RESEARCH ARTICLE

The Effect of different storage temperatures on complement protein (C3 and C4) levels: A single-centre study

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Abstract

Objective: To compare the effect of storage at 2-8°C and -40°C on temperature-sensitive serum complement protein levels, and to determine the optimal storage temperature that preserves sample integrity during transportation and storage.

Method: The comparative, laboratory-based, experimental study was conducted at the Immunology Laboratory, Indus Hospital and Health Network, Karachi, from August to November 2022. At the collection point, after centrifugation, a portion of each serum sample was transferred to a separate aliquot and frozen at -40°C, while the remaining serum was retained in the original tube at 2-8°C. Samples were transported to and stored in the laboratory at the same temperatures. They were assayed for temperature-sensitive serum complement proteins 3 and 4 by turbidimetry. Data was analysed using SPSS 21.

Result: There were 50 samples that were analysed. Coefficient of variation at the two temperatures was within 8% for 47(94%) C3 (p=0.730) and 46(92%) C4 (p=0.964) levels. Kappa coefficient value was 0.90 for C3, indicating near-perfect agreement, and it was 1.0 for C4, indicating perfect agreement. C3 and C4 levels did not differ significantly between samples stored at 2–8°C and −40°C (C3: mean difference 0.01 g/L; p=0.73; C4: mean difference 0.00 g/L; p=0.96).

Conclusion: Values for complement proteins 3 and 4 were not significantly different for samples stored at 2-8°C or -40°C.

Keywords: Complement system proteins, Temperature-sensitivity, Sample integrity, Temperature, Turbidimetry.

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Introduction

Complements are a large group of serum proteins that play an important role in the body's immune response. They serve as a key component of not only the innate immune system, but also the adaptive immune system.[1] In addition, they play an integral role in inflammation, including in chemotaxis, extravasation of leukocytes, and in increasing vascular permeability.[1]

Serum complement proteins are measured as part of the diagnostic workup in immunodeficiencies or for monitoring disease activity in some autoimmune diseases.[3,4]

Complement proteins are temperature-sensitive or heat-labile.[5] They are rapidly activated at higher temperatures and, therefore, prolonged exposure of samples to such temperatures may result in falsely low levels. Variable temperatures appropriate for sample storage for complement protein assays have been reported in the literature.[6,7] It is extremely important to store and transport blood or serum samples for complement level determination at appropriate temperatures to prevent falsely low levels.

The immunology laboratory of the Indus Hospital and Health Network (IHHN) in Karachi is situated at a drive of approximately 10 minutes from the collection point at the hospital. Blood samples are centrifuged and stored at 2-8ºC at the collection point until they are transported to the laboratory where they are also refrigerated at 2-8ºC till the day of testing.

The current study was planned to compare the existing sample storage temperature of 2-8ºC with -40ºC for complement protein 3 (C3) and complement protein 4 (C4) assays, and to determine the appropriate storage and transportation temperature for C3 and C4 assay samples.

Materials and Methods

The comparative, laboratory-based, experimental study was conducted at the Immunology Laboratory of IHHN, Karachi, from August to November 2022 after approval from the institutional ethics review board.

The sample was raised using convenience sampling technique, and comprised all blood samples requested for C3 and C4 testing by the clinicians regardless of the patients' age, gender or diagnosis. Samples were excluded

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if they were insufficient, haemolysed, lipaemic or icteric. The sample size of 50 was considered practical and feasible given the study design and available resources.

The blood samples were collected in yellow-capped gel and clot activator-containing tubes (serum separating tubes) at the collection point. Part of each sample was stored at -40ºC, while the rest was stored at 2-8ºC (Figure 1). The time taken to transport samples from the collection point to the immunology laboratory was around 10 minutes.

On the day of testing, which was 1-7 days after sample collection, all the samples were allowed to first attain room temperature. They were then transferred into sample cups and their complement levels were assayed by the principle of turbidometry.

