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# Assessment of Thyroid Hormone Level Alterations in Hepatitis B-Positive Serum Using a Quantitative Immunofluorescence Assay: A Randomized Controlled Trial

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**Abstract:** This retrospective study aimed to assess the differences in serum biomarker levels, specifically thyroid hormone (free T4 and Thyroid-Stimulating Hormone [TSH]) levels, according to the presence or absence of Hepatitis B Virus (HBV) infection. Using a quantitative immunofluorescence assay, we analyzed serum samples from patients with confirmed HBV infection at Dankook University Hospital in Cheonan Province, Republic of Korea, between August 2022 and September 2022. The study population was divided into two groups: Young individuals (<60 years) and older adults (≥60 years). Our findings indicated that differences in thyroid hormone levels between HBV-positive and negative participants were not significant. However, when considering age groups, TSH and free T4 levels in young individuals with HBV-positive serum were 1.78±0.09 μIU/mL (normal range: 0.4-5.0 μIU/mL) and 1.24±0.02 ng/mL (normal range: 0.8-1.9 ng/mL), respectively. In older adults with HBV-positive serum, the corresponding values were 2.22±0.17 μIU/mL and 1.24±0.07 ng/mL, respectively. Our results suggest that the influence of HBV on anti-pituitary hormone levels is age-dependent rather than virus related. Furthermore, the quantitative immunofluorescence method used in this study provides a simpler alternative to enzyme-linked immunosorbent assay or electrochemiluminescence immunoassay for assessing serological thyroid hormone levels in patients with HBV. These findings can aid in the interpretation of diagnostic test results and the timely detection of metabolic changes associated with HBV infection.

**Keywords:** Thyroid Hormone, Free T4, TSH, HBV, Serologic Study, Immunofluorescence

## Introduction

Hepatitis B is a well-known disease that causes acute liver deterioration and is associated with Acute-on-Chronic Liver Failure (ACLF) (Chen *et al*., 2019; 2022a Jafarpour and Azimzadeh, 2019) Certain metabolic pathological symptoms are caused by hepatitis B. In particular, thyroid dysfunction has been reported in many chronic illnesses, including severe liver diseases caused by hepatitis (Ma *et al*., 2022; Chen *et al*., 2022b). Of the various hormones related to the thyroid gland, two play a critical role in metabolism. Thyroid-Stimulating Hormone (TSH), is one of the most important thyroid hormones that function via the TSH receptor (Yang *et al*., 2022). Free T4, also called free thyroxine, is a hormone produced by the thyroid gland that regulates the metabolism of the body. Its functions include controlling the energy usage rate of cells and regulating the heart rate, body temperature, and energy levels (Jafarpour and Azimzadeh, 2019). There is some debate about the correlation between thyroid hormone levels and hepatitis B. Some argue that serum TSH level is negatively correlated with the severity of Hepatitis B Virus (HBV)-related ACLF; patients with increased TSH levels have a

high survival rate (Wu *et al*., 2018; Yang *et al*., 2022). Serum-Free T4 (FT4) level is negatively correlated with the severity of HBV-related ACLF. Some experts argue against the statement that patients with hepatitis B exhibit elevated TSH and FT4 levels that may impact thyroid hormone production (Cui *et al*., 2016; Zhang *et al*., 2022).

Very few studies using a quantitative immunofluorescence method, a Point-of-Care Test (PoCT), exist since the hormone levels are usually measured by Electrochemiluminescence Assay (ECLIA) or enzyme-linked Immunosorbent Assay (ELISA). However, an interest in the immunofluorescence-based quantitative assay as a rapid test is increasing after the coronavirus disease 2019 pandemic. Therefore, while our study is not the first on thyroid hormone level change in patients with hepatitis B, it aims to introduce an alternative quantitative measurement, this study aimed to assess the changes in the thyroid hormone level in hepatitis B-positive and negative serum using the immunofluorescence-based quantitative assay.

