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Evaluation of the Probable linkage between Cytomegalovirus and Type 2 diabetes

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ABSTRACT

Some recent investigations, have suggested a probable linkage between cytomegalovirus (CMV) and developing diabetes. But still there is not enough literature to assess the rate of this association. To this purpose, in the present study for detecting CMV genome, serum samples of 50 type 2 diabetic patients as the test group with an optimized PCR were tested. Sex, age and A1C parameter related to this group were recorded too. The specificity of the PCR test was 100% and its sensitivity was 100 copy of the viral genome in the sample. The related results were compared with the control group that was included 50 non-diabetic participants. The CMV genome did not find in None of these 100 samples. No related effects were observed between sex or amount of A1C and association of CMV genome in these patients. However, it seems that the age parameter is effective in this regard. Comparing these participants with some other similar research suggest that this association rate is not very high and the virus only in elderly age can be considered as a probable risk factor. Thus, for managing diabetes should be more concentrated on the-well-known factors such as obesity or unhealthy lifestyle.

1. Introduction

In addition to type 1 and post-transplant diabetes, cytomegalovirus (CMV) has been proposed as a triggering factor for type 2 of this metabolic disorder (Eltayib et al., 2014; Karim et al., 2014; Faraj et al., 2019; Yoo et al., 2019). This relatively new idea can be very effective to control the growing number of type 2 diabetes, but still, there is not enough literature in this regard. This study is planned to evaluate the rate of correlation between CMV and diabetes type 2.

Cytomegalovirus is a member of the Herpesviridae family. Its genome is in the form of double-stranded linear DNA. This virus is considered as one of the most common human pathogens because its related infections are often asymptomatic (Faraj et al., 2019; Cannon et al., 2012). CMV can be transferred from several different routes such as saliva, sexual contact, transplantation of solid organs, blood transfusion, etc. After first exposure that leads to infection, its genome as an external plasmid can remain forever in hematopoietic stem cells $CD34^{+}$ (Yoo et al., 2019; Cannon et al., 2012; Kumar and Herbein, 2014; Poole and Sinclair, 2015; Cannon et al., 2010). Whenever immune systems confront with a defect, the hidden virus can become again active.

There are some published reports that all convey the cytomegalovirus may have a role as a risk factor for developing type 2 diabetes. Some studies propose that lifelong infection

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with CMV will lead to chronic inflammation and also induce production of pro-inflammatory cytokines, that have harmful effects on the pancreatic β-cells. This in turn may cause defects in the cell's responses to insulin and consequently may develop the diabetic disease. Another line of research suggests that inducing of viperin (endoplasmic reticulum-associated, interferon-inducible virus inhibitory protein) production by CMV, is responsible for developing diabetes in these patients. The reason for that is the probable role of viperin to interact with the metabolism of glucose and lipid (Yoo et al., 2019). Derderian et al. (2017) directly show that there is a linkage between CMV infection and metabolic syndromes as it may be dependent on factors like Body Mass Index (BMI) and gender (Fleck-Derderian et al., 2017). Considering what has been mentioned by these studies and some more other, this can be concluded cytomegalovirus most probably have linkage with metabolic syndromes, but the remained question is the rate of this association. If the association rate between CMV and diabetic patients is too high, it can surely be one of the most important risk factors or maybe a causative agent. However, in a lower association rate it would mean that CMV is not a primary risk factor. In both situations awareness of this rate is very helpful.

Due to the importance of this hypothesis in managing diabetes, there is an essential need to have more literature in this field. A few clinical studies, during the last two decades, have used anti-IgG for evaluating exposure to CMV in diabetic groups (Lutsey et al., 2009; Chen et al., 2012; Faraj, et al., 2019; Izadi et al., 2012). However, finding DNA in peripheral blood can better reflect the active replication of the CMV genome and probable chronic inflammation or dysregulation of the immune system by CMV (Yoo et al., 2019). To the best of our knowledge, in Iran, no study specifically has been done to find the CMV genome in the serum of hyperglycemic patients. In this study using the PCR test in the serum samples of a relatively small group of diabetic patients, the genome of CMV has been traced then obtained results compared with a healthy (non-diabetic) control group. In addition, the age, sex, and A1C hemoglobin indexes are considered for studying the possible link to CMV infection.

2. Materials and Methods

2.1. Samples and study groups

In this study 50 diabetic serum samples from medical laboratories in Alborz province, Iran, randomly were collected. Similarly, 50 normal serum samples belong to non-diabetic persons were considered as the control group. Additional data include age, sex, and A1C index for each test sample were recorded too. For ethical considerations the identities of all participants were totally unknown and confidential.

