

## Original Article

# Evaluation of the presence of GAD65 and IA-2 Autoantibodies in Coxsackievirus B3 and B4 Infection in Type 1 Diabetic Children

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## Abstract

**Background and Aims:** It has been documented that in addition to the genetic susceptibility, environmental factors particularly viruses can also play an important role in the initiation or development of the pathogenesis of type 1 diabetes (T1D). However, findings from several epidemiological studies have shown conflicting results regarding the role of enteroviruses infections in this field of research. The purpose of the current study was to investigate a link between coxsackieviruses B3 and B4 infection and the development of T1D in children.

**Materials and Methods:** In this case-control study, 80 pediatric patients under 14 year of age with T1D and 80 non-diabetic children controls were enrolled between October 2017 to March 2018 from the Children's Medical Center in Tehran. Then, anti-GAD65 and anti-IA-2 autoantibodies were assessed in two groups using commercially available Enzyme linked immunosorbent assay (ELISA) kits. IgG antibodies of both Coxsackieviruses B3 and B4 were also measured by direct ELISA kits.

**Results:** The mean anti-GAD65 antibody titer in CV B3+ samples was  $4.26 \pm 2.46$  IU/mL, and was slightly higher than that found in the CV B3- samples with a mean titer of  $3.62 \pm 2.08$  IU/mL ( $p = 0.22$ ; 95% CI: -1.69 to 0.4). Also, the mean anti-IA-2 ELISA OD450 values in CV B3+ samples ( $0.260 \pm 0.155$ ) was similar with that of the CV B3- samples ( $0.260 \pm 0.160$ ) ( $p = 0.98$ ; 95% CI: -0.079 to 0.077).

**Conclusion:** This study showed that the titer of autoantibodies in CVB3+ or CVB4+ samples were not significantly different compared to CVB- samples. The results of this study suggest that there is still a need for further investigations to prove the association of coxsackieviruses and diabetes.

**Keywords:** Type 1 diabetes; Enterovirus; Coxsackievirus B3; Children; Coxsackievirus B4

## Introduction

**D**iabetes mellitus is a chronic progressive metabolic disorder characterized by hyperglycemia which results from either defects in pancreatic insulin secretion or targeted impairment of insulin action. Such a condition is associated with serious complications especially retinopathy, nephropathy,

neuropathy, and cardiovascular outcomes. Type I Diabetes mellitus (T1DM) is insulin-dependent and usually begins in childhood, while type II diabetes mellitus (T2DM) is noninsulin-dependent and often known as adult-onset diabetes (1).

T1D is one of the most common pediatric chronic diseases, particularly in children under 5 years of age (2). According to the report of International Diabetes Federation (IDF), 497100 children under 14 years of age were diagnosed with T1DM. T1DM occurs by the autoimmune destruction of pancreatic  $\beta$  cells involved in insulin production by autoreactive

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T lymphocytes. Genetic susceptibility plays a critical role in the development of T1DM. However, it has been suggested that environmental and other non-genetic factors may also contribute to the development of the disease (3, 4).

Type B Coxsackieviruses (CVB) are common human viral pathogens belonging to the Enterovirus genus within the Picornaviridae family. Infants, young children and immunocompromised patients are especially susceptible to acquiring CVB infection which can occasionally lead to infectious myocarditis, aseptic meningitis, and pancreatitis. In some clinical studies, it has been suggested that enterovirus infections, particularly CVB infection can be associated with the development of T1DM (5).

Several studies have proposed that humoral (antibody) and/or cellular (T cell) cross-reactivity between host proteins and CVB might be an explanation for this relationship (4, 6). Also some experiments suggested a role for CVB4 and CVB3 in the induction of autoimmune responses against pancreatic islets including the autoantigens glutamic acid decarboxylase (GAD-65) and anti-tyrosine phosphatase-like protein (IA-2) (7, 8).

However, available data are highly controversial, and it is still not clear whether CVB play a role in the development of T1DM.

Considering the controversial data, it is desirable to perform further studies on the relationship between CVB infection and development of the islet autoantibodies.

## Methods

**Study subjects:** A case-control study was performed on 80 diabetic children (as the target group) and 80 non-diabetic children (as the control group) referring to Children's Medical Center, Tehran University of Medical Sciences, over the 6-month period from October 2017 to March 2018. For the case group, the criteria for inclusion of the case group were twofold, namely being under 14 years of age and recent diagnosis of T1DM.

The diagnosis of type 1 diabetes was according to American Diabetes Association (ADA)

criteria (9). Fasting blood sugar (FBS)  $\geq$  126 mg/dL was used as the first criterion for screening pediatric patient with T1D.

