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ORIGINAL ARTICLE

The Association between Diabetes Mellitus Type 1 and Epstein-Barr virus Infection at Serological and PCR levels

[1]Nehal M. Harfoush*, [2] W. Zaghloul, [2]Wafaa K. Mowafy, [3]Nanees A. Salem, [1]Raghdaa Shrief
[1]Medical Microbiology and Immunology Department, Faculty of Medicine, Damietta University, Damietta, Egypt
[2]Medical Microbiology and Immunology Department, Faculty of Medicine, Mansoura University, Mansoura, Egypt
[3]Pediatric Department, Faculty of Medicine, Mansoura University, Mansoura, Egypt

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Corresponding Author:
Nehal Mohamed Harfoush
Assistant lecturer of Medical Microbiology & Immunology
Damietta Faculty of Medicine, Damietta University, Damietta, Egypt.
Tel.: +201008647628
prdt1919@hotmail.com,

ABSTRACT

Background: *Type 1 diabetes (T1D) is caused by the autoimmune system destroying pancreatic cells. Epstein-Barr virus (EBV) is a linear ds DNA virus. Most children get an early seroconversion infection with EBV and virus antibodies peak between 1-2 years of age.* ***Objective:*** *The aim was to detect Immunoglobulin M (IgM) and IgG antibodies against EBV in children recently diagnosed with T1D and finding the association between EBV infection and glutamic acid decarboxylase antibodies and zinc transporter 8 autoantibodies in those children.* ***Methodology:*** *This study included 50 children their age ranged from 1-18 years with new-onset T1D within 3 months of diagnosis and 50 controls from March to October 2022.* ***Result:*** *In the control group, 2% of participants had positive IgM compared to 18% of the patients' group (p = 0.008). The percentage of positive EBV IgM was significantly higher in positive (37.5%) than negative PCR patients (11.1%) (P = 0.042). Additionally, the percentage of positive GADA marker was statistically significant higher in positive (57.1%) than negative PCR patients (22.2%) (P = 0.017).* ***Conclusion:*** *There was significant difference as regards EBV IgM marker and GADA marker between positive and negative PCR patients.*

INTRODUCTION

Diabetes mellitus (DM) is considered a metabolic disorder characterized by variable degrees of impaired protein, lipid, and carbohydrate metabolism as well as persistent hyperglycemia. Type 1 diabetes mellitus (T1D), often known as juvenile diabetes, is characterized by beta cell loss brought on by an autoimmune mechanism resulting in an absolute lack of insulin. Diabetes mellitus type 1 is usually associated with glutamic acid decarboxylase antibodies, insulin, or islet cells, which identify the autoimmune pathways leading to beta cell loss[1].

The age at which childhood-onset T1D first appears is described by a dual distribution, with two peaks at 4-6 years old and early puberty (10-14 years old). In total, 45% of children are presented before the age of ten. Despite the fact that most autoimmune illnesses are commonest in women, no gender difference in the overall prevalence of childhood T1D was detected. Egypt accounts for around 25% of all childhood T1D cases in the Eastern Mediterranean and Middle Eastern regions. In Egypt, 8 out of 100,000 children under the age of 15 have T1D[2].

Glutamic acid decarboxylase (GAD) antibodies, antibodies to tyrosine phosphatase-like proteins such as insulinoma-associated protein (IA-2, ICA512), antibodies to islet cell autoantibodies (ICA), insulin autoantibodies (IAA), and antibodies to zinc transporter 8 (ZnT8) can all be found during the preclinical stage of T1D. Of patients with newly diagnosed T1D, 60–80% have autoantibodies against ZnT8. Furthermore, 26% of type 1 diabetic patients without antibodies to insulin, GAD, IA-2, or ICA had ZnT8 autoantibodies[3].