## Materials and Methods

### *Study Design and Participants*

We conducted a retrospective analysis of patients diagnosed with HBV from August 2022 to September 2022 on the basis of the results of Real-Time quantitative Polymerase Chain Reaction (RT-PCR) testing of blood samples at Dankook University Hospital in Cheonan province. Serum was extracted from the blood samples of the patients. The Clinical Research Review Committee of Dankook University approved this study (Institutional Review Board DKU, Certificate No. 2022-10-030) and all patients provided informed consent. A total of 200 patients were divided into two groups based on criteria such as age, sex, and infection status (positive or negative) (Karta *et al.*, 2022).

### *Data Collection*

Thyroid Hormone parameters (TSH and FT4 levels) were evaluated using an immunofluorescence quantitative analyzer (AnyLab F1, Z-Biotech, Republic of Korea). Serum TSH and FT4 levels were measured using AnyLab F TSH and AnyLab F FT4, respectively (Z-Biotech, Republic of Korea). These data were evaluated by the diagnostic test department at Dankook University according to the testing procedures of the AnyLab F device. Data on the inflammation parameters were obtained from the serum samples after confirmation of a positive or negative HBV diagnosis (Smieszek and Polymeropoulos, 2022).

### *Immunofluorescence-Based Quantitative Assay*

The detection buffer contains an antibody conjugated with a fluorescent dye that specifically binds to thyroid markers, such as TSH and FT4. When the detection buffer and specimen are mixed, the antibody in the detection buffer and the thyroid-related antigen in the specimen form an antigen-antibody complex. When this mixture is dropped into the sample well of the cartridge, it binds to the thyroid-marker antibody coated on the nitrocellulose membrane, which causes a sandwich immune response. The extent to which the immune response of the sandwich structure was converted into a fluorescence signal and the concentration were then calculated (AnyLab F1).

### *AnyLab F Assay*

The AnyLab F PoCT is a quantitative assay for measuring the concentration of total thyroid hormones, such as TSH and FT4, in serum, plasma, or whole blood using fluorescence immunoassay technology. The method uses a sandwich immuno-detection principle, such that the fluorescence-labeled detector antibody binds to the target protein in the sample. The sample is then applied onto a test strip and the fluorescence-labeled antigen-antibody complex is captured by a second antibody embedded in the solid phase. The signal intensity of fluorescence of the captured complex is directly proportional to the amount of antigen present. It thus enables the calculation of the sample antigen concentration via a pre-programmed calibration process. The test result is displayed on the reader as nanograms per deciliter (ng/dL) for FT4 and microIU per milliliter (µIU/mL) for TSH. A fluorescence-labeled control protein is included in the reaction and the control line's intensity is measured as a quality check.

All the reaction components and the meter required for assay performance are available from the manufacturer. The assay was performed according to the manufacturer's instructions. In brief, 100 µL of serum was mixed with a pre-measured volume of detection buffer containing fluorescence-labeled anti-monoclonal antibodies and anti-rabbit IgG. A small volume, 100 µL, of the mixture was then loaded into the sample well of the test strip and the cartridge was incubated at room temperature (15-30°C) for 15 min. The intensity of the captured fluorescence-labeled antigen-antibody complexes was measured using the supplied meter and the concentration of the targeted antigen in the sample was calculated. Assay accuracy and precision during the study were assessed using the internal quality control material supplied by the manufacturer.

### *Data Evaluation*

The 200 samples used in this study were anonymized serum samples provided by the laboratory for routine measurement of the total thyroid protein levels. The selected samples spanned the analytical range of the AnyLab F assay; the 200 samples had TSH concentrations between 0.1 and 100.0 µIU/mL and FT4 concentrations between 0.1 and 8.0 ng/dL. According to the Korean Society of laboratory medicine, the normal ranges for TSH and FT4 are 0.4-5.0 µIU/mL and 0.8-1.9 ng/dL, respectively.

### *Statistical Analysis*

Normally distributed continuous data were expressed as mean ± standard deviation and an independent t-test was used to detect the significance between the groups. All statistical analyses were performed using GraphPad Prism (version 7.00.159). A p-value <0.05 indicated statistical significance.