Furthermore, the glycosylated hemoglobin (HbA1C) content in blood samples was another criterion applied to confirm diagnosis T1DM (9). Children with other chronic disorders or with other forms of diabetes, as well as children over 14 years old were excluded from this survey. Informed consents were taken from parents of eligible children before enrollment. The protocol of the study was submitted to the Institutional Ethics Committee and in accordance with the Declaration of Helsinki, the ethical principles were followed.

### Sample collection and laboratory tests:

Following 8-12h of fasting, 2mL of venous blood was collected from each participant.

Fasting blood sugar (FBS)  $\geq$  126 mg/dL was used as the diagnostic criterion for diabetes.

Furthermore, the glycosylated hemoglobin (HbA1C) was measured for all the participants. Sera were separated from blood samples by centrifugation and stored at -20C.

**Determination of autoantibodies:** Measurement of serum levels of anti-IA-2 and anti-GAD65 autoantibodies was carried out using commercially available Enzyme linked immunosorbent assay (ELISA) kits (Medizym Diagnostic, Berlin, Germany), according to manufacturer's recommendations.

The concentrations of anti-IA-2 and anti-GAD65 auto-antibodies in each specimen were determined by measuring the absorbance at 450 nm using a microplate reader (Hiperion MPR 4+, Germany). Sera were considered to be positive for anti-GAD65 if antibody levels were  $\geq$ 5.0 IU/ml. Also, sera were considered to be positive for anti-IA-2 autoantibodies if their OD values were  $\geq$  0.304. The Medizym anti-IA2 shows a sensitivity of 71% and specificity of 98%, while the Medizym anti-GAD65 has a diagnostic sensitivity of 91% and a diagnostic specificity of 90% regarding patients with newly onset T1D. All samples (including positive and negative control) were tested in duplicate on two separate days.

**Table 1.** Demographic data and comparison of HbA1c, FBS, autoantibodies and anti-coxsackieviruses antibodies levels of diabetic patients and healthy controls.

Parameter	Diabetic Group	Non-diabetic Group	P value
<b>Gender, no. (%)</b>			
Male	40 (48.7%)	38 (51.3%)	0.752
Female	40 (48.8%)	42 (51.2%)	
<b>Age (Year)</b>			
Mean ± SD	9.05 ± 3.05	5.68 ± 2.32	< 0.0001**
<b>FBS (mg/dL)</b>			
Mean ± SD	255.2 ± 129.4	84.8 ± 8.2	< 0.0001**
<b>HbA1C (%)</b>			
Mean ± SD	10.05 ± 1.98	7.32 ± 0.40	< 0.0001**
<b>Anti-GAD65</b>			
Positive number (%)	30 (37.5%)	5 (6.3%)	< 0.0001**
Mean ± SD (IU/mL)	4.57 ± 2.26	2.81 ± 1.58	< 0.0001**
<b>Anti-IA-2</b>			
Positive number (%)	31 (38.8%)	10 (12.5%)	< 0.0001**
Mean ± SD (OD450)	0.285 ± 0.161	0.220 ± 0.084	0.002**
<b>Anti-CVB</b>			
<b>Anti-CVB3</b>			
Positive number (%)	8 (10.0%)	10 (12.5%)	0.80
Mean ± SD (OD450)	0.271 ± 0.05	0.277 ± 0.04	0.47
<b>Anti-CVB4</b>			
Positive number (%)	21 (26.3%)	28 (35.0%)	0.30
Mean ± SD (OD450)	0.322 ± 0.23	0.313 ± 0.20	0.78
<b>Anti-CVB3 &amp; B4 Coexistence</b>			
Positive number (%)	5 (6.3%)	4 (5.0%)	0.73

\*\* Highly statistically significant

**Anti-Coxsackieviruses IgG Analysis:** The levels of anti coxsackieviruses IgG in the sera of all participants were assessed using commercially available ELISA kit (mybiosource, USA) (7).

The synthetic peptides and secondary antibodies (anti-human IgG antiserum) also were used as described (7).

Optical density higher than the mean plus three standard deviation of each serum dilution of the control group were considered positive.

Also, anti-CVB3 IgG detection was carried out according to the manufacturer's instruction (MyBio-Source, USA).

**Statistical Analysis:** Analysis of all obtained data was done by using SPSS version 18 software (Chicago, IL, USA). Figures were performed using the GraphPad Prism software package, version 7.0 (GraphPad Software, Inc., San Diego, CA, USA). The Student's t test was used to compare the continuous variables between the two groups, and the chi-square test was used to compare the categorical variables. Data were considered statistically significant when p value of less than 0.05. Logistic regression was used to estimate odds ratios (OR) and 95% confidence intervals (CI).

