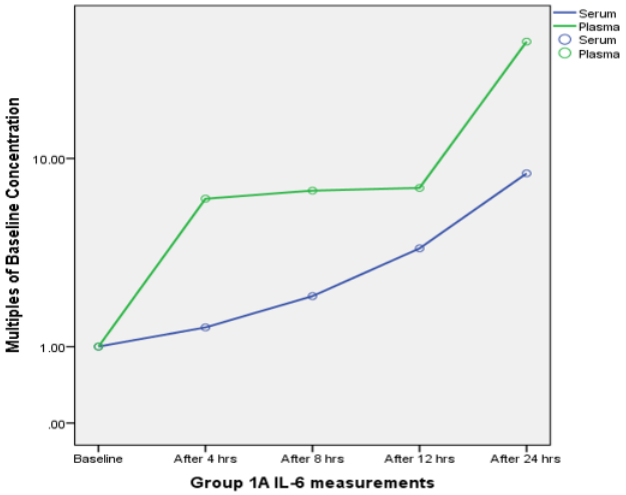

Figure 4: Multiples of baseline concentrations of IL-6 at various time points under different storage conditions.
Figure 4A: Centrifuged but unseparated samples stored at room temperature (25°C).

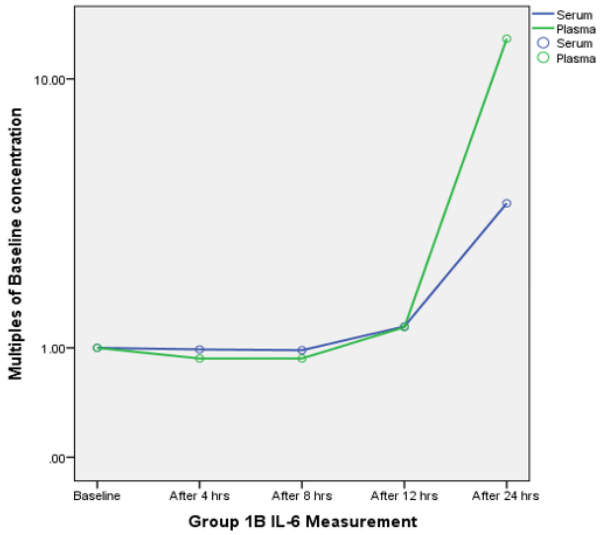

Figure 4B: Centrifuged and separately aliquoted samples stored at room temperature (25°C).

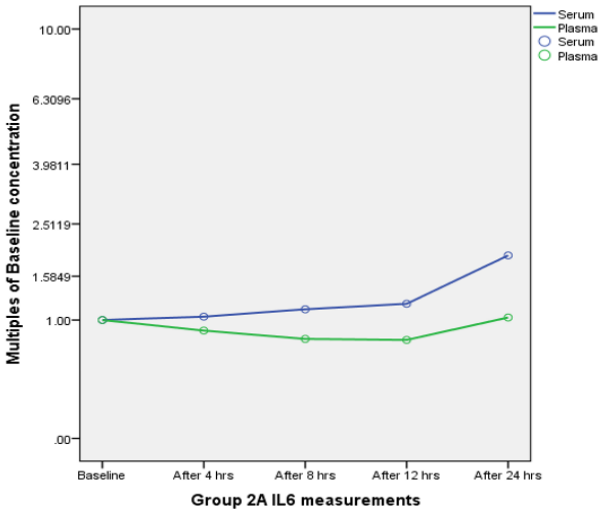

Figure 4C: Centrifuged but unseparated samples stored at 2°C - 8°C.

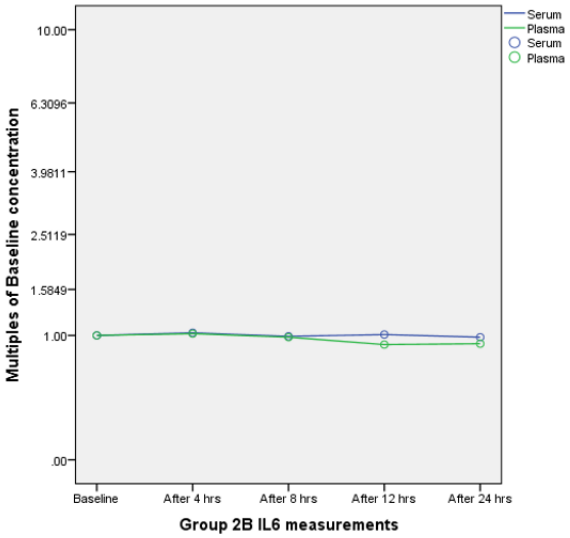

Figure 4D: Centrifuged and separately aliquoted samples stored at 2°C - 8°C.

DISCUSSION

This study was intended to find the effect of blood sample handling and storage procedures on measurement of IL-6 levels among RT-PCR confirmed COVID-19 patient samples. 28 paired serum and plasma samples were grouped into four groups and different handling procedures were performed. To simulate the daily working state, samples in group 1A and 2A were not separated from blood cells. Results indicated that baseline IL-6 values were not significantly different between serum and plasma samples among subgroups. Though the difference is not statistically significant, baseline serum IL-6 levels were found to be slightly higher than corresponding IL-6 values measured in plasma up to concentrations of 11 pg/mL, after which the plasma IL-6 values overtakes corresponding serum measurements. The increases in IL-6 concentration were found to be significantly higher with increase in storage temperature and time, without separation. Also this difference is more prominent in plasma than that in serum especially for samples stored at room temperature.