## Results

### *Demographic and Clinical Characteristics*

The patients were divided into two age groups: Young individuals (0-59 years) and older adults (≥60 years). The mean ages were 48±11 years and 67±10 years for the young individuals and older adults, respectively. Among the 200 participants, 140 were included in the young individual group (age, 0-59 years), while 60 were included in the older adult groups (age, >60 years) (Table 1 and Fig. 1).

In all, 104 patients diagnosed with hepatitis B were included in this study: 79 (76%) were young individuals and 25 (24%) were older adults. A total of 96 healthy individuals were included in this study, of which 61 (41%) were young individuals and 35 (59%) were older adults (Table 2 and Fig. 1).

### *Thyroid Hormone Parameters in Differentiating HBV-Positive Patients from HBV-Negative Patients*

In HBV-positive individuals, the TSH and free T4 levels were 2.27±0.16 µIU/mL and 1.22±0.02 ng/dL, respectively. In contrast, in HBV-negative individuals, the respective values were 2.00±0.12 µIU/mL and 1.23±0.02 ng/dL (p-value <0.005). The thyroid hormone level was not significantly altered when comparing HBV patients and healthy participants (Fig. 2).

### *Levels of Thyroid Hormone Parameters in Young Individuals and Older Adults Infected with Hepatitis B*

In young HBV-positive individuals, the TSH and FT4 levels were 2.22±0.17 µIU/mL and 1.21±0.02 ng/dL, respectively. In contrast, in older HBV-positive adults, the respective values were 2.44±0.44 µIU/mL and 1.24±0.07 ng/dL (p-value <0.005). The thyroid hormone changes caused by hepatitis B infection were unrelated to the changes caused by age (Fig. 3).

### *Levels of Thyroid Hormone Parameters in Male and Female Patients Infected with HBV*

In Figure 4, in HBV-positive males, the TSH and FT4 levels were 2.07±0.20 µIU/mL and 1.52±0.04 ng/dL, respectively, whereas, in HBV-negative males, the respective values were 1.91±0.14 µIU/mL and 1.60±0.04 ng/dL (p-value <0.005). In the case of HBV-positive females, the TSH and FT4 levels were 2.53±0.26 µIU/mL and 1.49±0.06 ng/dL, respectively, whereas, in HBV-negative females, the respective values were 2.47±0.35 µIU/mL and 1.41±0.06 ng/dL (p-value <0.001). The results indicate that the TSH levels increased slightly in both sexes after HBV infection, while the FT4 level increased in females but decreased in males after HBV infection.

**Table 1:** Demographic features and laboratory findings of participants based on age

| | Young individuals (age, 0-59 years) | | | Older adults (age, ≥60 years) | | |
|---|---|---|---|---|---|---|
| Parameter | Hepatitis B negative (n=61) | Hepatitis B positive (n=79) | $P_1$ | Hepatitis B negative (n=35) | Hepatitis B positive (n=25) | $P_2$ |
| Sex | | | | | | |
| Male (%) | 43 (70%) | 44 (56%) | NS | 18 (51%) | 15 (60%) | *NS* |
| Female (%) | 18 (30%) | 35 (44%) | NS | 17 (49%) | 10 (40%) | NS |
| TSH (µIU/mL) | 1.91±0.13 | 2.22±0.17 | <0.005 | 2.18±0.26 | 2.44±0.44 | <0.050 |
| Free T4 (ng/dL) | 1.24±0.02 | 1.21±0.02 | <0.005 | 1.21±0.03 | 1.24±0.07 | <0.005 |

NS, Non-Significant; TSH, Thyroid-Stimulating Hormone

**Table 2:** Demographic features and laboratory findings of participants based on hepatitis B infection

| | Hepatitis B negative | | | Hepatitis B positive | | |
|---|---|---|---|---|---|---|
| Parameter | Young individuals (aged 0-59 years) (n=61) | Older adults (aged ≥60 years) (n=35) | $P_1$ | Young individuals aged 0-59 years) ((n=79) | Older adults (aged ≥60 years) (n=25) | $P_2$ |
| Sex | | | | | | |
| Male (%) | 43 (70%) | 18 (51%) | NS | 44 (56%) | 15 (60%) | NS |
| Female (%) | 18 (30%) | 17 (49%) | NS | 35 (44%) | 10 (40%) | NS |
| TSH (µIU/mL) | 1.91±0.13 | 2.47±0.37 | <0.0001 | 2.22±0.17 | 2.44±0.44 | <0.0080 |
| Free T4 (ng/dL) | 1.24±0.02 | 1.21±0.03 | <0.0300 | 1.21±0.02 | 1.24±0.07 | <0.0001 |

