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Detection of Epstein-Barr virus and Hashimoto's autoimmune in patients with a thyroid disorder

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Abstract. Hypothyroidism's most common cause is Hashimoto's thyroiditis (HT). Because Epstein-Barr virus is a common pathogen that causes autoimmune diseases to be prevalent worldwide and because it stays in the body for the duration of life, which explains why autoimmune diseases have a chronic course and are frequently accompanied by symptomatic exacerbations, the aim of this research is to determine the relationship between Epstein-Barr virus (EBV) and Hashimoto's autoimmune disease in patients with a thyroid disorder. 120 samples were collected from the governorate of Najaf (60 with thyroidectomy and 60 as controls) to identify the presence of EBV. The proportion of patients with EBV was 27 (45%) compared to the control group, which was all negative. This study detects the autoimmune disease (Hashimoto thyroiditis) in all 160 samples using the anti-thyroperoxidase (TPO) test, which yields a positive result in 40% of patients (24), a negative result in 60% (36), and a negative result in 100% (60) of control samples.

1 Introduction

The thyroid is part of the endocrine system responsible for the synthesis and release of the thyroid hormones thyroxine and triiodothyronine (T3 and T4). In the front of the neck, the thyroid gland is situated. and has a butterfly-like aspect due to its bilobular structure [1]. The thyroid gland contains two categories of cells: follicular and parafollicular [2]. Thyroid hormones are synthesised in the follicular cells of the thyroid gland [3]. Multiple stages are involved, including iodide capture, organisation, coupling, storage, and secretion. The iodine element is necessary to create thyroid hormones. and is regarded as the rate-limiting phase in thyroid hormone production [4]. The most frequent autoimmune condition that affects people is Hashimoto thyroiditis (HT), which frequently leads to hypothyroidism or persistent lymphocytic thyroiditis, which is The most common autoimmune condition in people, Hashimoto thyroiditis (HT), frequently causes hypothyroidism or recurrent lymphocytic thyroiditis, leading to degeneration of the thyroid tissue and hypoactivity.

HT is defined as anti-thyroglobulin (Tg) antibodies and thyroid peroxidase (TPO) that are present in tissue-specific autoimmune disorders [5]. HT is considered, alongside Graves' disease (GD), to be a thyroid autoimmune disease (AITD) whose incidence has significantly risen in recent years [6]. The herpes virus family includes the double-stranded DNA virus

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known as the Epstein-Barr virus (EBV). Around 95% of adults worldwide contract the disease at some point in their lives and carry it for the rest of their lives. Depending on the patient's age, a primary EBV infection results in a clinical course that varies greatly [7]. EBV infection is associated with several lymphoid and epithelial malignancies as well as post-transplant lymphoproliferative diseases. A number of autoimmune illnesses have also been connected to EBV infections. [8]. Multiple studies have shown a connection between EBV infection and multiple sclerosis (MS). It was also implied that increased anti-EBV antibody titres could predict the development of MS [9]. EBV has a latent phase and a lytic cycle. The reactivated virus has been associated with several incapacitating autoimmune symptoms and is capable of causing the generation of thyroid antibodies [10].

2 Methods

2.1 Ethical Consideration

After the protocol was authorised by the Ethical Review Board for human studies at the Faculty of Nursing/University of Kufa/Iraq (No. 10-04-01/2015), all subjects submitted their written informed consent prior to enrollment [11, 12].

