The Effect Of Storage Duration On The Stability Of Immunochromatographic Test (Ict) Hepatitis B Surface Antigen (Hbsag) Test Results In Normal And Moderately Hemolyzed Serum

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ABSTRACT

The examination of Hepatitis B Surface Antigen using the immunochromatographic test is a widely utilized screening method for diagnosing Hepatitis B. Test accuracy is significantly influenced by preanalytical factors, including storage duration and specimen condition. This study aimed to determine the effect of storage duration on the stability of Hepatitis B Surface Antigen test results in normal and moderately hemolyzed serum. A quantitative laboratory experimental design was applied. Samples were divided into normal and moderately hemolyzed serum groups, then examined at zero, four, and eight hours of storage. Result stability was assessed based on the test line intensity score on the immunochromatographic cassette. Data were analyzed using the two-way analysis of variance test. Results demonstrated that the mean intensity score in normal serum decreased from a strong to a weak line category as storage duration increased. In moderately hemolyzed serum, the intensity decline was more profound, approaching a negative category at eight hours. The statistical analysis showed that storage duration, serum condition, and their interaction significantly affected result stability. In conclusion, prolonged storage and hemolysis deteriorate result stability, emphasizing the necessity of stringent preanalytical factor control in laboratories.

Keywords: Hemolysis; Hepatitis B Surface Antigens; Immunochromatography; Preanalytical Phase; Specimen Handling

INTRODUCTION

Hepatitis B is an infectious disease that remains a serious global health challenge today, with high morbidity and mortality rates [4, 11]. The infection caused by the hepatitis B virus (HBV) can progress to chronic liver disorders, cirrhosis, and life-threatening hepatocellular carcinoma [11, 12]. In the effort to reduce transmission rates and determine the appropriate clinical management of patients, establishing an early diagnosis plays a highly crucial role [2, 11]. One of the primary screening methods most frequently relied upon in healthcare facilities is the Hepatitis B Surface Antigen (HBsAg) test [2, 3]. Among the various available modalities, the Immunochromatographic Test (ICT) or rapid test method has become a popular choice because its procedure is simple, time-efficient, economical, and does not require complex special instrumentation, making it highly ideal for basic laboratory services as well as mass screening in the field [1, 2].

Although the ICT method offers various operational conveniences, the reliability and accuracy of the final test results remain highly dependent on three critical laboratory phases: the pre-analytical, analytical, and post-analytical phases [13, 20]. Various literatures indicate that errors in the pre-analytical phase are the largest contributor to the inaccuracy of laboratory results, encompassing the processes of collection, handling, transportation, storage, and the assessment of specimen conditions prior to testing [8, 13, 18]. One of the pre-analytical parameters that frequently affects specimen quality is the storage duration of the serum before the analysis is conducted [17, 19]. The storage of specimens for a certain duration outside of optimal conditions can disrupt the stability of biological components within the serum [6, 17]. In immunoserological testing, delays or prolonged storage of serum can potentially reduce the structural stability of both antigens and antibodies, thereby affecting the sensitivity of the immunological reaction on the chromatographic membrane [17, 18].

In addition to the storage time factor, the physical integrity of the sample, such as hemolytic conditions, is also a pre-analytical constraint most frequently encountered in laboratory specimens [9, 10, 16]. Hemolysis, characterized by the rupture of erythrocyte cell walls and the release of intracellular components including hemoglobin into the serum or plasma, is generally triggered by improper phlebotomy techniques, specimen transportation factors, or delays in the testing process [9, 10]. The presence of free hemoglobin and alterations in the physicochemical characteristics of the serum are strongly suspected to inhibit the specific formation of antigen-antibody complexes, as well as obscure visual interpretation due to changes in the intensity of the color line on the test strip [10, 15].

A review of previous studies indicates that evaluations regarding the effect of pre-analytical factors on laboratory test results have been widely published, both focusing independently on the impact of specimen storage duration [6, 17] and discussing interferences due to hemolysis in isolation [9, 10, 16]. However, literature that comprehensively examines the simultaneous interaction between time degradation factors and hemolysis interference particularly at a moderate degree of hemolysis on the stability of ICT HBsAg diagnostic performance is currently still very limited [18, 19]. Most previous clinical studies only looked at the result status in a binary manner (positive/negative) without measuring periodic fluctuations in the decline of reading line intensity, even though the combination of these two pre-analytical factors is highly likely to exert a multiplicative effect that accelerates the decline in sample quality [17, 25].

The novelty of this study lies in an experimental evaluation approach that simultaneously combines two critical pre-analytical variables, namely variations in storage duration (0 hours, 4 hours, and 8 hours) and differences in the physical condition of the serum (normal serum compared to moderately hemolyzed serum). Through a standardized semi-quantitative intensity scoring method (scores 0 to 3), this study is designed to precisely map the degradation patterns of test result stability. Furthermore, statistical analysis using the Two-Way ANOVA test is applied to prove the significance of the interaction between delay time and serum condition on the decreased sensitivity of the ICT HBsAg test kit. This combined approach is highly essential to provide empirical and accurate pre-analytical tolerance limits for Medical Laboratory Technology (MLT) practitioners in healthcare facilities [22, 25].