NS, Non-Significant; TSH, Thyroid-Stimulating Hormone

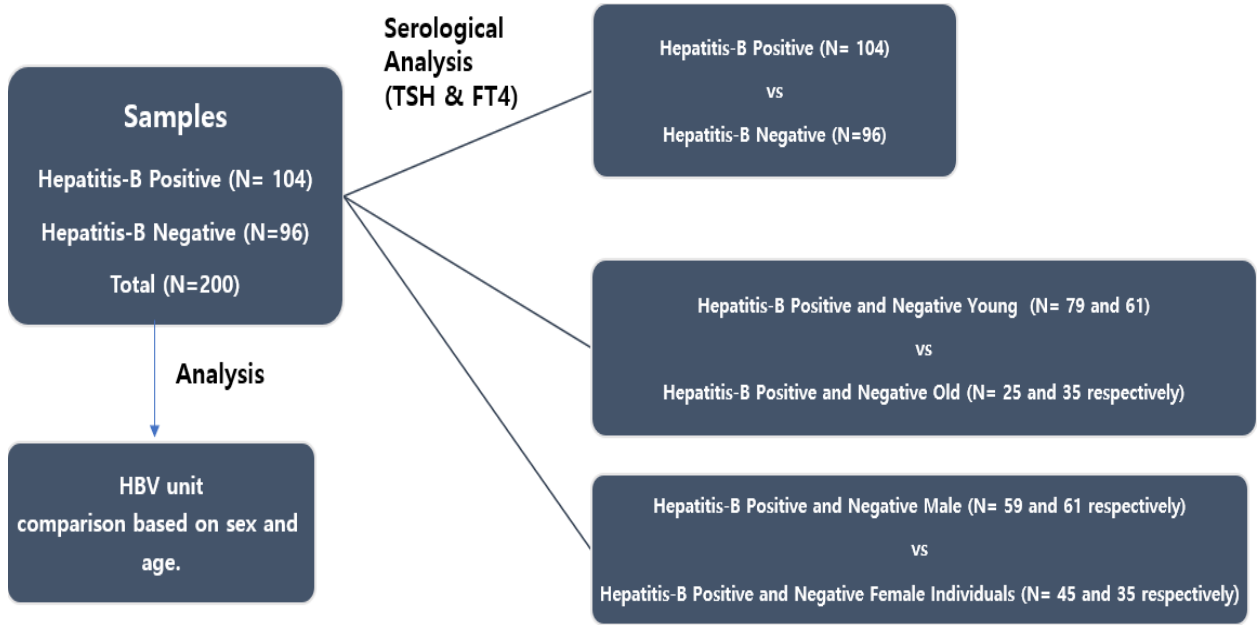



**Fig. 1:** Flowchart of the study methodology

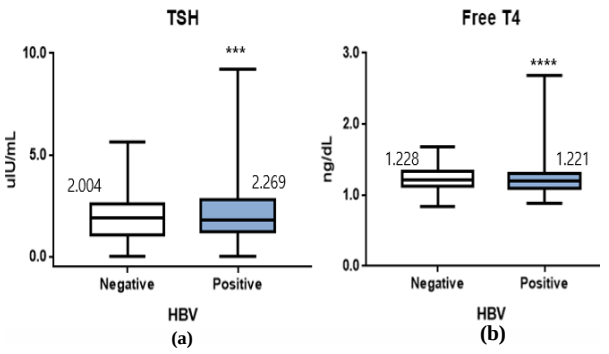



**Fig. 2:** Comparison of thyroid hormone (Thyroid-Stimulating Hormone [TSH] and free T4) levels based on Hepatitis B Virus (HBV) infection; (a) TSH (p-value: 0.0004) and (b) Free T4 (p-value <0.0001)