2.2 Individuals

The study was a case-control study conducted on 120 individuals between February and September of 2022, dividing them into two groups: 60 individuals who underwent a thyroidectomy and 60 individuals who appeared to be healthy [13, 14]. 5 ml of blood were collected from the individual, and the epidermis around the arm was sterilised with 70% ethyl alcohol before the blood was divided into two portions as follows: The first portion (2 ml) from both groups was transferred into anticoagulant tubes and promptly frozen at -20 degrees Celsius for use in a molecular study. After allowing the blood to coagulate for 30 minutes at room temperature, the remaining volume (3 ml) was transferred into a gel tube for serum separation. then centrifugated the blood for 5 minutes at 4000 rpm. After that, the serum was gathered in five sterile Appendrofe tubes and kept at -20 degrees Celsius to determine the presence of TPO antibodies. Detection of Hashimoto's autoimmunity by Cobas Elecsys anti-thyroid peroxidase (TPO) Principle immunoassay for the in vitro quantification of thyroid peroxidase antibodies in human plasma and serum. The anti-TPO test is used as a diagnostic aid for autoimmune thyroid diseases.

2.3 Procedure

The anti-TPO antibodies (labelled with ruthenium complex) were incubated with 20 L of sample. The sample's anti-TPO antibodies compete with those that have been ruthenium-labelled for biotinylated TPO antigen after the addition of streptavidin- and biotinylated TPO during incubation. Following the aspiration of the reaction mixture into the measuring cell, the magnetic attraction of the microparticles to the electrode surface was used by biotin and streptavidin to bind the whole complex to the solid phase. Then, unbound compounds were removed using ProCell or ProCell M. In order to determine the results, a master curve provided by the reagent barcode or e-barcode was compared to a calibration curve produced via 2-point calibration, particularly for the instrument. Chemiluminescent emission is induced by applying a voltage to the electrode and is detected by a photomultiplier.

2.4 EBV Molecular Detection

Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR) was used for the molecular identification of EBV. Using Favorgen or Taiwan kit.

2.5 DNA extraction

2.5.1 Preparation before use

Ensure that everything is RNase-free when working with this system. Preparation of wash buffer W1 by dissolving, preparation of wash buffer W2 by dissolving, and added When the tube is first opened, add 1 ml of VNE buffer to the lyophilized carrier RNA tube, fully mix by vortexing, and then transfer the mixture to the VNE buffer. A VNE buffer containing carrier RNA needs to be kept at 4 °C. RNase-free water needs to be heated to 70 °C for the elution step.

General Procedure:

1. 150 µl of the sample (serum, plasma, fluid from the body, or cell culture supernatant) were transferred into an unprovided microcentrifuge tube.
2. Divide the sample volume into many tubes if it exceeds 150 µl.
3. 570 µl of VNE buffer (with additional carrier RNA) was incorporated in the sample, vortexed to thoroughly combine it, and then left to sit at room temperature for 10 minutes.
4. Ensure that carrier RNA has been introduced to the VNE buffer before using it for the first time.
5. 570 µl of ethanol (96–100%) were added to the sample liquid, and it was well mixed by plus-vortexing.
6. Incorporate a collection tube (supplied) with a VNE column. Added ethanol to the sample mixture and transferred up to 700 µl to the flow-through, the utilised collection tube, and the VNE Column were all discarded after the flow-through was centrifuged at 8,000 x g for 1 minute.
7. 8,000 x g of centrifugation for one minute after adding ethanol to the remaining transferring a sample of the combination to the VNE column.
8. The collection tube and the flow-through should be thrown away. The new collection tube (supplied) should be combined with the VNE column.
9. Added 500 µl of Wash Buffer 1 with additional ethanol , and the VNE Column was centrifuged at 8,000 x g for 1 minute before the flow-through was discarded. Combine the used collection tube with the VNE column.
10. Ensure that Wash Buffer 1 has ethanol (96–100%) added when the container is first opened.
11. Then added 750 µl of Wash Buffer 2 with additional ethanol, and centrifuged the VNE Column at 8,000 x g for 1 minute before the flow-through was discarded.
12. the used collection tube and the VNE column together.
13. When you first open Wash Buffer 2, make sure that ethanol (96–100%) has been added.
14. Replicate step 7. 750 µl of Wash Buffer 2 (ethanol added) were added, and the VNE Column was centrifuged at 8,000 x g for 1 minute before the flow-through was discarded. Combine the used collection tube with the VNE column.
15. For an additional 3 minutes, the VNE column was centrifuged at full speed (around 18,000 X g). Toss the collection tube and the flow-through into the trash.
16. Essential Step 1 By taking this action, the remaining liquid won't interfere with the ensuing enzymatic reactions.