2.2. DNA Extraction and Optimization of PCR test

Using the DNG-plus method, the DNA of total samples were extracted. PCR assay with Glycoprotein B as the target gene, CMV257F (5' CGG TGG AGA TAC TGC TGA GGT C 3'), and CMV257R (5' CAA GGT GCT GCG TGA TAT GAA C 3') as specific forward and reverse primers respectively were used to detect CMV genome in both test and control samples (12). The amplicon size was 257 bp. Then the assay was optimized and validated.

2.3. Evaluation of specificity and sensitivity of PCR test

In order to determine the sensitivity of the PCR test, the genome copy number was calculated for a suspension of the CMV genome. Then a micro serial dilution up to 10^{-6} with that was provided. Each dilution was evaluated to obtain the level of test sensitivity. In terms of specificity, besides online checking, the DNA of humans, mice, both types of *herpes simplex virus*, *Staphylococcus aureus, adenovirus, hepatitis B, and Saccharomyces cerevisiae* were selected to evaluate the test specificity. The Thermal cycler program for PCR amplification was adjusted as below:

First Denaturation 94℃ for 3 min, Denaturation 94℃ for 30 sec, Annealing 66□ for 30 sec, 40 Cycle extension 72℃ for30 sec, and Final Extension 72℃ for 7 min.

2.4. Analysis of PCR products

Finally, the electrophoresis of the reaction product was carried on the 1.5% agarose gel in TBE 0.5 x buffer; the obtained results were

compared between the test and control groups. To visualize the amplicon bands ethidium bromide was used. The relationship between PCR results and other indexes like age, sex, and A1C were studied too.

3. Results

3.1. Characteristics of the test and control groups

The groups were selected randomly. The age was ranged between 20 to 80 years old. 14 cases (28%) of the total 50 persons in the test group, were men and 36 cases (72%) were women. The range of mentioned indexes in the test group in details was as below (Tables1-2).

Overall distributions of A1C and age indexes based on percentage are demonstrated in tables 3 and 4 respectively.

3.2. Optimized mixture of the PCR test

After optimization of the PCR mixture the following list showed the best result in terms of amplicon band clarity:

MgCl2: 1.5 mM, dNTP:0.2mM, Forward primer:0.2 µM, Reverse primer0.2 µM, Taq DNA pol :1.5 U.,10Xbuffer:1x (2.5 µM). The result of optimized test can be seen in figure 1.

3.3. Specificity and Sensitivity of the test

The optimized test showed 100% specificity. As can be seen in figure 2 the amplicon band just is appeared for the CMV genome.
The level of test sensitivity, is 100 copy of virus DNA in reaction. The result of the test sensitivity is demonstrated in figure 3. Amplicon bands up to sixth well reaction, are visible, which is equal to 100 DNA copy in the test mixture (figure 3).

3.4. Results of using the PCR test for both case and control groups

In this step, none of the samples in the study or control groups were positive of the CMV genome. The results were the same for all of the age, sex, and A1C categories.

Table 1. Indexes related to the men participants

A1C / Age	5-6	6.1-7	7.1-8	8.1-9	9.1-10	10.1-11	11.1-12	Total
20-30	---	---	---	---	---	---	---	---
31-40	---	---	---	---	---	---	---	---
41-50	---	1	---	---	1	1	---	3
51-60	---	1	---	---	---	---	---	1
61-70	1	5	2	---	---	---	---	8
71-80	1	1	---	---	---	---	---	2
Total number	2	8	2	---	1	1	---	14

Table 2. Indexes related to the women participants

A1C / Age	5-6	6.1-7	7.1-8	8.1-9	9.1-10	10.1-11	11.1-12	Total
20-30	---	---	5	---	---	---	---	5
31-40	---	3	---	---	---	---	---	3
41-50	---	---	5	---	---	---	---	5
51-60	---	---	---	10	---	---	---	10
61-70	---	---	---	---	8	---	---	8
71-80	---	---	---	---	---	2	3	5
Total	---	3	10	10	8	2	3	36

Table 3. Overalldistribution of A1C index based on percent in the test group

A1C	5-6	6.1-7	7.1-8	8.1-9	9.1-10	10.1-11	11.1-12	Total
Percentage	4%	22%	24%	20%	18%	6%	6%	100%