Serum C3 and C4 values of each patient were recorded for both samples stored at different temperatures. The levels were categorised into low, normal, and high. The reference range of C3 was 0.81-1.57g/L, <0.81g/L was considered low, and >1.57g/L was considered high. The reference range of C4 was 0.13-0.39g/L, <0.13g/L was considered low, and >0.39g/L was taken as high. These reference intervals were in line with manufacturer's recommendation (Binding Site Group Ltd., Birmingham, United Kingdom, as used on Optilite IE 700).

Data was analysed using SPSS 21. Data normality was assessed using the Shapiro-Wilk test. Comparison of C3 and C4 levels stored at the two different temperatures was done using paired t-test. Mean±standard deviation values and coefficient of variation (CV) were calculated, and CV <8.0% was considered acceptable.[8,9] Agreement between categorical results was assessed using the Cohen's kappa coefficient, while agreement between continuous measurements was evaluated using Bland-Altman analysis.[10] P<0.05 was considered statistically significant.

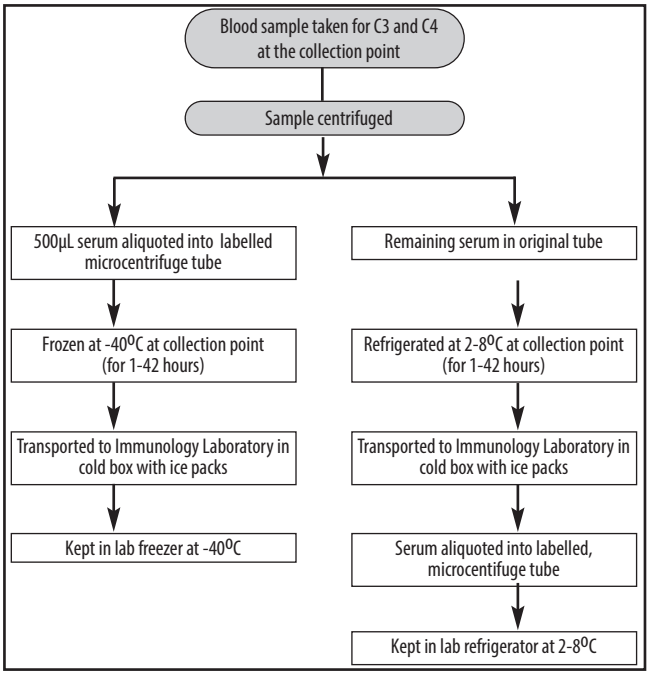

Figure-1: Procedure of specimen handling, including storage and transport.

Results

There were 50 samples that were analysed. CV at the two temperatures was within 8% for 47(94%) C3 (p=0.730) and 46(92%) C4 (p=0.964) levels (Table).

The mean C3 level at 2–8ºC was 1.04±0.35 g/L (range: 0.12–1.76 g/L), while at -40ºC it was 1.05±0.38 g/L (range: 0.10–1.96 g/L). The mean C4 level at 2–8ºC was 0.25±0.11 g/L (range: 0.01–0.76 g/L), while at –40ºC, it was 0.25±0.10 g/L (range: 0.01–0.61 g/L) showing similar mean values at both temperatures.

The mean difference between the two temperatures for C3 was 0.01g/L (95% confidence interval [CI]: -0.04-0.06, p=0.73), and for C4 it was 0.00g/L (95% CI: -0.03-0.03, p=0.96).

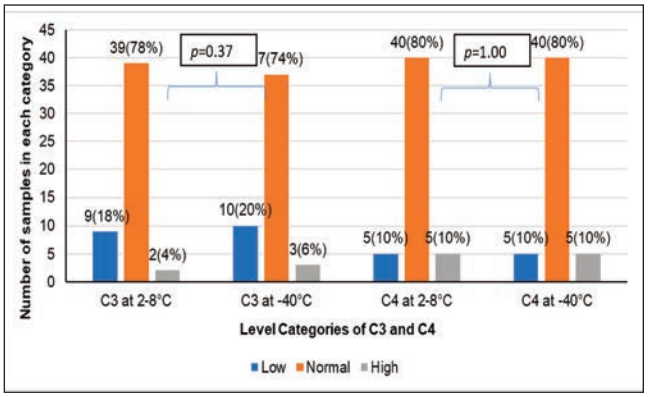

Figure-2: Distribution of serum C3 and C4 concentration categories (low, normal, and high) at storage temperatures of 2-8 °C and -40°C.