17. The elution tube (supplied) and VNE column combined to add 50 µl of warmed, RNase-free water to the VNE column's membrane centre. Spend two minutes in the VNE column.
18. Essential Step 1 Make sure the RNase-free water is applied to the membrane centre and fully absorbed for efficient elution.
19. The nucleic acid was eluted after a 2-minute centrifugation.
20. Nucleic acid should be kept at -70 °C.

The condition reaction was as the fallowing table:

Table 1. The condition reaction of RT-qPCR

Step	Temperature	Duration	Cycles
Initial denaturation	95 C̊	2 min	1
Denaturation	95 C̊	20 sec	44 cycles
Annealing	59 C̊	1 min	
Extension and data collection	72 C̊	1min	

Table 2. GoTaq® 1-Step RT-qPCR Reaction Mix.

Component	Volume	Concentration
GoTaq® qPCR Master Mix, 2X	10 µl	1X
Forward Primer (20X)	1.5 µl	200 nM
Reverse Primer (20X)	1.5 µl	200 nM
Supplemental CXR Reference Dye (if required)	0.3 µl	300nM
DNA template	6.7 µl	100

Table 3. Prime used in this study

primer	**Details**	**Reference**
F-EBV (TC-70):	5_-CTT GGA GAC AGG CTT AAC CAG ACT CA-3_	[15]
R-EBV (TC-72):	5_-CCA TGG CTG CAC CGA TGA AAG TTA T-3_	

2.6 Statically Analysis

Statistical information was documented as mean standard error (SE). The Graph Pad Prism version 7 programme was used to conduct a statistical test. The statistical significance threshold was set at a P-value of 0.05 [16, 17].

3 Results

3.1 Detection of EBV by RT-qPCR

In this study, EBV virus was detected by real-time PCR in all samples (patient and control), with 45% (27/60) of patient samples being positive and 55% (33/60) being negative, while all control samples were negative (Figures 1 and 2).

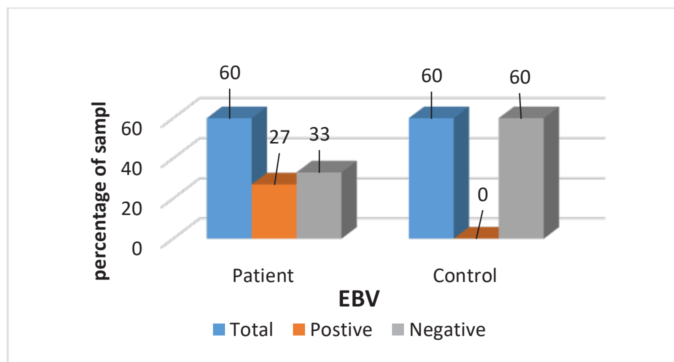

Fig. 1. Detection of EBV by RT-qPCR

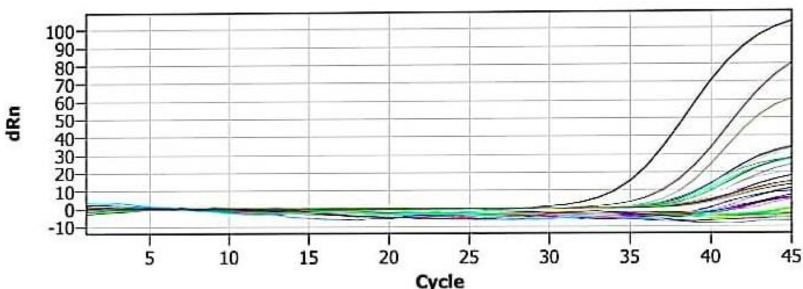

Fig. 2. Real-time PCR amplification of commercial standards of EBV

3.2 Distribution of the sample with age group.

Figure (1) shows the distribution of the sample to five ages group (≥30 , 31-40 , 41-50 , 51-60 , <60) and it turns out that the higher age group in infection was (41-50) , while the lowest age group was (<60) (figure 3)