**Table 2.** Association of CVB3 and CVB4 antibodies with GAD65 and IA-2 autoantibodies.

	Anti-CV B3			Anti-CV B4		
	Anti-CV B3 <sup>+</sup>	Anti-CV B3 <sup>-</sup>	p value	Anti-CV B4 <sup>+</sup>	Anti-CV B4 <sup>-</sup>	p value
Anti-GAD65						
Mean ± SD (IU/mL)	4.26 ± 2.46	3.62 ± 2.08	0.22	3.90 ± 2.37	3.59 ± 2.02	0.39
Anti-IA-2						
Mean ± SD (OD450)	0.260 ± 0.155	0.260 ± 0.160	0.98	0.268 ± 0.149	0.256 ± 0.163	0.65

## Results

**Demographic characteristics of participants:** Out of 160 participants included in the study, 82 (51.3%) were females and 78 (48.8%) were males with mean age of  $7.36 \pm 3.19$  years (range, 1–14 years). The mean age in diabetic and healthy groups was  $9.05 \pm 3.05$  and  $5.68 \pm 2.32$ , respectively, and the results of the independent sample t-test showed that this difference was statistically significant ( $p < 0.0001$ ). According to Chi-square test results, diabetic and healthy groups were not statistically significant different in terms of sex distribution ( $p=0.75$ ) (Table 1).

The age distribution of participant in the two groups is shown in Table 2.

**Serum Levels of FBS and HbA1c:** The mean FBS level in children with T1D ( $255.2 \pm 14.47$  mg/dl) were significantly higher than in healthy children group ( $84.83 \pm 0.92$  mg/dl) ( $p < 0.0001$ ).

In the present study, it was observed that the mean HbA1c value of diabetic children was statistically significant higher than that of healthy children, the mean value of diabetic children being  $10.06 \pm 0.22\%$  and that of non-diabetic children being  $7.32 \pm 0.04\%$  ( $p < 0.0001$ ) (Table 1).

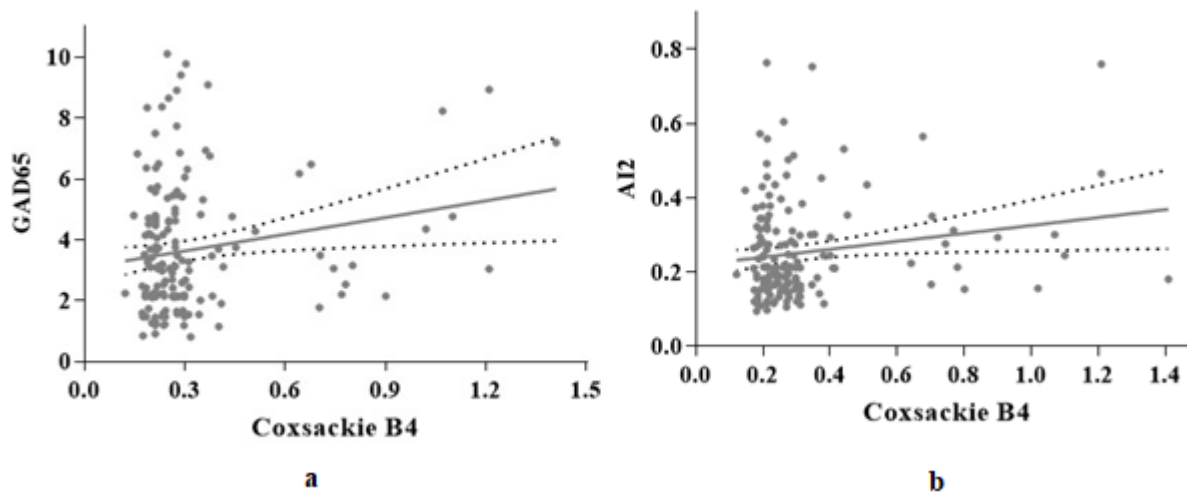
**Analysis of anti-CV IgG:** Among 160 participants, 18 (11.3%) were positive for anti-CV B3 IgG and 49 (30.6%) were positive for anti-CV B4 IgG. Positivity percentages of anti-CV B3 IgG detection in diabetic and non-diabetic children were 10.0% and 12.5%, respectively ( $p = 0.61$ ; Odds Ratio [OR]=0.77; 95% confidence interval [CI]: 0.29-2.08).

