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EFFECT OF LONG-TERM STORAGE ON STABILITY OF ADENOSINE DEAMINASE (ADA) ACTIVITY IN PERITONEAL FLUID SAMPLE

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Abstract

Introduction: Adenosine deaminase (ADA) activity in peritoneal fluid is a well-established diagnostic marker for tuberculous peritonitis. However, data on the stability of peritoneal fluid ADA during extended storage are limited, which has implications for retrospective studies and clinical research.

Aim: To evaluate the effect of long-term storage in different temperature(room temperature, 2-4°C,-20°C,-80°C) on ADA activity in peritoneal fluid samples and is to determine the ideal storage temperature and time.

Materials and Methods: This prospective observational study included 31 peritoneal fluid samples were enrolled in the study form Central Biochemistry Laboratory(CBL) after completion of routine requested diagnostic tests. Samples were immediately processed at baseline(<1 hour of sample collection) and compared with values obtained after storage at room temperature(22-24°C), 2-4°C,-20°C,-80°C for 1 day,7 days,30 days,60 days, and 180 days. ADA was estimated using the enzymatic method(Erba Manneim, india) on an automated analyzer. Repeated-measures ANOVA with Bonferroni correction was used for statistical analysis.

Results: Mean baseline ADA activity was 53.75±14.37 U/L. ADA activity showed a significant decline of 11.79 ± 4.42 IU/L($p<0.01$) at Day 180 stored in room temperature(RT). These data indicate marked loss of enzyme activity during prolonged ambient storage. Slight variation in the ADA activity at 2-4°C as compared to RT. ADA activity remained stable from day 1 to 180 days at -20°C and -80°C of storage (mean percentage change is <5.8 from baseline; $p>0.05$).

Conclusion: Peritoneal fluid ADA activity is stable from day 1 to 180 days when stored at -20°C and -80°C. The ADA activity is deteriorated significantly in RT and slightly deteriorated in 2-4°C. Ideal recommended storage temperature and time is -20°C, and -80°C

Keywords: Adenosine Deaminase; Peritoneal Fluid; Specimen Storage; Tuberculosis, Peritoneal; Biomarkers

INTRODUCTION

In India, extrapulmonary tuberculosis (EPTB) has risen from 16-18% of notified TB cases in 2017-2018 to 24% in 2022, with tuberculous peritonitis cases contributing roughly 13% of EPTB[1]. Estimation of adenosine deaminase (ADA) activity in peritoneal fluid increased because it is a reliable accurate biochemical marker for diagnosing tuberculous peritonitis[2]. ADA is considered one of the key enzymes of purine catabolism involved in the breakdown of adenosine to inosine. Its activity is rapidly increased in peritoneal tuberculous effusions due to activation and proliferation of T-lymphocyte at sites of mycobacterial infection[3]. Peritoneal fluid ADA activity cutoff of 30 IU/L shows high sensitivity between 95-100% and specificity between 92-97% for diagnosing peritoneal tuberculosis[4,5]

Long-term storage of biological samples is required in clinical research and retrospective studies. However, enzyme stability during different storage conditions remains a critical concern because it can lead to inaccurate results and misdiagnosis[6]. Previous pleural fluid investigations have shown that ADA activity is stable for up to approximately 2.6 years at -80 °C, followed by a gradual decline in enzyme levels. Although multiple studies have characterized the stability of ADA in pleural fluid under various storage conditions, comparable data for long-term storage of peritoneal (ascitic) fluid ADA remain scarce.[7,8,9].

This study aimed to systematically evaluate the effect of long-term storage at different storage temperature like room temperature, 2-4°C,-20°C, -80°C on ADA activity in peritoneal fluid samples and establish the guidelines for the maximum storage time and best storage conditions for providing reliable data.