World Health Organization classifies T1D into two categories: autoantibody-positive (immune-mediated) diabetes (type 1A) and idiopathic diabetes with beta-cell loss (type 1B), based on the presence of detectable serum autoantibodies. The majority of patients (70–90%) have autoimmune T1D (type 1A diabetes mellitus), and their loss of β-cells is a result of T1D-related autoimmunity, which also occurs concurrently with the development of T1D-associated autoantibodies. Idiopathic T1D, also known as type 1B diabetes mellitus, affects a smaller proportion of patients when no immune responses or autoantibodies are found and the origin of β-cell loss is unknown; this kind of diabetes has a significant genetic component[4].

Epstein-Barr virus, also known as Human Herpes Viruses (HHV) 4, is a member of the Lymphocryptovirus genus within the Gammaherpesviridae family. Most initial EBV infections are subclinical over the world. There are two primary EBV antibodies, IgM and IgG, that are globally prevalent and detected in all population groups. Ninety to ninety-five percent of adults have positive EBV antibody tests[5].

Real-time polymerase chain reaction (PCR) is a common and widely-used technique to quantify EBV loads. It uses the laser scanning in a closed tube or 96-well plate to measure the buildup of amplified products[6].

Epstein-Barr virus has been linked to the emergence of autoimmune diseases due to its ability to evade the immunity and modulate the immune response, making it a plausible candidate for the start and development of autoimmune disorders. Subsequently, it has been proposed that it is connected to the start of T1D. Studies on the association between EBV and the onset of T1D, however, may be extremely scarce[7].

The aim of the present work was finding out the association between EBV infection and T1D by detection of EBV DNA by Real-time PCR and IgM and IgG antibodies against EBV as well as GADA and ZnT8 autoantibodies by ELISA in children recently diagnosed as TID.

METHODOLOGY

Study Population

This study included 50 patients with new-onset T1D within 3 months diagnosis their age ranged between 1-18 years as a patient group as well as 50 children attended the same hospital for a cause other than diabetes as a control group. All participants' data were collected from their files including demographic parameters and risk factors. Blood samples were collected to detect DNA of EBV by Real time PCR and IgM and IgG antibodies against EBV, glutamic acid decarboxylase antibodies and Zinc transporter 8 autoantibodies using ELISA.

Exclusion criteria

Patients with DM type 1 more than 3 months, malignancies, autoimmune diseases or with other viral infections were excluded.

Blood Sampling

Blood samples were collected from both groups of the study in coagulates gel tubes and sent immediately to Medical Microbiology and Immunology Department then were left to clot at room temperature followed by centrifugation at 3000 rpm for 15 minutes to separate the serum. The serum samples were put in 3 tubes and stored frozen at -20˚C until used.

Molecular Identification of EBV by Real-Time Polymerase Chain Reaction:

DNA isolation from serum was performed using the QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions then EBV detection of DNA and amplification of the Epstein–Barr nuclear antigen 1 (EBNA-1) and beta-globulin genes as an internal control by real-time PCR; QauntStudio5 PCR System (Applied Biosystems, Waltham, MA, USA) were performed[8].

Enzyme-linked Immunosorbent Assay (ELISA):

Immunoglobulin M and G for EBV, glutamic acid decarboxylase antibodies and zinc transporter 8 autoantibodies were detected by ELISA technique (Demeditec Diagnostics/Germany) according to the kit instructions.

Statistical analysis and data interpretation:

Collected data was revised, coded, tabulated and introduced to a PC using Statistical package for Social Science (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.). Data were presented and analysed depending on the type of data for each parameter.

RESULTS

Statistical analysis of sociodemographic data (age and sex) of the studied groups showed no statistically difference among the studied groups regarding the sex and age. Most of patients in the patients group were male (52%) while were female in the control group (54%). According to age groups, most of patients in the patients group ranged from 6-10 years (46%), on the other hand, most of patients in the control group were ≤ 5 years (42%) (Table 1).