Anti-CV B4 IgG was detected in 26.3 % and 35.0 % in diabetic patients and healthy control, respectively ( $p = 0.23$ ; OR=0.66; 95% CI: 0.33-1.30) (Table 1).

**Analysis of autoantibodies:** Among 160 participants, 41 (25.6%) were positive for anti-IA-2 IgG and 35 (21.8 %) were positive for anti-GAD65 IgG. A statistically significantly higher positivity rate of anti-IA-2 IgG was seen in diabetic group (38.8%) compared with non-diabetic group (12.5%) ( $p < 0.0001$ ; OR=4.42; 95% CI: 1.98-9.86). Furthermore, the mean anti-GAD65 IgG level in diabetic children ( $4.57 \pm 2.26$  IU/mL) was significantly higher than in the control group ( $2.81 \pm 1.58$  IU/mL) ( $p < 0.0001$ ) (Table 1).

**Assessment of association between levels of autoantibodies and anti-CV IgG:** As shown in Table 2, the mean anti-GAD65 antibody titer in CV B3<sup>+</sup> samples was  $4.26 \pm 2.46$  IU/mL, and was slightly higher than that found at the CV B3<sup>-</sup> samples with a mean titer of  $3.62 \pm 2.08$  IU/mL ( $p = 0.22$ ; 95% CI: -1.69 to 0.4). Also, the mean anti-IA-2 ELISA OD450 values in CV B3<sup>+</sup> samples ( $0.260 \pm 0.155$ ) was similar with that of the CV B3<sup>-</sup> samples ( $0.260 \pm 0.160$ ) ( $p = 0.98$ ; 95% CI: -0.079 to 0.077).

The mean titer of anti-GAD65 antibody in CV B4<sup>+</sup> samples ( $3.90 \pm 2.37$  IU/ml) was not shown significantly difference compared to CV B4<sup>-</sup> samples ( $3.59 \pm 2.02$  IU/ml) ( $p = 0.39$ ; 95% CI: -1.03 to 0.412) (Table 2). However, linear regression analysis showed that anti-CV B4<sup>+</sup> antibodies levels were positively correlated with the levels of anti-GAD65 antibodies ( $p=0.01$ ;  $r=0.18$ ; 95% CI: 0.03 to 0.33) (Fig. 1a).



**Fig. 1.** Correlation between the levels of anti-CVB4 IgG antibodies and a) anti-GAD65 antibodies; b) anti-IA-2 antibodies

The mean anti-IA-2 ELISA OD450 values in CV B4+ samples ( $0.268 \pm 0.149$ ) was slightly higher than that of the CV B4- samples ( $0.256 \pm 0.163$ ). However, this difference was not statistically significant ( $p = 0.65$ ; 95% CI: -0.066 to 0.041) (Table 3). Nevertheless, similar to anti-GAD65, linear regression analysis revealed that anti-CV B4+ antibodies levels were positively correlated with the levels of anti- IA-2 ( $p = 0.02$ ;  $r = 0.17$ ; 95% CI: 0.01 to 0.32) (Fig. 1b).

## Discussion

Type 1 diabetes is a genetic autoimmune disorder and several studies suggested the involvement of an environmental factors (10). A significant numbers of viruses are associated with T1D (11). Also, coxsackie B viruses have been detected in the pancreatic islets T1D patients (2, 12). Furthermore, several studies indicated the role of CVB4 and CVB3 as the causative agents of T1D (13, 14).

In this study, assessment of association between levels of pancreatic autoantibodies and anti- CVB IgG was carried out.

In our study, we found that the mean titer of autoantibodies in CVB3+ or CVB4+ samples were not shown significantly difference compared to CVB- samples. The results of this study are similar to those of Yoon et, al.

They showed that the incidence of diabetes in the CVB4 infected group after the epidemic was not different from the control group. They concluded that viral infections alone cannot cause diabetes and a genetic background is needed.

Maha and colleagues studied the two groups of diabetic patients. In the first group, the disease was recently diagnosed and the second group had diabetes for more than one year. They showed that although the increase in IgM antibody against CVB4 in the first group was greater than that of the second group, there was no difference between the two groups for IgG antibodies. In our study, ELISA was used to measure IgG against CVB3 and CVB4, and the results was similar to that of Maha et, al. In conclusion, the results of this study suggest that there is still a need for further investigations to prove the association of coxsackie-viruses and diabetes.

## Conclusion

This study has shown that the titer of auto-antibodies in CVB3+ or CVB4+ samples were not significantly different compared to CVB- samples.

The results of this study suggest that there is still a need for further investigations to prove the association of coxsackieviruses and diabetes.

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## Conflict of interest

No conflict of interest is declared.

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