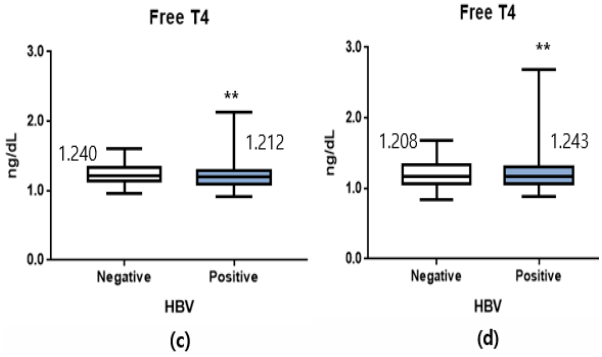
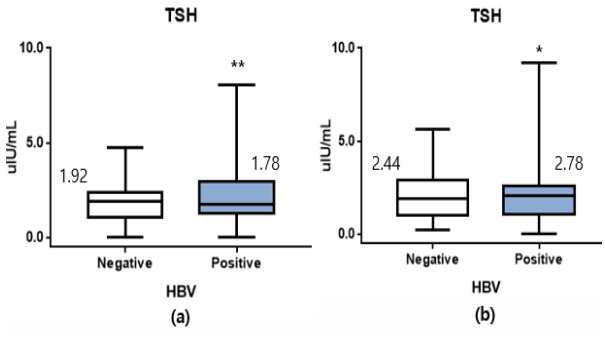
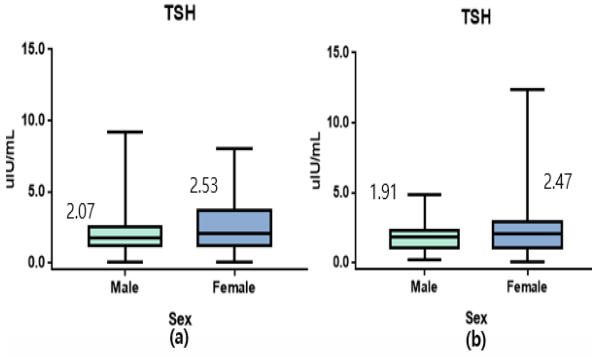



**Fig. 3:** Comparison of thyroid hormone (Thyroid-Stimulating Hormone [TSH] and free T4) levels between the age groups; TSH level in young individuals (a) (0-59 years), (b) TSH level in old individuals (≥60 years), (c) Free T4 level in young individuals (0-59 years), and (d) Free T4 level in older individuals (≥60 years)



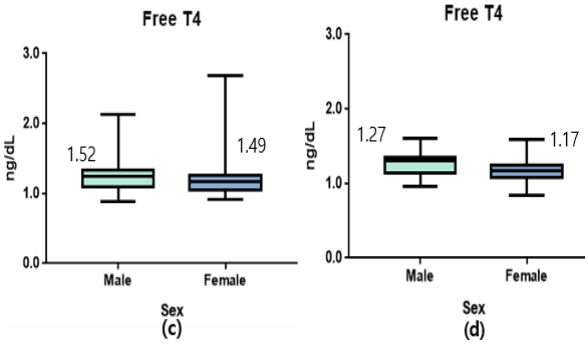



**Fig. 4:** Comparative analysis of thyroid hormone levels, specifically Thyroid-Stimulating Hormone (TSH) and free T4, based on sex in both HBV-positive and HBV-negative groups; (a) TSH level in the Hepatitis B Virus (HBV)-positive group, (b) TSH level in the HBV-negative group, (c) Free T4 level in the HBV-positive group, (d) Free T4 level in the HBV-negative group