Based on the background and problems presented, this study aims to determine and analyze the effect of storage duration on the stability of ICT HBsAg test results in normal and moderately hemolyzed serum. Through achieving these objectives, the results of this study are expected to provide theoretical contributions in the form of new scientific references in the field of immunoserology, as well as practical benefits as a guideline and evaluation material for specimen handling in clinical laboratories to ensure the validity of Hepatitis B diagnostic results [11, 12, 25].

METHODS

This quantitative laboratory experimental study utilized a post-test only factorial design (2x3) to evaluate the effect of the physical condition of the serum (normal and moderately hemolyzed) [9, 10] as well as the storage delay duration (0, 4, and 8 hours at room temperature) [6, 17] on the stability of the HBsAg Immunochromatographic Test (ICT) results [1, 2]. Conducted at the Immunoserology Laboratory of Medical Laboratory Technology (MLT), this study utilized 50 residual clinical specimens of HBsAg-positive human serum, selected through a purposive sampling technique based on specific inclusion and exclusion criteria [14, 22]. Procedurally, the serum was separated via centrifugation [14], a portion was mechanically simulated to undergo moderate hemolysis [9, 16], and each group was subsequently stored according to its assigned treatment duration before being tested using the HBsAg ICT kit [11, 12]. The stability of the results was measured semi-quantitatively through visual macroscopic observation of the test line intensity, utilizing a scoring system ranging from 0 (negative) to 3 (strong line) [1, 2]. Subsequently, the obtained data were statistically analyzed using the Shapiro-Wilk normality test, followed by the parametric Two-Way ANOVA test (at a significance level of $\alpha = 0.05$) to observe the main effects as well as the simultaneous interaction effect of the two independent variables [19, 23]. The entire experimental series was conducted in strict compliance with research ethics principles, which included guaranteeing the anonymity of patient identities, maintaining the integrity of the purpose of specimen usage, and implementing Biosafety Level 2 (BSL-2) occupational safety standards, including the management of infectious waste [11, 12, 20].

RESULTS

This study evaluated the effect of storage duration and the physical condition of the specimen on the stability of the ICT HBsAg test results. The examination was conducted using the Immunochromatographic Test (ICT) method with variations in storage duration of 0 hours,

4 hours, and 8 hours on two specimen groups, namely normal serum and moderately hemolyzed serum.

The macroscopic observation data of the test line intensity on the ICT HBsAg cassette were converted into a scoring system ranging from 0 to 3. The distribution of the mean intensity scores in the normal serum group is presented in Table 1.

Table 1. ICT HBsAg Test Results on Normal Serum Based on Storage Duration

Storage Duration	Mean Intensity Score	Result Category
0 hours	3.0 ± 0.00	Strong line
4 hours	2.3 ± 0.46	Moderate line
8 hours	1.8 ± 0.50	Weak line

Source: Primary Data (2026)

Based on Table 1, the mean intensity score of the ICT HBsAg test results on normal serum showed a declining trend as the storage duration increased, decreasing from a strong line category (3.0 ± 0.00) at 0 hours to a weak line (1.8 ± 0.50) at 8 hours.

Furthermore, the observation results for the serum group with moderate hemolysis conditions are presented in Table 2.

Table 2. ICT HBsAg Test Results on Moderately Hemolyzed Serum Based on Storage Duration

Storage Duration	Mean Intensity Score	Result Category
0 hours	2.0 ± 0.00	Moderate line
4 hours	1.3 ± 0.46	Weak line
8 hours	0.6 ± 0.49	Negative*

Source: Primary Data (2026)

Note: A mean score of 0.6 indicates that most of the results visually fall within the negative to weak line range.

Table 2 shows that the mean intensity score in moderately hemolyzed serum started at a moderate line category at the 0-hour mark and continuously declined until it reached a near-negative category at 8 hours of storage. A direct comparison between the two groups is outlined in Table 3.

Table 3. Comparison of Mean ICT HBsAg Intensity Scores between Normal and Moderately Hemolyzed Serum

Storage Duration	Normal Serum (Mean ± SD)	Moderately Hemolyzed Serum (Mean ± SD)
0 hours	3.0 ± 0.00	2.0 ± 0.00
4 hours	2.3 ± 0.46	1.3 ± 0.46
8 hours	1.8 ± 0.50	0.6 ± 0.49

Source: Primary Data (2026)

To test the significance of the data, a prerequisite normality test (Shapiro-Wilk) was conducted, the results of which are presented in Table 4.

Table 4. Normality Test Results

Variable	p-value	Interpretation
Normal serum group	0.200	Normal
Moderately hemolyzed serum group	0.189	Normal

Source: Primary Data (2026)

Because both data groups had a p-value > 0.05, meaning the data were normally distributed, testing proceeded using the parametric Two-Way ANOVA test. The results of the multiple comparison analysis are presented in Table 5.