Previously, most studies used serum for cytokine measurements [2]. The manufacturer of many ELISA and immunoassay kits for cytokine analyses also recommends using serum samples for the analyses. However, several studies indicated that coagulation activation is linked to inflammation in a process called immunothrombosis [3]. When blood is drawn into plastic tubes, complement and coagulation activation by the plastic surface leads to the activation of immune-competent cells and platelets, in part due to their recognition of this foreign surface. This phenomenon of activation by foreign surfaces is termed bioincompatibility [4]. Complement is rapidly activated by plastic and other foreign surfaces and participates in the activation of platelets and leukocytes [5]. Hence coating plastic tubing with heparin effectively reduces complement activation and cytokine release [6]. Many studies have shown significant differences in cytokine measurements between different sample types. While some demonstrated that basal concentrations were higher in plasma than that of serum, some studies have not [7-10]. Our study did not exhibit a statistically significant difference in baseline serum and plasma measurements of IL-6 indicating that the complement and coagulation activation were kept minimum possibly due to the enhanced quality of the clot activator tube used.

There is a delicate balance between production and degradation of cytokines *in-vitro*. Various stimuli like coagulation had been confirmed as one of the factors which may induce the release of cytokine [11]. Several studies have demonstrated that activation of blood cells by Factor VIIA, XA, thrombin can induce release of IL-6 from them [12]. Whenever blood is withdrawn in vacutainer tubes, it initiates coagulation pathway with activation of blood cells. This chain reaction is only going to increase further with increased time. We hypothesize this as one of the possible reason for elevation of IL-6 levels with increase in storage time. As any kind of biological reaction requires appropriate temperature, this could act as a potential influencing factor for further increase in IL-6 levels. In our study, the levels of IL-6 were higher compared to baseline, with increase in storage time. This gets even pronounced at room temperature samples than that of refrigerated samples. In fact there has been an increase of more than five times the baseline concentration of IL-6, for samples stored at room temperature with prolonged storage time.

Our study is consistent with the results from other studies that cold storage can maintain stability of cytokines like IL-6 [13]. Many studies have recommended EDTA as an anticoagulant which could stabilize the whole blood through sequestering of calcium ions, in turn stabilizing cytokine levels [14]. However, we used only heparin containing tubes for sample collection as per the recommendations of our equipment manufacturer.

Practically, most of the labs process samples without separation, even after centrifugation. So we intended to see the influence of the contact of serum/plasma with cells, on IL-6 values for the sample. Our results had indicated that there has been an increase in IL-6 levels of not more than two times the baseline value up to 8 hours in case of refrigerated samples (Group 2A - 2B), and up to 12 hours in case of samples stored at room temperature (Group 1B) irrespective of separation status of sample. However un-separated serum or plasma samples kept at room temperature do show a drastic increase in IL-6 levels as early as 4 hours (Group 1A). Samples centrifuged and separated soon after collection and stored at 2 degree - 8 degree had shown values close to the baseline even after 24 hours for both serum and plasma. We hypothesized that sample separation post centrifugation may not have any direct influence like that of storage temperature or processing time, on IL-6 levels, but may influence them in activation of immune cells with time. This is especially seen if the samples remain unseparated, irrespective of presence of additive like heparin or its absence. This could possibly explain the disproportionate increase of IL-6 levels in un-separated plasma samples stored at room temperature compared to that of serum samples (Group 1A). This also confirms the production of cytokines from blood samples in unseparated specimen with increase in storage temperature.

We had intended to simulate the practical working state in labs in our study design in order to document the changes in IL-6 levels. Also not many studies are available documenting the changes of the cytokines in SARS CoV-2 infected patient samples. These we consider as advantages of our study. There are potential limitations in our study too. Firstly we wanted to avoid too much of blood sample collection from a single patient hence each subgroups consisted of different participants. Secondly we felt that sample size was much smaller in each subgroup.

CONCLUSION

Based on our study results we can conclude that measurement of cytokine like IL-6 is highly sensitive and any kind of pre-analytical errors may lead to erroneous results. Separated serum or heparinized plasma kept at 2 degrees to 8 degrees can keep IL-6 values stable up to 24 hours. If refrigeration is unavailable, separated serum or plasma is recommended for measuring IL-6 levels as soon as possible.

FUNDING

No funding was received & the study was done out of academic interest.

AUTHORS CONTRIBUTIONS

Dr.Srivatsan.R is the primary author who led the study & collated the data..Dr.Marquess Raj & Dr.Srivatsva.P researched on the disease & shared expertise on interpretation of the findings.

Mr.Giri Babu N & Mr.Ramesh.P.G helped with organising the study & study material.

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AUTHORS INFORMATION

The primary author is a biochemist with 7 years experience in lab medicine practice.

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