Table 4. Overalldistribution of Age index based on percent in the test group

Age	20-30	31-40	41-50	51-60	61-70	71-80	Total
Percentage	10%	6%	16%	22%	32%	14%	100%

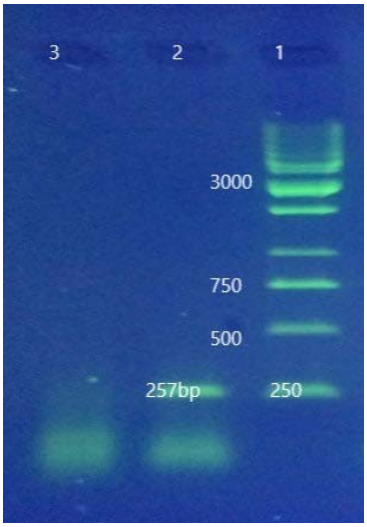

Figure1. The result of the test optimization: 1: 1kb DNA ladder (Fermentas), 2: Positive control (CMV genome), 3: Negative control

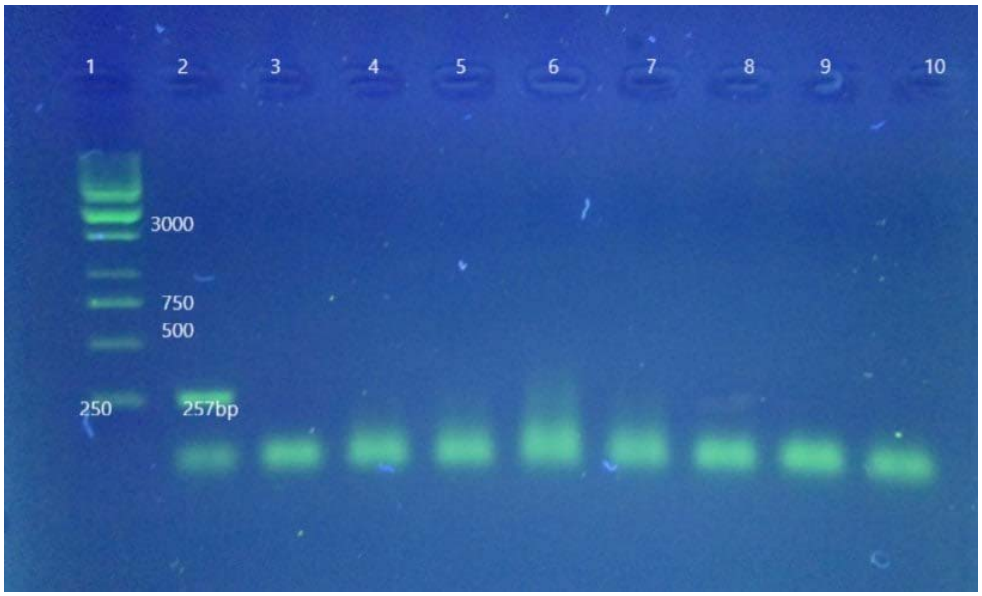

Figure 2. The specificity of the PCR test. 1: 1kb DNA ladder (Fermentas), 2: CMV,3: Human,4: Mouse, 5: HSV1, 6: HSV2, 7: HBV, 8: Adenovirus, 9: Saccharomyces cerevisiae 10: Negative control