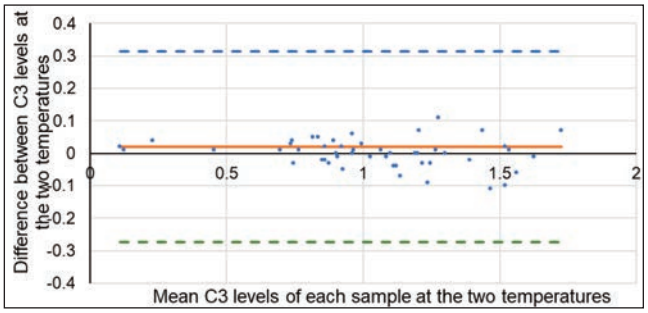

Figure-3: Bland-Altman plot showing agreement between C3 concentrations measured after storage at 2-8 °C and -40°C. The central solid line represents the mean difference (bias=0.02 g/L) between paired values. The dashed lines show the 95 % limits of agreement (mean ± 1.96 standard deviation [SD]). Of the total 59 samples, 46(92%) paired observations fell within these limits, indicating minimal bias and strong agreement between the two temperature conditions.at storage temperatures of 2-8 °C and -40°C.

For C4, all the 50 paired samples remained within the same category at both temperatures, while only minor variation was observed for a few C3 results (Figure 2). No significant association was found between storage temperature and analyte category ($p>0.05$).

Kappa coefficient value was 0.90 for C3, indicating near-perfect agreement, and it was 1.0 for C4, indicating perfect agreement.

Agreement between C3 values at 2-8°C and -40°C was

Table: C3 and C4 levels in samples stored at two different temperatures.