Table 5. Statistical Test Results of the Effect of Storage Duration on ICT HBsAg Result Stability

Variable	p-value	Interpretation
Storage duration	0.000	Significant
Serum condition	0.000	Significant
Interaction of the two variables	0.003	Significant

Source: Primary Data (2026)

Table 5 shows that the storage duration variable, serum condition, and the interaction between the two variables each produced a p-value < 0.05, indicating a meaningful statistical effect. The proportion of intensity decline is visualized through percentages in Table 6.

Table 6. Percentage Distribution of ICT HBsAg Intensity Decline

Storage Duration	Normal Serum	Moderately Hemolyzed Serum
0 hours	100%	100%
4 hours	76.7%	60.0%
8 hours	60.0%	30.0%

Source: Primary Data (2026)

DISCUSSION

The results of this study prove that the stability of HBsAg diagnostic test results using the Immunochromatographic Test (ICT) method is highly influenced by pre-analytical factors, specifically including storage duration, the integrity of the specimen's condition (hemolysis), and the interaction effect between the two [8, 18].

Prolonged specimen storage consistently triggered a decline in the test line intensity across all sample groups. This decline in stability aligns with the concept of molecular degradation, wherein prolonged serum storage outside optimal temperatures potentially causes changes in protein conformational structures and a reduction in the biological activity of antigens within the serum [6, 17]. Based on the principle of the ICT method, the formation of a strong test line relies on the specific complex binding between the HBsAg antigen in the patient's sample and the monoclonal antibodies conjugated to the nitrocellulose membrane [1, 2]. When the stability of antigen viability decreases due to prolonged delay times, the antigen-antibody binding affinity weakens, thereby causing the failure of optimal color band formation [17, 19].

In addition to the time factor, the physical condition of the specimen in the form of moderate hemolysis exerts a highly significant initial degradation impact. The data showed that at the 0-hour point, moderately hemolyzed serum was only capable of producing a moderate-

level line intensity, which was substantially lower compared to normal serum. Hemolysis causes erythrocyte membrane lysis, releasing free hemoglobin and various intracellular materials into the serum compartment [9, 10]. The presence of free hemoglobin and cellular debris increases fluid viscosity and triggers a steric hindrance effect, which physically and chemically interferes with the capillary flow of the sample along the chromatographic membrane [10, 16]. Beyond hindering the immunological reaction, the dense red pigment from free hemoglobin creates visual background obscurity, which masks the interpretation of line intensity on qualitative and semi-quantitative screening devices [16, 22].

Furthermore, statistical analysis (Two-Way ANOVA) confirmed the presence of a significant simultaneous interaction between storage duration and hemolytic conditions in accelerating sample degradation. Specimens that have undergone hemolysis proved to be profoundly more susceptible to stability changes as the delay time increased [6, 17]. The percentage decline in test line intensity demonstrated that at an 8-hour storage time, the reaction strength in normal serum remained at 60.0%, whereas in hemolyzed serum, it plummeted drastically to 30.0%, which clinically poses a risk of generating false negative interpretations. Biochemical alterations resulting from erythrocyte lysis are believed to disrupt the serum microenvironment, triggering the release of proteolytic enzymes that progressively accelerate the denaturation of viral antigens over time [5, 9].

These findings are highly relevant and corroborate recent literature reviews highlighting that the majority of errors in laboratory medicine diagnostics (up to more than 70%) originate from failures in the pre-analytical phase [13, 23, 24]. The clinical implications of this study underscore the critical importance of adhering to standardized guidelines for specimen collection and management [14, 25]. The utilization of fresh serum (without unnecessary time delays) and the rejection of visually hemolyzed specimens constitute fundamental measures that must be strictly implemented by medical laboratory technologists to guarantee the accuracy, validity, and reliability of diagnostic results for infectious diseases [9, 12, 20].

CONCLUSION

This study concludes that pre-analytical factors, particularly the combination of storage duration and the physical condition of the specimen, have a crucial effect on the stability of HBsAg Immunochromatographic Test (ICT) results. The longer the serum is stored, a consistent decline in stability occurs, characterized by a visual reduction in test line intensity, wherein this degradation process is proven to occur faster and more prominently in specimens with a moderate hemolysis condition compared to normal serum. The interaction effect of these two variables confirms that specimens undergoing red blood cell lysis possess a significantly higher susceptibility to testing time delays. The main limitations in this study include the evaluation of the degree of hemolysis, which was not quantitatively measured based on free hemoglobin levels; the reading of line intensity, which still relies on subjective visual interpretation without digital instrumentation; and the scope of the research, which has not yet explored more extreme variations in temperature and storage duration. Based on these findings and limitations, it is recommended for laboratory practitioners and institutions to tighten pre-analytical procedures to minimize the risk of hemolysis, and to prioritize the testing of fresh serum specimens as soon as possible without time delays to guarantee diagnostic validity. For

the development of future research, it is recommended to design studies with more comprehensive time and temperature variations, utilize digital intensity readers for result objectivity, and employ more advanced comparative diagnostic methods such as Enzyme-Linked Immunosorbent Assay (ELISA) or Chemiluminescence Immunoassay (CLIA).

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