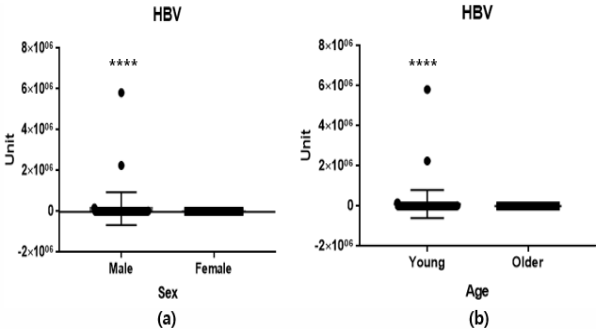



**Fig. 5:** Comparison of Hepatitis B Virus (HBV) DNA levels based on sex and age, (a) The HBV DNA levels based on sex in the HBV-positive group; (b) the HBV DNA levels based on age in the HBV-positive group

### *Levels of Hepatitis B DNA by Sex and Age Among Hepatitis B-Positive Patients*

According to Wang, the amount of hepatitis B DNA influences thyroidal dysfunction through mitochondrial stress (Wang *et al*., 2020). Figure 5 shows the amounts of hepatitis B virus DNA levels (HBV DNA levels) in HBV-positive individuals. Interestingly, the HBV DNA level in male patients was 140256±105119 IU/mL, while that in female patients was 55.93±30.58 IU/mL, indicating a significant difference. Furthermore, a major difference was noted between the young individuals (104697±78641 IU/mL) and older adults (262.3±217.2 IU/mL). The respective trend was not correlated when Fig. 5 was compared with Figs. 2-3. Regarding sex and age differences among HBV-positive patients, the TSH and FT4 values showed minor differences. Thus, the relationship between the thyroid hormone and hepatitis B surface antigen levels was not significant.

## Discussion

This study focused on the analytical and diagnostic performance of two thyroid hormone assays on POC platforms to assess their analytical and diagnostic quality in clinical use. The POC assay results showed no significant changes in serological thyroid hormone levels after HBV infection.

Some studies state that thyroid hormones and liver function impairment by HBV infection are barely related (Yu *et al*., 2007; Mansour Ghanaei *et al*., 2011; Chi *et al*., 2017). The FT4 level increased, while the TSH level remained within the normal range (Chen *et al*., 2019; Ma *et al*., 2022). However, no significant difference was observed among infection, age, and sex. Furthermore, the thyroid hormone level was not significantly correlated with the quantitative trend of the HBV DNA levels. Therefore, determining a significant relationship between HBV infection and thyroid hormone levels is challenging.

While there may be a relationship between thyroid hormone levels and HBV infection, the exact mechanism underlying this relationship is not well understood. Some researchers propose that HBV may impact thyroid hormone metabolism by influencing the production, transport, or clearance of these hormones. Others suggest that the immune response to the virus could also be a factor involved in the disruption of thyroid hormone levels, although this theory is not universally accepted. Further research is needed to fully understand the connection between thyroid hormones and hepatitis B infection (Huang *et al*., 2019).

Our study had several limitations. Owing to the study design and retrospective nature of data collection, other thyroid biomarkers, such as total T3 (a known initial indicator of thyroid metabolic activity (Chen *et al*., 2015; Huang *et al*., 2019) and free T3 (which is very important in cellular metabolism regulated by thyroid hormones (Wu *et al*., 2015; Chi *et al*., 2017; Jafarpour and Azimzadeh, 2019), affecting the liver and neurons (Chen *et al*., 2015; Cui *et al*., 2016), were not examined. However, since the biomarkers were measured after confirmation and hospitalization of the patients with hepatitis B, they were deemed useful for monitoring and predicting the progress of thyroid activity. Furthermore, owing to the hospital's administrative approach and temporary operations, only patients with quantitatively confirmed positive and negative hepatitis B diagnoses were managed. Therefore, the presence or absence of other respiratory illnesses in inpatients and the criteria for severe or non-severe hepatitis B could not be determined based on the symptoms. Future research should explore other potential biomarkers, including cardiac and inflammation makers, for predicting the relationship of these biomarkers with hepatitis B status.

This study did not specifically analyze the thyroid values based on the quantitative results of HBV DNA quantification (HBV qPCR). Instead, it compared the thyroid values between patients infected with HBV and those not infected with HBV using the HBV DNA quantification method. While HBV DNA quantification testing can be used to monitor the effectiveness of antiviral therapy, this study did not consider the analysis of virus treatment. The absence of information regarding the treatment status of the virus or the condition of the treatment drugs is a limitation of this study. A more in-depth discussion of the thyroid test results based on the quantitative values of HBV DNA will be addressed in ongoing additional research.