Table 1: Comparison of the demographic data (age and sex) between the patients and control groups

Variables	Patients group (n= 50)	Control group (n= 50)	Test of significance	P value
Sex				
Males (n= 49)	26 (52%)	23 (46%)	$\chi2$= 0.360	0.548
Females (n= 51)	24 (48%)	27 (54%)		
Age groups				
≤ 5 years (n= 33)	12 (24%)	21 (42%)	$\chi2$= 3.688	0.158
6-10 years (n= 40)	23 (46%)	17 (34%)		
11-18 years (n= 27)	15 (30%)	12 (24%)		

P: probability. Categorical data expressed as Number (%). $\chi2$: Chi-square test

Comparison of the markers' distribution between patients and control groups were illustrated in table 2. There was statistically significant difference between the studied groups regarding EBV IgM marker (p =0.008); the number of participants with positive IgM was higher in the patients group (18%) than in the control group (2%). Most of participants in patients and control groups did not have EBV IgM marker, 82% and 98%, respectively. There was no significant difference between the studied groups regarding other markers (EBV IgG, GADA, ZnT8 autoantibodies and EBV DNA).

Table 2: Comparison of the markers' distribution (EBV IgM, EBV IgG, GADA, ZnT8 autoantibodies and EBV DNA) between the patients and control groups

Marker	Patients group (n= 50)	Control group (n= 50)	Test of significance	P value
EBV IgM				
Negative (n= 90)	41 (82%)	49 (98%)	**FET = 7.111**	**0.008***
Positive (n= 10)	9 (18%)	1 (2%)		
EBV IgG				
Negative (n= 51)	24 (48%)	27 (54%)	$\chi 2$= 0.360	0.548
Positive (n= 49)	26 (52%)	23 (46%)		
GADA				
Negative (n= 67)	34 (68%)	33 (66%)	$\chi 2$= 0.045	0.832
Positive (n = 33)	16 (32%)	17 (34%)		
ZnT8 autoantibodies				
Negative (n= 88)	43 (86%)	45 (90%)	$\chi 2$= 0.739	0.538
Positive (n = 12)	7 (14%)	5 (10%)		
EBV DNA				
Negative (n= 68)	36 (72%)	32 (64%)	$\chi 2$= 0.735	0.391
Positive (n= 32)	14 (28%)	18 (36%)		

P: probability. Categorical data expressed as Number (%). $\chi 2$: Chi-square test.
FET: Fischer's exact test. *: significant value < 0.05.

There was statistically significant difference between positive PCR patients and negative PCR patients as regards EBV IgM marker (P = 0.042); the percentage of positive EBV IgM marker was statistically significant higher in positive PCR patients (37.5%) than negative PCR patients (11.1%). Additionally, there was statistically significant difference between positive PCR patients and negative PCR patients as regards GADA marker (P = 0.017); the percentage of positive GADA marker was statistically significant higher in positive PCR patients (57.1%) than negative PCR patients (22.2%). On the other hand, there was no statistically significant difference between the patients as regards the relation between EBV DNA (PCR) and other markers (EBV IgG and ZnT8 autoantibodies) (Table 3).

Table 3: Relation between EBV DNA and other markers (EBV IgM, EBV IgG, GADA and ZnT8 autoantibodies) in the patients group

Markers	Items	EBV DNA				Significance test
		Negative (36)		Positive (14)		
		No	%	No	%	
EBV IgM	Negative	32	88.9	9	62.5	X^2=4.134,
	Positive	4	11.1	5	37.5	**P 0.042***
EBV IgG	Negative	20	55.6	4	28.6	X^2=2.941,
	Positive	16	44.4	10	71.4	**P 0.084**
ZnT8 autoantibodies	Negative	32	88.9	11	78.6	X^2=0.891,
	Positive	4	11.1	3	21.4	**P 0.345**
GADA	Negative	28	77.8	6	42.9	X^2=5.649,
	Positive	8	22.2	8	57.1	**P 0.017***

*= significance

In attempt to find a relation between the presence or absence of EBV IgM and other markers in our study (EBV IgG, GADA and ZnT8 autoantibodies) in patients group, we found no statistically significant difference between the EBV IgM and other markers (EBV IgG, GADA and ZnT8 autoantibodies) as shown in table 4. The number of patients who were EBV IgM marker positive was 9. All patients with positive ZnT8 autoantibodies did not have EBV IgM marker.

