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Research Article

The role of *Herpes Simplex Viruses* in triggering type 2 diabetes

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ABSTRACT

Recently, some common viruses, like *Herpes Simplex Virus (HSV)*, have been proposed as a risk factor to develop type 2 diabetes. However, still there is not enough literature to determine its importance as a triggering or risk factor. This case-control study is designed to evaluate the Attendance rate of the *HSV* genome in serum samples of diabetic patients. The study and control groups included 50 serum samples of diabetic type 2 and 50 non-diabetic respectively. The detecting PCR test for HSV genome types 1 and 2 with common DNA polymerase gene region, as the target gene, was optimized. Demographic parameters like Sex, age, and A1C were recorded too. Amplicon size was 454 bp. The specificity and sensitivity of the PCR reaction were100% and 50 Copy/reaction respectively. The analysis results showed that neither the genome of *HSV-1* nor 2 was found in those 100 serum samples. In addition, there was no relation between sex or A1C level and *HSV* genome presence. But it seems the relatively young age of this group is effective for obtaining these negative results. The small size of this population with negative PCR results, clearly show that HSV infection cannot be a first-order risk factor, but due to time-consuming mechanisms of probable effects, this may affect elderly populations, as obtained results of some studies. Therefore to prevent or manage type 2 diabetes, still should be more focused on the conventional risk factors such as obesity and malnutrition.

1. Introduction

This project is trying to study the linkage between infectious diseases and metabolic disorders. In this regard, lately has been suggested that *Herpes simplex viruses*, one of the most common viral groups, and type 2 diabetes, one of the most prevalent metabolic diseases, are in connection. In some studies, these viral infections have been introduced as a risk factor or triggering item for diabetes type2 (Woelfle et al., 2022). However, this idea is somehow controversial and still needs more evidence to determine the exact role of these infections.

Eight viruses belonging to the *Herpesviridae* family are suspected to trigger diabetes type 2 include *herpes simplex viruses (HSV) 1 and 2, varicella-zoster virus (VZV), Epstein–Barr virus (EBV), cytomegalovirus (CMV) and human herpesviruses (HHV) 6, 7, and 8* (Dworzański et al., 2019).

The prevalence of Type 2 diabetes has been estimated very high. It was about 9.3% in 2019, and these statistics rapidly reached 10.5 % in 2021, which also results in an abundant mortality rate, mainly because of cardiovascular diseases (Saeedi et al., 2019; sun et al., 2021).

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The well-established risk factors for that are high BMI and obesity, age, high blood pressure, and elevated level of blood lipid. In addition, some environmental factors such as viral and bacterial infections are assumed to be guilty of triggering diabetes. But the proof of the last one is not easy. Since it is not clear if these infections are colonized in the body before the advent of diabetes or after it.

In one aspect should be said that diabetes will reduce the power of the immune system and this will facilitate the establishment of different infections. For instance, during the covid-19 pandemic, that demonstrated diabetes obviously precedes the infections, and this can be a risk factor for initiation of infection (Landstra and de Koning, 2021; Leon-Abarca et al., 2021; Rezaei et al., 2021).

on the other hand, some other studies suggest that herpes simplex virus type 1 and 2 or cytomegalovirus or even covid-19 virus are risk factors for developing diabetes (Sun et al., 2005; Mohammed and Salloom, 2021; Rathmann et al., 2022). Due to the inflammation caused by these infections which can damage body organs this idea can make sense (yoo et al., 2019). However, most of these research projects are cross- sectional studies and can not show the precedence of diabetes or infections (Ghane et al., 2018; Eltayib et al., 2014; Faraj et al., 2019). Besides that these results are usually distorted by demographic factors like age and etc (Lutsey et al., 2009; Chen et al., 2012).

The present study assay the association rate of HSV 1 and 2 in diabetic patients in comparison with a non-diabetic group. Although this case-control study does not prove the preceding infection in diabetic patients, this can demonstrate the accuracy level of this idea, for the conclusion that if infections can be an established risk factor for diabetes, or in what conditions can affect the body's metabolic health situation.

These data can be considered helpful for managing diabetes type 2.

2. Materials and Methods

2.1. Samples and study groups

The samples for the study group include: 50 diabetic serum samples that were randomly collected from Persian medical laboratories. Age, sex and A1C index for this group were recorded. The control group, similarly include 50 non- diabetic patients, that were selected equally in terms of age and sex for each case of the test group. Due to ethical considerations the identities of all participants were totally unknown, and samples were extracted from their routine test samples of these participants, and no other extra sampling was done.

2.2. Extraction of DNA and Optimizing the PCR test

For DNA extraction both boiling and DNG-plus methods were used. The extracted DNA was kept at -20 for usage in the next steps. The common gene region of DNA polymerase between both types of HSV was considered as the target gene. The sequences of forward *(HSVF)* and reverse primers *(HSVR),*are shown in table 1 (Akbarian et al., 2015). Afterward, the test was optimized in terms of reaction content and thermal profile.

Table 1. sequences of primers

primer	sequence
(HSVF)	5'-acctaccggcatacaagctca-3'
(HSVR)	5'-aagtggctctggcctatgtcc-3'

2.3. Determining specificity and sensitivity of the PCR test

To evaluate and determine the sensitivity of the PCR test, the genome copy number was calculated for a suspension of the HSV genome. Then a micro serial dilution up to 10^{-5} 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, cytomegalovirus, Staphylococcus aureus, adenovirus, hepatitis B, and *Saccharomyces cerevisiae* was selected to evaluate the test specificity. The Thermal cycler program for PCR amplification was adjusted as below:

First Denaturation 94°C for 2 min, Denaturation 93°C for 20 sec, Annealing 70 for 20 sec, Extension 70°C for 20 sec. The number of cycles was 40.