MATERIALS AND METHODS

This prospective observational study was conducted in the Department of Biochemistry at a tertiary care Dr Panjabrao Alias Bhausaheb Deshmukh Memorial Medical College in Amravati, Maharashtra, India over a 1-year period (Jun 2025 to Jun 2026). The study protocol was approved by the Institutional Ethics Committee (Reference No:

PDMMC/SS/Ethical/7929/2025) and conducted in accordance with the Declaration of Helsinki. Under waiver of informed consent because leftover samples was used after required diagnostic tests processed.
Thirty one peritoneal fluid samples were enrolled in the study from Central Biochemistry Laboratory (CBL) after completion of routine requested diagnostic tests.
Inclusion criteria: All samples received in the CBL with in the 1 hour of sample collection were included.
Sample size calculation assumed a clinically significant difference of 10% change in ADA activity, with 80% power and alpha=0.05, requiring a minimum of 30 samples.
Exclusion criteria: Grossly hemolyzed, clots, contaminated samples, and insufficient sample volume (< 5 mL)were excluded form the study.
All samples were collected in sterile plain tubes, transported to the laboratory within 1 hour of collection at ambient temperature (20-25°C), and processed immediately upon arrival. Left over samples were aliquoted into 20 Micro-centrifuge tubes(MCT) each tube pipetted for 0.5 mL and tubes were kept in different storage temperature like room temperature, 2-4°C, -20°C, -80°C for time point at day 1, at day 7, at day 30, at day 60, at day 180.

ADA Estimation Method
ADA activity was measured using the enzymatic method, adapted for automated analysis on the Roche Cobas C311 analyzer. The principle involves the adenosine undergo deamination to get inosine. This inosine is converted to hypoxanthine in presence of purine nucleoside phosphorylase(PNP) which is converted to uric acid and hydrogen peroxide in presence of xanthine oxidase. This hydrogen peroxide reacts N-Ethyl-N-3-methylaniline and 4-aminoantipyrine in presence of peroxidase to get quinone dye, measured spectrophotometrically at 540 nm.
Baseline ADA activity was measured within 1 hour of sample collection. Remaining aliquot samples were measured at predetermined time intervals at day 1, at day 7, at day 30, at day 60, at day 180 for all storage temperature like room temperature, 2-4°C, -20°C, -80°C.
At each time point all aliquots at four different temperature, samples were thawed at room temperature, mixed gently, and analyzed immediately. All measurements were performed in duplicate by the same technician using the same reagent lot to minimize inter-assay variability.
Accuracy and precision of ADA was ensured by Internal quality control and External quality assurance. Coefficient of variation (CV) for intra-assay and inter-assay precision was maintained at <4.7%.

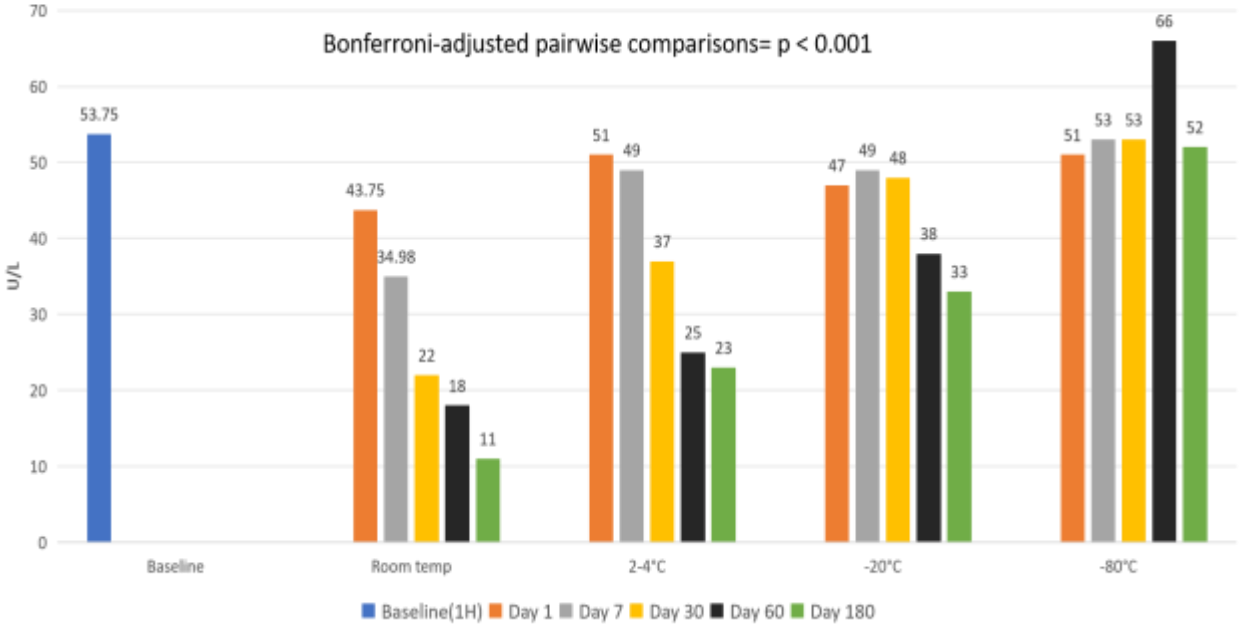
Statistical Analysis
Data were analyzed using SPSS version 26(IBM Corp., Armonk, NY, USA). Continuous variables were expressed as mean±standard deviation (SD) and mean percentage change. Comparison of ADA activity across storage time points with different temperature was performed using repeated-measures ANOVA with Bonferroni post-hoc correction. A p-value <0.05 was considered statistically significant.