Sample	C3				C4			
No.	2-8°C	-40°C	Mean±SD	CV (%)	2-8°C	-40°C	Mean±SD	CV (%)
1	1.08	1.09	1.08±0.01	1.1	0.32	0.31	0.32±0.01	2.2
2	1.47	1.57	1.52±0.07	4.5	0.48	0.49	0.49±0.01	1.5
3	1.02	1.03	1.02±0.01	1.0	0.25	0.23	0.24±0.01	5.9
4	1.23	1.26	1.24±0.02	1.5	0.23	0.22	0.23±0.01	3.1
5	1.09	1.13	1.11±0.03	3.1	0.27	0.26	0.27±0.01	2.7
6	0.25	0.21	0.23±0.03	11.9	0.06	0.04	0.05±0.01	28.3
7	0.95	1.96	1.46±0.71	49.0	0.18	0.34	0.26±0.11	43.5
8	0.9	0.91	0.91±0.01	0.8	0.31	0.32	0.32±0.01	2.2
9	1.1	1.17	1.14±0.05	4.4	0.3	0.32	0.31±0.01	4.6
10	1.1	1.1	1.1±0.00	0.0	0.35	0.35	0.35±0.00	0.0
11	1.3	1.3	1.3±0.00	0.0	0.25	0.29	0.27±0.03	10.5
12	0.77	0.76	0.77±0.01	0.9	0.17	0.16	0.17±0.01	4.3
13	1.76	1.69	1.73±0.05	2.9	0.17	0.17	0.17±0.00	0.0
14	1.47	1.4	1.44±0.05	3.4	0.21	0.2	0.21±0.01	3.4
15	0.96	0.96	0.96±0.00	0.0	0.4	0.4	0.40±0.00	0.0
16	0.86	0.89	0.88±0.02	2.4	0.4	0.41	0.41±0.01	1.7
17	1.41	1.52	1.47±0.08	5.3	0.33	0.35	0.34±0.01	4.2
18	0.76	0.72	0.74±0.03	3.8	0.27	0.27	0.27±0.00	0.0
19	1.19	1.28	1.24±0.06	5.2	0.26	0.28	0.27±0.01	5.2
20	0.70	0.69	0.70±0.01	1.0	0.76	0.61	0.69±0.11	15.5
21	0.84	0.79	0.82±0.04	4.3	0.29	0.29	0.29±0.00	0.0
22	1.38	1.4	1.39±0.01	1.0	0.22	0.23	0.23±0.01	3.1
23	0.93	0.91	0.92±0.01	1.5	0.25	0.25	0.25±0.00	0.0
24	0.85	0.87	0.86±0.01	1.6	0.17	0.18	0.18±0.01	4.0
25	0.9	0.9	0.9±0.00	0.0	0.26	0.25	0.26±0.01	2.8
26	1.54	1.53	1.54±0.01	0.5	0.21	0.2	0.21±0.01	3.4
27	0.91	0.87	0.89±0.03	3.2	0.24	0.21	0.23±0.02	9.4
28	0.87	0.85	0.86±0.01	1.6	0.16	0.15	0.16±0.01	4.6
29	0.86	0.81	0.84±0.04	4.2	0.25	0.26	0.26±0.01	2.8
30	1.19	1.19	1.19±0.00	0.0	0.14	0.13	0.14±0.01	5.2
31	0.73	0.76	0.75±0.02	2.8	0.3	0.3	0.30±0.00	0.0
32	0.97	0.96	0.97±0.01	0.7	0.14	0.13	0.14±0.01	5.2
33	0.13	0.12	0.13±0.01	5.7	0.25	0.25	0.25±0.00	0.0
34	0.90	0.95	0.93±0.04	4.2	0.22	0.22	0.22±0.00	0.0
35	1.62	1.63	1.62±0.01	0.6	0.11	0.11	0.11±0.00	0.0
36	1.53	1.59	1.56±0.05	3.0	0.29	0.3	0.30±0.01	2.4
37	1.20	1.20	1.20±0.00	0.4	0.27	0.28	0.28±0.01	2.6
38	0.84	0.86	0.85±0.01	1.5	0.2	0.18	0.19±0.01	7.4
39	1.01	0.98	1.00±0.02	2.3	0.11	0.11	0.11±0.00	0.0
40	0.46	0.45	0.46±0.01	1.6	0.22	0.23	0.23±0.01	3.1
41	0.75	0.72	0.73±0.02	3.2	0.03	0.03	0.03±0.00	0.0
42	1.53	1.51	1.52±0.01	0.9	0.19	0.18	0.19±0.01	3.8
43	1.2	1.23	1.22±0.02	1.8	0.37	0.34	0.36±0.02	6.0
44	1.33	1.22	1.27±0.08	6.1	0.41	0.42	0.42±0.01	1.7
45	1.07	1.06	1.07±0.01	0.9	0.35	0.34	0.35±0.01	2.0
46	1.10	1.14	1.12±0.03	2.9	0.36	0.36	0.36±0.00	0.2
47	1.27	1.26	1.26±0.01	1.0	0.33	0.33	0.33±0.00	0.0
48	1.24	1.17	1.21±0.05	4.1	0.19	0.19	0.19±0.00	0.0
49	0.99	0.93	0.96±0.04	4.4	0.21	0.23	0.22±0.01	6.4
50	0.12	0.10	0.11±0.02	13.6	0.01	0.01	0.01±0.00	0.0

CV: Coefficient of variation, SD: Standard deviation.