Table 4: Relation between EBV IgM and other markers (EBV IgG, GADA and ZnT8 autoantibodies) in the patients

Markers	Items	EBV IgM				Significance test
		Negative (41)		Positive (9)		
		No	%	No	%	
EBV IgG	Negative Positive	22 19	53.7 46.3	2 7	22.2 77.8	X^2=2.922, **P 0.087**
GADA	Negative Positive	28 13	68.3 31.7	6 3	66.7 33.3	FET, **P 0.999**
ZnT8 autoantibodies	Negative Positive	34 7	82.9 17.1	9 0	100.0 0.0	FET, **P 0.450**

*= significance

The relation between EBV IgG and other markers was illustrated in table 5. About 61.5% of patients with positive EBV IgG did not have GADA while 38.5% of positive EBV IgG patients had GADA.

Table 5: Relation between EBV IgG with other markers (GADA and ZnT8 autoantibodies) in the patients

Markers	Items	EBV IgG				Significance test
		Negative (24)		Positive (26)		
		No	%	No	%	
GADA	Negative Positive	18 6	75.0 25.0	16 10	61.5 38.5	X^2=1.039, **P 0.310**
ZnT8 autoantibodies	Negative Positive	20 4	83.3 16.7	23 3	88.5 11.5	FET, **P 0.907**

*= significance

The relation between ZnT8 autoantibodies with GADA was illustrated in table (6). There was no statistically significant difference between positive and negative ZnT8 autoantibodies patients as regards other markers. The percentage of patients with positive ZnT8 autoantibodies and positive GADA was 42.9%.

Table 6: Relation between ZnT8 autoantibodies with GADA in the patients

Markers	Items	ZnT8 autoantibodies				Significance test
		Negative (43)		Positive (7)		
		No	%	No	%	
GADA	Negative Positive	30 13	69.8 30.2	4 3	57.1 42.9	FET, **P 0.793**

DISCUSSION

Diabetes Mellitus is among the oldest illnesses that humans have ever faced. It is a metabolic disease characterized by chronic hyperglycemia and varying degrees of defective metabolism of proteins, lipids, and carbohydrates. Although the genesis and etiology of diabetes vary widely, abnormalities in insulin responsiveness, insulin production, or both often occur along the course of the disease[9].

Numerous environmental variables, such as viruses, have been suggested as possible risk factors that could impact Type 1 diabetes development. Human T1D has been associated with multiple viruses, such as the mumps, rotavirus, Epstein-Barr virus (EBV), and cytomegalovirus (CMV). The Epstein-Barr virus can

infect a variety of cell types mainly, B cells and epithelial cells[10].

The 100 participants in this study were split into two groups: 50 children, aged 1 to 18 years, who were diagnosed with T1D within three months of the initial diagnosis, and 50 children, aged 1 to 18, who attended Mansoura University Children's Hospital (MUCH) for a reason other than diabetes and were appropriately selected in terms of age, sex, and place of residence.

To see if type 1 DM and EBV infection were related, the two groups were compared regarding zinc transporter 8 autoantibodies, IgM, IgG antibodies and glutamic acid decarboxylase antibodies by ELISA and EBV DNA in whole blood using real-time PCR.

The two groups under study did not significantly differ in terms of age. The majority of patients (46%) in the cases group belonged to the 6–10 age range. This is the same finding detected in the study done by Gomes *et al.*[11] who found that the age at time of diagnosis of T1D patients was 11 (6–16) years. The age range of 10 to 14 years old has been documented as the greatest incidence of T1D[12].

Despite the fact that most autoimmune illnesses are more prevalent in women, there is no gender difference in the overall prevalence of childhood T1D[13]. Type 1 diabetes is more prevalent in men in certain studies[14-15]. However, no statistical difference was detected between the number of male and female T1D cases in our study, despite the fact that there were more male cases than female ones. Additionally, a study done by Jasim *et al.* has found that the female rates are higher than males in the first type of diabetes[16].