RESULTS
Baseline ADA activity
Peritoneal fluid ADA activity at baseline (1 hour) showed considerable variability, with values ranging from 4.34 to 266.00 IU/L in 31 samples. The mean baseline ADA was 53.75 ± 14.37 IU/L, and this same baseline value was used to compare across all four storage temperatures (room temperature, 2–4°C, −20°C, −80°C) and six storage time points(Day 1, Day 7, Day 30, Day 60, Day 180).
Changes in ADA activity over time at different storage temperatures
The ADA activity at each time point and temperature is summarized in Table 1 and Figure 1. At room temperature, mean ADA declined steadily from the baseline 53.75 ± 14.37 IU/L to 43.75 ± 12.51 IU/L Day 1, 34.98 ± 10.92 IU/L at Day 7, 22.08 ± 6.86 IU/L at Day 30, 18.34 ± 7.42 IU/L at Day 60, and 11.79 ± 4.42 IU/L at Day 180. These data indicate marked loss of enzyme activity during prolonged ambient storage.

Temperature	Time	Mean ±SD(IU/L)	Mean % Change vs Baseline	Post-Hoc pairwise comparisons
Baseline*	1h	53.75±14.37	--	F(1.45,42.14)=16.96 p<.001
RT	Day 1	43.75±12.51	-17.2	--
RT	Day 7	34.98±10.92	-35.6	--
RT	Day 30	22.08±6.86	-46.8	--
RT	Day 60	18.34±7.42	-65.7	--
RT	Day 180	11.79±4.42	-80	--
2-4°C	Day 1	51.60±14.04	-4.3	--
2-4°C	Day 7	49.80±13.65	-9.7	--
2-4°C	Day 30	37.30±10.63	-33	--
2-4°C	Day 60	25.52±7.23	-48	--
2-4°C	Day 180	23.21±7.41	-62	--
-20°C	Day 1	47.30±11.86	-2.3	--
-20°C	Day 7	49.03±12.88	-6.7	--
-20°C	Day 30	48.04±13.07	-17.4	--
-20°C	Day 60	38.98±10.50	-25.5	--
-20°C	Day 180	33.39±8.96	-34.3	--
-80°C	Day 1	51.95±13.90	-2.9	--
-80°C	Day 7	53.02±14.27	-2.2	--
-80°C	Day 30	53.00±14.30	-3	--
-80°C	Day 60	66.82±19.17	24.3	--
-80°C	Day 180	52.46±14.15	-5.8	--

[Table/Fig-1]**: The Peritoneal fluid ADA activity at different storage times and temperatures. ADA activity are presented hear as range, mean ±SD(IU/L), mean percentage change vs baseline for four storage temperature (room temperature,2-4°C,-20°C,-80°C) and six storage time(base line, at day 1, at day 7, at day 30, at day 60, at day 180).**

[Table/Fig-2]: This Bar graph showing mean peritoneal fluid ADA values at different storage conditions and time point.