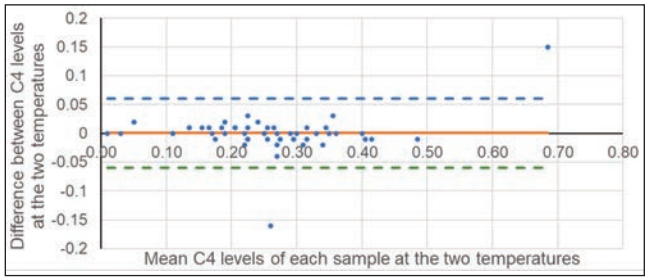

Figure-4: Bland-Altman plot showing agreement between C4 concentrations measured after storage at 2-8 °C and -40 °C. The mean difference (bias=0.0002g/L) is shown by the central line, with dashed lines representing the 95% limits of agreement. Of the 50 samples, 48 (96%) paired observations lay within these limits, indicating excellent consistency between the two temperature conditions.

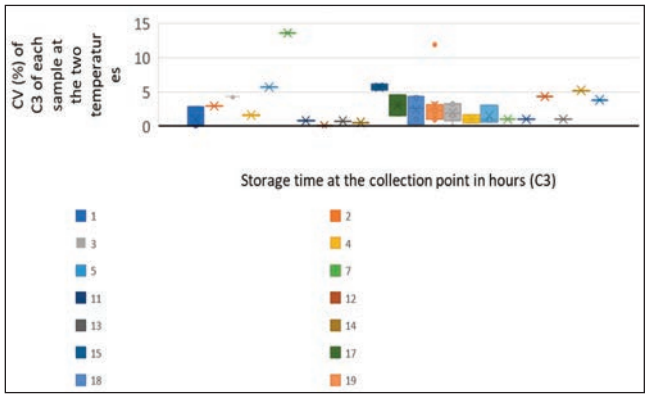

Figure-5: Relationship between storage time at the collection point and the coefficient of variation (CV) for C3 concentrations measured at 2-8 °C and -40 °C. Each box represents the distribution of CV values across different storage durations (1-42 hours). Two outliers with CVs >10% were observed at 7 and 19 hours, but no consistent trend was noted between storage duration and assay variability.

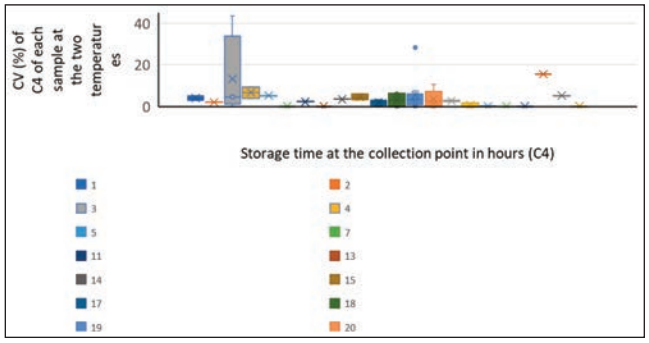

Figure-6: Relationship between storage time at the collection point and the coefficient of variation (CV) for C4 concentrations measured at 2-8°C and -40°C. Four outliers with higher CVs were observed at storage times of 3, 19, 20 and 39 hours. No systematic trend between CV and storage time was apparent, indicating stable assay performance over the studied period.

showed that the mean difference (bias) was 0.002g/L (95% CI: -0.11-0.12g/L), with 46(92%) samples being within these limits, demonstrating negligible systematic bias between measurements obtained at the two storage temperatures (Figure 3).

Agreement between C3 values at 2-8ºC and -40ºC was showed that the mean difference (bias) was 0.0002g/L (95% CI: -0.06-0.06g/L), with 48(96%) samples lying within these limits, confirming excellent agreement and no proportional bias between results at the two storage temperatures (Figure 4).

The duration of sample storage at the collection point before transportation to the laboratory ranged from 1 to 42 hours. The mean storage time in the main laboratory was 17.28±10.09 hours, the median was 18.5 hours (IQR: 13.5–20.0 hours), and the mode was 19 hours.

The relationship between storage duration and measurement variability for C3 showed that two outliers with CVs >10% were noted at 7 and 19 hours, but the overall distribution demonstrated no consistent association between storage time and precision of results (Figure 5), indicating that storage duration within 1-42 hours did not materially affect assay reproducibility for C3.