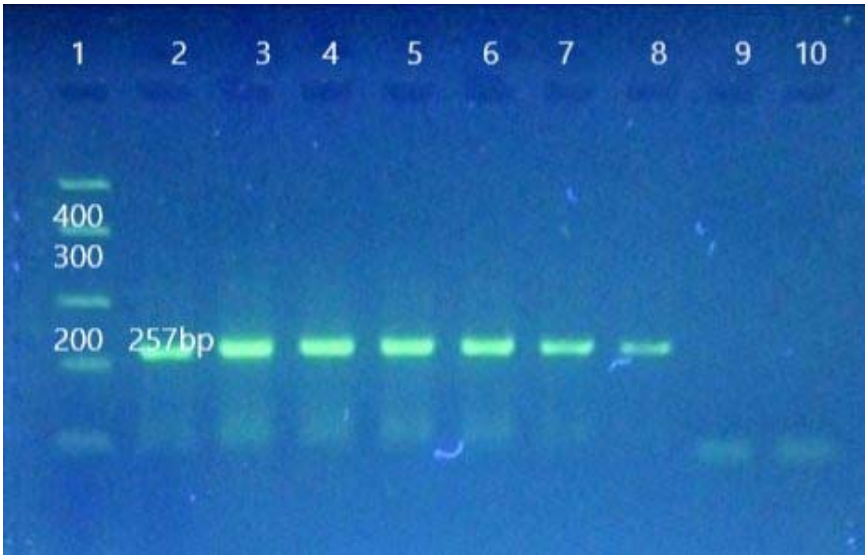

Figure 3. The result of sensitivity of the PCR test: 1: low range DNA ladder, 2: Positive control (CMV) (257 bp) 3: reaction /10000000 copy, 4: reaction/ 1000000 copy,5: reaction/ 100000 copy,6: reaction/ 10000 copy 7: reaction /1000 copy,8: reaction/ 100 copy,9: reaction:10 copy,10: Negative control

4. Discussion

The controversial idea of linking between infections and both types of diabetes has been increasingly discussed by scientific communities. During the current pandemic of COVID-19 lots of studies have pointed to the possible role of this virus in enhancing the risk of diabetes or even cause it (Toniolo et al., 2019; Marchand et al., 2020; Mukherjee et al., 2020; Hussain et al., 2020). The effects of different viruses such as *CMV, HSV, EBV, HCV, enteroviruses* and etc. on developing diabetes have been also reported (Toniolo et al., 2019; Hyöty et al., 2002; Vanni et al., 20016). Actually, denying the bidirectional relation between infections and diabetes is not easy. Because diabetes can weaken the immune system against microbial invaders, so diabetic people are more prone to be defected by infections. The other side of this relatively new idea is the role of microbial inflammation or other related mechanisms to develop diabetes. Like many other novel hypotheses, there are both for and against groups. For instance, Lee et al. (2013) findings did not support concurrent viremia and developing type 1 diabetes in young children (Lee et al., 2013). Similarly, Lutsey et.al (2009) reported no meaningful association was found between pathogen organisms and type2 diabetes in their study group (Lutsey et al., 2009). It seems that in this field the results are inconsistent.

Although there is a relation between infectious agents and diabetes, the inconsistency of different results should be rationalized. In the present study, the CMV genome was not detected in any of the participant's serum samples in the test or control groups. To explain the obtained results some factors such as the type of applied method, size of the study group, sex, A1C index, and age of participants should be considered for possible effects. In terms of method, some studies have been compared serological techniques and molecular methods for detection cytomegalovirus. Despite, ELISA can be a very powerful confirmative test, but it is more proper for showing previous exposure with the agent. However, detecting the genome in peripheral blood by PCR can better reflect the active replication of the virus and the chance of chronic inflammation (Yoo et al., 2019; Sanousi et al., 2016; Lakzayi et al., 2020), which are the key points in this sort of research. The second item, which can be considered as a limitation too, is the small size of the community sample in comparison with the relatively huge number of participants in some other studies. However, the main purpose of this research is to evaluate the strength of the linkage between CMV and diabetes. Therefore, if this rate of incidence is so high, this can be shown better in a small size population. The third and fourth items are sex

and A1C indexes of participants. The results show that both sexes are equal in this regard. As well as A1C hemoglobin doesn't show any significant effect. As the population characteristics demonstrates, around 60% of this group has an A1C index more than 7. This shows that the level of blood sugar had not been controlled properly by the majority of this group during the last three months. In spite of that, none of them were positive for the presence of the CMV genome. Last but not the least, is the age index. It seems that age has a significant effect on the obtained results. Lutseyet al. (2009) in their study with 1000 participants, aged between 45-84, did not observe any association between cytomegalovirus or some other pathogens and diabetes type 2. They concluded no etiological role can be defined for those agents (Lutsey et al., 2009). However, another study in 2012, that all of its participants were above 85, in 17 % of cases association between cytomegalovirus and diabetes mellitus was observed but 7.9% of the studied population without having infection with the CMV was diabetic. Researchers in this study suggested that infection with this virus can be a possible risk factor to cause type 2 diabetes in the elderly (Chen et al., 2012). Comparing these two studies show that age is an important factor. It appears that a long period of chronic inflammation is needed for CMV to develop diabetes. Back to the present study, show that its participants are relatively young and the age range is between 20-80. Apparently, CMV infection in this range of age does not have a significant role, but in elder people, this may be an environmental risk factor.

Considering the obtained results in this study, the association rate between CMV and diabetes type 2 is not very high. Therefore, this cannot be a primary risk factor. In order to control diabetes should be more concentrated on the typical risk factors such as obesity, poor nutrition, and unhealthy lifestyle.Determining the role of other probable risk factors still demands more studies.

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