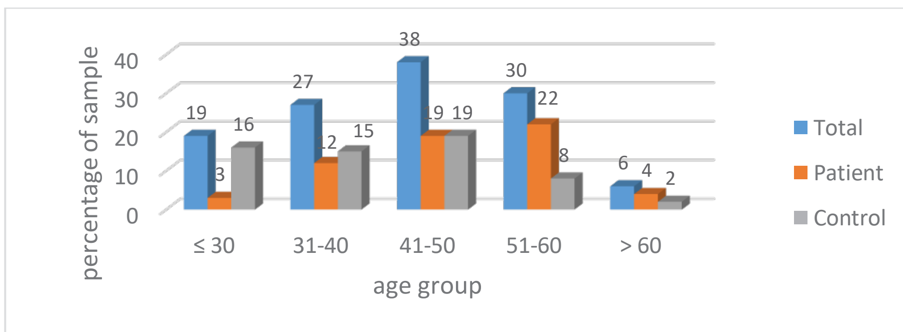

Fig. 3. Distribution of sample according to age group.

3.3 Distribution of the sample according to sex

By collecting samples, it was found that the number of male patients was 15 ompared with 21 as controls. On the other hand, the number of female patients was 45 compared with 39 in the control group (Figure (4))

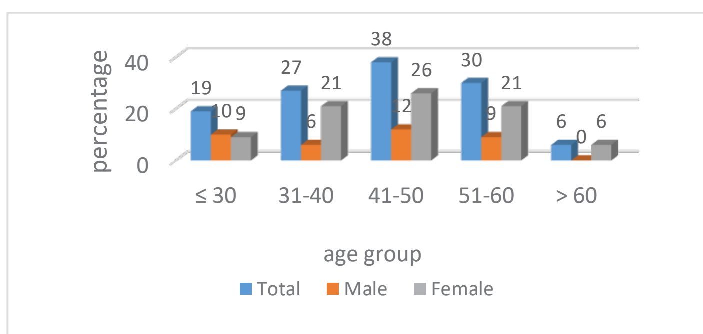

Fig. 4. Distribution of sample according to sex

3.3 Detection of Hashimoto's autoimmune disease among patients with thyroid disorders

Autoimmune disease was detected by using the COBAS E 411 system for all samples; the result for patients was 40% (24/60) positive and 60% (36/60) negative, while the result for all controls was negative. where the result showed a significant difference between the patient and the control Table (4)

Table 4. Detection of Hashimoto Autoimmune Disease among patient with thyroid disorder:

Auto-immune	Group	Patient (N=60)		P-value	Control (N=60)	
		Positive (%)	Negative (%)		Positive (%)	Negative (%)
Gender	Male	6 (10)	9 (15)	0.6	0	60 (100)
	Female	18 (30)	27 (45)		0	60 (100)

3.4 Correlation between EBV infection and Hashimoto's autoimmune disease in patients with thyroid disorders

The percentage of patients who were positive for EBV and Hashimoto autoimmune disease was (25%, 15/60), while the percentage of patients with Hashimoto autoimmune disease who were not infected with EBV was (15%, 9/60). In contrast, the percentage of patients who tested positive for EBV but were not infected with Hashimoto was 20% (12/60), and the percentage of patients who were not infected with EBV and Hashimoto was 40% (24/60) Table (5)

Table 5. Correlation between EBV infection and Hashimoto's autoimmune disease in patients with thyroid disorders

Parameter	Group	TPO (N=60)		P-value	Control (N=60)	
		Positive (%)	Negative (%)		Positive (%)	Negative (%)
EBV	Positive	15 (25)	12 (20)	0.02	0	0
	Negative	9 (15)	24 (40)		0	60 (100)