The relationship between storage duration and measurement variability for C4 showed four outliers at 3, 19, 20 and 39 hours, but no trend in variability across storage durations was observed (Figure 6).

Discussion

Sample storage temperature is a crucial pre-analytical variable for laboratory tests for serum analyte measurements. Determining an optimal sample storage temperature is even more crucial for analytes that are temperature-sensitive. Such analytes include serum complement proteins that are heat-labile[6] and are rapidly inactivated as temperature increases.

The IHHN is a non-profit healthcare centre providing services to all patients free of cost. Thus, most of the analytes in its immunology laboratory are run in batches for which serum samples need to be stored for a few days. Consequently, storage temperature needs to be determined and carefully adjusted to prevent falsely low complement levels at high temperatures.

The current study showed that retaining primary tubes at 2-8ºC for up to 42 hours does not affect the integrity of complement proteins. These findings are useful for forming guidelines for sample transportation to outreach laboratories where the transportation time can be up to several days.

Studies evaluating the effect of storage temperature on serum complement levels, to our knowledge, are scarce. Yang S et al.[6] analysed the effect of short- and long-term storage at room temperature and at 80ºC. They found that storing samples for >4 hours at room temperature caused C3 and C4 degradation, while long-term storage of samples at -80ºC did not affect sample integrity. The current study checked the effect of 2-8ºC and -40ºC storage temperatures over a short period before and after transportation to the laboratory. The study found that the practice of storing samples at 2-8ºC before processing (for up to 7 days) did not affect sample integrity compared to a lower temperature of -40ºC.

Lee DH et al.[7] examined the effect of storage temperature on the protein profile of human serum using mass spectrophotometer proteomic analysis, and reported that the levels of C3, C4, and certain other proteins, decreased with increasing storage temperature from -80ºC to -20ºC, 4ºC and room temperature. Thus, they concluded that serum should be stored at -80ºC for optimal conservation of various proteins. Since the primary objective of the current study was to determine storage temperature from the point of collection down to batch testing in laboratory, the study did not check sample integrity at room temperature or at -80ºC.

Sinosich et al.[11] reported that C3 and C4 levels decreased after 24 and 48 hours of storage at room temperature, while they were stable for a longer period when stored at 4ºC. The current samples were stored for up to 7 days at 2-8ºC and -40ºC with no change in sample integrity.

There were a few outliers for both C3 and C4 in the current study (Figures 3 and 5). However, as the study did not find any correlation between these values and sample storage time or mean C3 and C4 concentrations, it was concluded that they were due to random errors. Moreover, the result category (normal, low, and high) at both temperatures remained the same for six out of seven of these samples. The study also observed that C3 and C4 outliers did not show a decreasing level at higher storage temperatures. This again favoured a random error rather than sample degradation.

The current study has limitations. First, the sample size of 50 was based on a convenience sampling approach, which may have limited the generalisability of the results. The study was restricted to a single-centre setting, and results may vary in different healthcare environments. The samples were only assayed once during each of the two temperature storage conditions due to cost restraints, and the complement levels were not assessed at room temperature. Also, complement split products in the

samples were not assessed to ascertain complement activation. Finally, the study did not investigate the effects of storage at room temperature beyond 7 days.

Conclusion

Serum C3 and C4 values were not significantly different whether the samples were stored after collection in the refrigerator at 2-8°C or in the freezer at -40°C. This is a significant finding because storing samples in the refrigerator is easier, is less labour-intensive, and requires a shorter time for the samples to be brought to room temperature before testing.

Disclaimer: The text was presented as a poster at the Conference of Pakistan Association of Pathologists in 2023.

Conflict of Interest: None.

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Author Contribution:

HMH: Concept, design, data analysis and writing.
AF: Performed complement assays and compiled data.
SAJ: Performed complement assays, compiled data and data analysis.
SA: Design, data analysis, critically reviewed data and proofreading.