The existence of Epstein Barr virus (EBV) increases the risk of seven autoimmune diseases, including inflammatory bowel disease, celiac disease, and diabetes and EBV infection has a critical role in diabetes type1 development[17-18].

Presence of EBV IgM indicates recent initial infection while, EBV IgG antibodies indicates past infection[19].

Detection of EBV antibodies, IgM or IgG indicates EBV infection and inflammation. This inflammation has a role in the development and pathogenesis of T1D in different mechanisms. Chronic infection results in increased processing and presentation of viral antigens which may have mimicry with host proteins. This leads to a continuous acquisition of autoreactive events, leading to tissue damage and virus-mediated self-tissue destruction ended by different diseases including T1D[7].

In our present work, EBV IgG was detected in 52% and 46% of patients and control groups, respectively, without statistically significant difference between them. Similarly, Mohammed and Sabr stated that the T1D patients with positive EBV IgG were higher than the patients without EBV IgG [20] and in another study has done by Jasim *et al.*[16] , 86% of T1D patients had EBV IgG antibody. It has been known that over 95% of population have antibodies against EBV antigens[21].

There was statistically significant difference between the studied groups regarding EBV IgM marker in this work; 18% of patients had EBV IgM and only 2% in control group had EBV IgM. Most of participants in patients and control groups did not have EBV IgM, so they did not have acute EBV infection or EBV reactivation.

Another study completed by Mohammed and Sabr[20] revealed that only seven out of 56 T1D patients were positive for anti-EBV IgM antibody and there was significant differences between the patient and the control groups. In agreement, a study was done on 180 type 1 diabetic patients and 150 healthy individuals showed only 25 positive samples for the EBV IgM antibody[16].

Epstein-Barr virus reactivation-induced IgM autoantibodies have the potential to damage the healthy tissue and trigger the classical complement system, which aids the development and worsens autoimmune disorders such as Graves' disease as a result of antibodies production by EBV reactivation[22].

Seven cases in our work were positive for both IgM and IgG. That means that these children were in acute stage of EBV. IgM antibody appears first after exposure to the virus then tends to disappear after about four to six weeks while IgG emerges during acute infection with the highest level at two to four weeks, then drops slightly, stabilizes, and persists for life[23].

Real-time polymerase chain reaction (PCR) is a standard and popular method to estimate EBV loads. It is quick, sensitive, consistent and advantageous due to using of closed tubes or wells decreasing the risk of carry-over contamination[24].

In this work, the percentage of T1D patients with negative PCR was higher than the percentage of patients with positive PCR (72% vs. 28%). In accordance, Jasim *et al.*[16] has reported that the number of diabetic patients with PCR negative was double the number of PCR positive patients. Additionally, Mohammed and Sabr[20] releaved that 15 T1D patients (26.76%) had EBV genome detected by PCR.

Following a primary infection, the typical pattern of antibody development to EBV-specific antigens is the expression of IgM antibodies to the viral capsid antigen (VCA IgM), which are quickly replaced by IgG antibodies to this antigen (VCA IgG), which last for years. Antibodies to the early antigen (EA), membrane antigen and EBV nuclear antigens (EBNA) start to emerge a few weeks after acute infection and last a lifetime[25].

The positive PCR indicates that the patient is currently infected with EBV[26]. In this study, 71.4% of PCR positive patients had EBV IgG and only 37.5% of PCR positive patients had EBV IgM which might indicate poor immune response. Young children

(especially <4 years old), immunosuppressed, or immunodeficient children may not have specific antibodies. Additionally, the viral antibodies level may not reach the lower limit of detection[27]**.** EBV infection increases cytotoxicity and destruction of the immune cells by causing inflammatory cytokines to be released[28].

The clinical manifestations of type 1 diabetes can be diagnosed, predicted, and differentiated using anti-islet autoantibodies against insulin such as glutamic acid decarboxylase (GADA), insulinoma-associated antigen-2 (IA-2A), and the recently identified zinc transporter 8 (ZnT8A) autoantibodies[29].