4 Discussion

Up to 10% of the global population [18] is affected by autoimmune thyroid disorders (AITDs) such as Hashimoto's thyroiditis (HT), Lymphocytic One of HT's histological characteristics

is the infiltration of the thyroid gland, which leads to the creation of antibodies that set off an immune response. Antibodies to either thyroglobulin or thyroperoxidase [19] are the most prevalent antibodies in this condition. Multiple factors, The development of HT is thought to be significantly influenced by a variety of factors, such as genetics, viral illnesses, the environment, and others. EBV is the most prevalent of the viruses associated with the onset of numerous autoimmune illnesses. Once a patient is infected with the virus, the virus can stay dormant within B-cells until it finds an appropriate activation trigger, which makes the infection permanent [20]. In the literature, the issue of whether thyroid surgery is safe for older patients is still up for debate. Additionally, because there is no established age cutoff for older people, Different research defines different age ranges, which makes comparison analysis difficult [21].

In this study, the relationship between HT and EBV is demonstrated; in figure (1), patients with EBV have a greater number of B lymphocytes than healthy controls, as The majority of the time, EBV infection affects children and remains dormant in B lymphocytes. In accordance with the investigation by X. Bian et al., compared to healthy controls, patients with T1DM had a considerably higher EBV antibody titer [22].

In a separate study, Homayouni et al. (2017) identified 65.8% of EBV infections with the EBNA1 gene using nested PCR. Their investigation yielded several significant findings: (a) age was significantly linked with the frequency of EBV; and (b) in young patients, particularly, the presence of high levels of EBV and its gene products may contribute to the formation of thyroid tumours [23]. In figure (3) of the distribution of patients by age group, the oldest age group was 41–50, and in table (4), female patients outnumbered male patients. Between 2005 and 2013, high amounts of EBV and its gene products, especially in young patients, may have played a role in the development of thyroid tumours [24]. 75.6% of cases were categorised into the younger cohort when cases were grouped based on age (>65 years), 16.3% of those in the older age cohort (65 to 74 years), and (Echanique et al. 2019) found that males made up 20.3% of the population, compared to women's (79.3%).

Observed twenty-four female and six male patients who underwent total thyroidectomy. According to numerous sources, almost all thyroid disorders are more prevalent in females, which accounts for this difference in gender distribution. And located About two patients are aged 12–30, followed by six patients aged 31–40. 12 patients are between the ages of 41 and 50, while 10 patients are older than 50[25]. Also, the prevalence of Hashimoto thyroiditis is highest in women between the ages of 30 and 60, and the likelihood of having the ailment rises with advancing age. Women are more likely than men to be affected by this condition. However, it is crucial to remember that this disease can be identified in individuals of any age, even infants [26]. This study found a significant association between Hashimoto's autoimmune disease and EBV, with a higher proportion of infected patients (25%) than uninfected patients (15%). (P-value 0.02) In accordance with Table 5, anti-EBV antibody titers are higher in patients with autoimmune thyroiditis and EBV infection in their sera than in control individuals. [27]. On the other hand, it was revealed that every test for EBV antibodies except for EA IgG, which was positive in 20 HT cases but just four controls, was negative. were found to be positive in both cases and controls [28]. When viral nuclear RNA (EBER) positivity was assessed, Hashimito's thyroiditis (80.7%) and Grave's disease (62.5%) subjects both had a high prevalence of EBV. Acute EBV infection can co-occur with autoimmune hypothyroidism and that thyrotoxicosis can occur just after infectious mononucleosis due to primary EBV infection [29].

5 Conclusion

Our findings revealed that EBV infection, one of the causes of autoimmune diseases such as Hashimoto thyroiditis, can lie dormant within B-cells until a sufficient trigger induces its

activation and growth. It was also discovered that people between the ages of 51 and 60 are more susceptible to developing hashimoto's thyroiditis. The proportion of females with Hashimoto was greater than that of males, and the result was statistically significant when compared to the control sample

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