Samples stored at 2-4°C retained higher ADA activity than room temperature at corresponding time points, with mean values of 51.60 ± 14.04 IU/L at Day 1, 49.80 ± 13.65 IU/L at Day 7, 37.30 ± 10.63 IU/L at Day 30, 25.52 ± 7.23 IU/L at Day 60, and 23.21 ± 7.41 IU/L at Day 180. Storage at -20°C preserved ADA even better, with mean values of 47.30 ± 11.86 IU/L at Day 1, 49.03 ± 12.88 IU/L at Day 7, 48.04 ± 13.07 IU/L at Day 30, 38.98 ± 10.50 IU/L at Day 60, and 33.39 ± 8.96 IU/L at Day 180.

The best stability was observed at -80°C. Mean ADA remained close to baseline up to Day 30, with values of 51.95 ± 13.90 IU/L at Day 1, 53.02 ± 14.27 IU/L at Day 7, and 53.00 ± 14.30 IU/L at Day 30. At Day 60 there was a transient increase (66.82 ± 19.17 IU/L), and at Day 180 mean ADA was 52.46 ± 14.15 IU/L, indicating minimal decline over six months of storage.

Percentage change in ADA relative to baseline

To express stability more directly, percentage change in ADA activity was calculated relative to baseline for each sample and storage condition. At room temperature, mean percentage changes were -17.15% at Day 1, -35.63% at Day 7, -46.83% at Day 30, -65.68% at Day 60, and -80.73% at Day 180, confirming severe loss of activity over time. At 2-4°C, ADA declined more slowly but still showed substantial reduction by later time points (approximately -4% at Day 1, -10% at Day 7, around -33% at Day 30, -48% at Day 60, and about -62% at Day 180).

At -20°C, mean percentage change remained close to baseline during the first month (approximately -2% to -7% up to Day 7 and Day 30), with a moderate fall by Day 60 (around -25%) and Day 180 (about -34%). In contrast, samples stored at −80°C exhibited only minimal change throughout follow-up, with mean percentage changes near zero at Day 1, Day 7, and Day 30, a positive shift at Day 60 (about +24%), and a small decline at Day 180 (around -5.8%), indicating excellent long-term preservation of ADA activity.

Repeated-measures analysis: Mauchly's test of sphericity was significant($p \leq 0.001$). After Greenhouse–Geisser correction, the main effects of temperature and time on raw ADA were not statistically significant($p = 0.343$).

In contrast, baseline ADA (1h) had a strong modifying influence. The temperature × baseline interaction was highly significant, $F(2.31, 67.07) = 36.35$, $p < 0.001$, $\eta p^2 = 0.556$, and the time × baseline interaction was similarly significant, $F(1.57, 45.40) = 34.12$, $p < 0.001$, $\eta p^2 = 0.541$. The three-way interaction among temperature, time, and baseline ADA was also significant, $F(1.45, 42.14) = 6.99$, $p = 0.004$, $\eta p^2 = 0.194$, showing that the pattern of change over time across storage temperatures depended strongly on the initial ADA level.

Multivariate omnibus testing supported these findings, with a significant interaction effect (Wilks' $\Lambda = 0.347$, $F(3, 27) = 16.96$, $p < 0.001$, $\eta p^2 = 0.653$). The between-subjects effect of baseline ADA was highly significant, $F(1, 29) = 2707.45$, $p < 0.001$, $\eta p^2 = 0.989$, with Group 2 plateauing at a higher mean response ($\bar{x}$=37.49) compared to Group 1 ($\bar{x} = 26.19$).

Trend analysis and temporal patterns

Within-subject contrasts showed that linear and higher-order trends for time alone and temperature alone were not statistically significant. However, the interaction between time and baseline ADA displayed significant linear, quadratic, cubic, and fourth-order trends, with F-values of 37.791, 4.299, 12.286, and 50.077, respectively, indicating that the shape of ADA change over time differed according to baseline ADA. The temperature × baseline interaction showed a

significant linear trend, F = 14.928, p = 0.001, and several components of the temperature × time × baseline interaction were also highly significant, confirming that temporal trajectories of ADA across temperatures were strongly baseline-dependent.

Graphically, room temperature and 2-4°C showed severe downward decay over time, at -20°C showed a delayed decay pattern at day 30 time point, and -80°C largely resisted decay and even exhibited a peak at Day 60($\bar{x}$ = 66.82 IU/L). Overall, the hierarchy of stability across temperatures, supported by Bonferroni-adjusted pairwise comparisons, placed -80°C as the most stable condition, followed by -20°C, 2-4°C, and room temperature.