Antibodies to GAD are found in 70 % of cases with type 1 diabetes at the time of diagnosis [29]**.** Anti-GAD positivity in persistently non-diabetic (PND) adults is associated with HLA risk haplotypes and thyroid autoimmunity but not with clinical parameters of diabetes**.** ZnT8 autoantibodies are present in 60–80% of newly diagnosed T1D patients. In addition, 26% of people with type 1 diabetes who do not have antibodies to GAD, insulin, ICA, or IA-2 have ZnT8 autoantibodies[30]**.**

Zinc transporter-8 (ZnT8), encoded by SLC30A8 gene, is a secretory granule membrane protein highly specific to the pancreatic beta cells that identified as an autoantigen in T1D[31]**.** According to other earlier studies, zinc transporter 8 (ZnT8) is linked to both childhood and acute onset type 1 diabetes patients, and it is thought to be a more precise indicator of autoimmune-mediated beta-cell death[32-33]**.**

Only twelve participants (7 patients and 5 control) in our work were positive ZnT8 autoantibodies. On consistent, Garnier *et al.*[34] detected 110 out of 516 patients suffered from T1D with positive ZnT8A**.**

On the other hand, in the study done by Gomes *et al.*[11] Znt8A was detected in 68.7% of recent-onset T1D patients and 48.9% of the entire patient cohort**.** In 302 patients with T1D at time of diagnosis, the positivity of ZnT8A was 62% by radio binding assay and electrochemiluminescence[35]**.** Additionally, the study of 268 children with type 1 diabetes detected 117 patients with positive ZnT8A[36]. It has been concluded that, the ZnT8A detection increases T1D diagnosis rate and has high affinity and high predictive value for T1D development[37]**.**

Regarding the relation between PCR and ZnT8A in our work, nearly half of T1D patients who had positive ZnT8A were positive for PCR. This finding is in line with a study done by Ollig et al.[38] suggested that activation and proliferation of B cells by EBV infection is associated with increased intracellular zinc concentration**.**

Glutamate decarboxylase (GAD) is a neuroendocrine enzyme that facilitates the conversion of glutamate into gamma-aminobutyric acid (GABA) that has the potential to decrease pancreatic inflammation, guard β-cells from autoimmune damage, and enhance the regrowth of new β-cells in the context of T1D[39]**.** Antibodies to GAD instruct the immune system to eliminate pancreatic cells[40].

Our result regarding GADA revealed that third of participants in both patients and control groups were positive for GADA. This contradicts what was stated previously in a study conducted by Jasim *et al.*[16] where they has found that the T1D patients who had GADA were nearly double number of T1D patients who did not have GADA**.**

There was no statistically significant difference between positive and negative ZnT8 autoantibodies patients as regards other markers. All patients with positive ZnT8 autoantibodies did not have EBV IgM marker**.** The percentage of patients with positive ZnT8 autoantibodies and positive GADA was 42.9%.

This finding was in the same line with the study done by Gomes *et al.*[11] who observed that ZnT8A was similarly associated with GAD65A in 30.2% of patients**.**

CONCLUSION

Depending on the present results, there was statistically significant difference between positive PCR patients and negative PCR patients as regards EBV IgM marker (P = 0.042) and GADA marker (P = 0.017).

Recommendations

This study has certain limitations because of its limited sample size and single center design. Larger studies are needed to estimate true response relationships and to detect different mechanisms underlying this connection.

Ethical Approval

An ethical approval was taken from the Institutional Research Board (IRB) (MDP.21.11.84) before performance of this study. Oral informed consent was obtained from the parents of all participant children as well as the approval of the mangers of Mansoura University Children's Hospital (MUCH) in which the study was conducted. Confidentiality and personal privacy were respected at all levels of the study.

Declarations:

Consent for publication: Not applicable

Availability of data and material: Data are available upon request.

Competing interests: The author(s) declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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