Nevertheless, the significance of our study lies in the fact that it provides biomarker data using a quantitative immunofluorescence method, which offers a distinct approach compared to the commonly used Chemiluminescence Assay (CLIA) or RT-PCR in hospital settings. Point-of-Care Testing (PoCT) methods have historically been less reliable due to lower sensitivity when predicting quantitative biomarker data in human samples, despite their competitive pricing. However, advancements in detection technology have improved the immunofluorescence quantitative assay, rendering it highly advantageous, given its heightened sensitivity and specificity. Therefore, this PoCT platform can serve as a viable alternative to RT-PCR and CLIA methods. In terms of cost and time, the PoC platform may offer advantages. PoC tests are generally designed to be cost-effective, providing rapid and accessible results at the PoC. These tests often have low upfront equipment costs and may not require specialized laboratory infrastructure. Additionally, the PoC platform can potentially reduce overall healthcare costs by enabling fast diagnosis and appropriate treatment decisions, leading to improved patient outcomes and resource utilization. If direct RT-qPCR of serum samples can be successfully achieved, it may present a competitive alternative to the PoC platform. This approach could enable rapid and sensitive detection of target nucleic acids directly from the serum samples, similar to the advantages offered by the PoC platform. However, several factors, including equipment, expertise, cost-effectiveness, immediate availability, etc., need to be considered to assess the competitiveness of direct RT-qPCR and the PoC platform. This assay employs antibodies that are labeled with fluorescent substances to identify and quantify specific antigens in a sample. The fluorescent labeling feature enables visualization of the antigen-antibody complex and this makes it a very precise and sensitive method of detecting and measuring the levels of antigens in a given sample (Michel *et al*., 2020). This type of test is particularly useful in diagnosing and monitoring a range of diseases, including autoimmune conditions, cancer, and metabolic and infectious diseases. Additionally, immunofluorescence quantitative assays are relatively easy to perform and can provide results in a relatively short amount of time (Shurrab *et al*., 2022).

Further studies should be conducted using immunofluorescent-based quantitative assays to detect other metabolic hormones in serological samples from patients with hepatitis B. It would also be useful to examine the changes in thyroid hormone levels in patients with hepatitis A and B and establish a correlation between the two conditions. Thus, efforts to solve these challenges of the quantitative PoCT system will continue through the application of new reader technologies or improvement in existing technologies.

## Conclusion

In summary, this study evaluated the performance of two thyroid hormone assays using PoC platforms and found no significant changes in serological thyroid hormone levels before and after HBV infection. The relationship between HBV infection and thyroid hormone levels remains uncertain, with conflicting findings in previous research. The exact mechanism behind this association is not fully understood, warranting further investigation. Despite limitations such as excluding certain thyroid biomarkers and the inability to determine the presence of other respiratory illnesses or the severity of hepatitis B, the quantitative immunofluorescence method used in this study provided valuable biomarker data. Immunofluorescence quantitative assays offer improved sensitivity and specificity, making them valuable for diagnosing and monitoring various diseases. Future studies using this assay should explore additional metabolic hormones in patients with hepatitis B and investigate the correlation between thyroid hormone levels and both hepatitis A and B. Continuous efforts to enhance the quantitative PoC systems will help overcome the challenges involved in the use of diagnostic technologies.

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## Author's Contributions

**Hyeokjun Yun and Bo Kyeung Jung:** Concepted the work and written the manuscript.

**Kyung Cheol Min and Jae Kyung Kim:** Designed the work and collected the data.

## Ethics

The study was conducted in accordance with the declaration of Helsinki and approved by the institutional review board of the clinical research review committee of Dankook University (institutional review board DKU, certificate No. 2022-10-030).

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## Nomenclature

TSH: Thyroid-stimulating hormone
HBV: Hepatitis B Virus
FT4: Free T4

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