DISCUSSION

This study evaluated the effect of storage temperature and duration on the biochemical stability of peritoneal fluid ADA in a tertiary care setting. The baseline ADA values demonstrated substantial variation, reflecting the heterogeneous clinical background of effusions and underscoring the importance of maintaining sample integrity to avoid misclassification.

Our descriptive and percentage-change analyses revealed a clear, clinically relevant hierarchy of stability. ADA activity stored at room temperature showed progressive and pronounced decline, with mean activity falling by approximately 81% relative to baseline after 180 days, consistent with expectations that ambient conditions promote enzymatic degradation. Miller KD, et al. Reported similar results that progressive decline in ADA activity at ambient temperature[10] Refrigerated storage at 2–4°C slowed this process but still resulted in loss of roughly half of baseline activity by six months, indicating that simple refrigeration is insufficient for long-term preservation. Antonangelo et al. reported that pleural fluid ADA remained stable for up to 28 days when samples were stored at 4°C or -20°C, with no significant distortion of enzyme activity, supporting the use of refrigerated or frozen storage for short- to medium-term preservation[11]

Freezing at -20°C offered better stability, with minimal changes in the first month and moderate decline thereafter, aligning with prior pleural fluid studies that support refrigerated or −20°C storage for short to medium durations. However, deep freezing at −80°C provided the most robust preservation: ADA remained essentially unchanged up to Day 30, showed a transient increase at Day 60, and returned to near-baseline values at Day 180, with mean percentage changes clustering within ±10% of baseline. These findings extend previous observations in pleural fluid ADA by demonstrating that peritoneal fluid ADA can be reliably preserved for at least 6 months at −80°C.

Inferential analysis emphasised that the main effects of time and temperature alone were not statistically significant after sphericity correction, whereas interactions involving baseline ADA were strongly significant. This suggests that samples with different initial ADA levels do not behave uniformly under storage, and that high-ADA specimens may be particularly sensitive to pre-analytical conditions. The significant time × baseline and temperature × baseline interactions, together with the three-way interaction, indicate that both the rate and magnitude of ADA change over time depend on baseline concentration and storage temperature. This has direct practical implications: laboratories should recognise that single, generic storage recommendations may not be appropriate across the full spectrum of ADA values.

From a pre-analytical perspective, these data reinforce broader evidence that incorrect storage temperature and prolonged delays can distort analyte concentrations and compromise diagnostic accuracy. For ADA in peritoneal fluid, the results support immediate analysis whenever possible; when delays are unavoidable, short-term storage at 2-4°C or −20°C may be acceptable, but long-term storage should be at −80°C to minimise non-biological variation. The exploratory low-ADA subgroup analysis, which showed similar qualitative patterns but lower absolute values, suggests that the advantages of -80°C storage apply both to low-ADA and higher-ADA specimens.

Limitations include the focus on a single parameter(ADA) and single sample type.

Future work should extend this stability assessment to other key biochemical parameters like protein, LDH, glucose, cholesterol in pleural and peritoneal fluids, and examine storage time and temperature.

CONCLUSION

Peritoneal fluid ADA activity is highly sensitive to storage temperature and duration. In this study, room temperature and simple refrigeration at 2-4°C were associated with substantial decline in ADA over 6 months, while freezing at -20°C provided intermediate stability, preserving most activity for the first month but allowing moderate loss thereafter. Deep freezing at -80°C gives highly stable values, maintaining ADA close to baseline even at 180 days, and repeated-measures analysis showed that these patterns were strongly influenced by baseline ADA levels.

For clinical laboratories, these findings underline the importance of appropriate pre-analytical handling of peritoneal fluid samples. Whenever feasible, ADA should be measured as soon as possible after collection; if analysis must be delayed, storage at −80°C should be considered the preferred option, especially for specimens with high baseline ADA where diagnostic decisions are most critical. Adopting such temperature and time specific protocols can improve the reliability of ADA-based interpretation in peritoneal effusions, reduce pre-analytical errors, and support more accurate differentiation of underlying disease processes.

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