2.4. Amplicon

In this step, the reaction product was electrophoresed on the 1.5% agarose gel in TBE

0.5 x buffer. For visualizing the amplicon bands Syber green was used.

The achieved results were compared between the test and control groups. In addition, the correlation between indexes like age, sex, A1C and the existence of HSV genome in specimens were considered too.

3. Results

3.1. Demographic information of the control and study groups

The case group was selected randomly and the control group was selected based on that. The age was ranged from 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 detail was as below (Table2).Overall distributions of A1C and age indexes based on percentage are demonstrated in table 3.

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 the 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 *HSV* genome. The level of test sensitivity is 50 copies of the viral DNA in reaction. The result of the test sensitivity is demonstrated in figure 3. Amplicon bands up to 5th well reaction, are visible, which is equal to 50 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 *HSV* genome. The results were the same for all of the age, sex, and A1C categories (figure 4 -8).

If H_0 regarding the group of men and women is such that there is no relationship between the index and age, then based on the Pearson test, the results showed that:at the statistical level of 95%, there was no correlation between A1C index and men's age($P>0.05$), but a correlation was observed between women's blood sugar level and their age ($P<0.05$). Since the purpose of this research is not the relationship between these variables, we refrain from further explanation in this field.

Table2: Indices related to the participants

A1C / Age	5-6		6.1-7		7.1-8		8.1-9		9.1-10		10.1-11		11.1-12		Total number	
	M	W	M	W	M	W	M	W	M	W	M	W	M	W	M	W
20-30	--	--	--	--	--	5	--	--	--	--	--	--	--	--	--	5
31-40	--	--	--	3	--	--	--	--	--	--	--	--	--	--	--	3
41-50	--	--	1	--	--	5	--	--	1	--	1	--	--	--	3	5
51-60	--	--	1	--	--	--	--	10	--	--	--	--	--	--	1	10
61-70	1	--	5	--	2	--	--	--	--	8	--	--	--	--	8	8
71-80	1	--	1	--	--	--	--	--	--	--	--	2	--	3	2	5
Total number	2	--	8	3	2	10	--	10	1	8	1	2	--	3	14	36

(M= Men, W=Women)

Table 3. Overall distribution of A1C and Age indices based on percent in the test group

A1C / Age	5-6		6.1-7		7.1-8		8.1-9		9.1-10		10.1-11		11.1-12		Total	
Percentage	**M**	**W**	**M**	**W**	**M**	**W**	**M**	**W**	**M**	**W**	**M**	**W**	**M**	**W**	**M**	**W**
20-30	--	--	--	--	--	10	--	--	--	--	--	--	--	--	--	10
31-40	--	--	--	6	--	--	--	--	--	--	--	--	--	--	--	6
41-50	--	--	2	--	--	10	--	--	2	--	2	--	--	--	6	10
51-60	--	--	2	--	--	--	--	20	--	--	--	--	--	--	2	20
61-70	2	--	10	--	4	--	--	--	--	16	--	--	--	--	16	16
71-80	2	--	2	--	--	--	--	--	--	--	--	4	--	6	4	10
Total	4	--	16	6	4	20	--	20	2	16	2	4	--	6	28	72

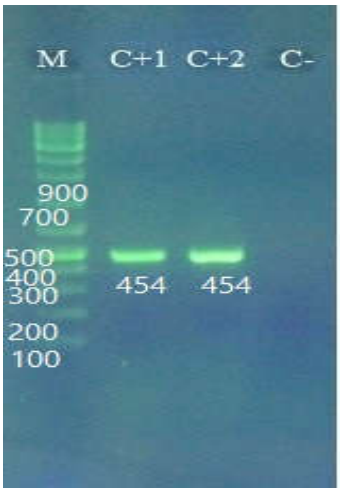

Figure 1: Optimized PCR test. M: DNA Ladder (bioflux) marker size 1Kb. C+1: (positive control) 454 bp PCR product from Herpes Simplex Virus I. C+2: (positive control) 454 bp PCR product from Herpes Simplex Virus II. C- Negative control.

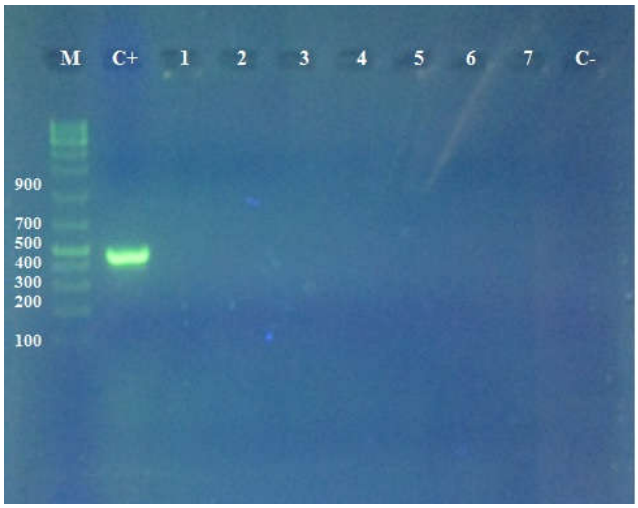

Figure 2: Specificity of the optimized PCR test. M: Size marker of 1 Kb DNA Ladder (bioflux) company. C+: (positive control) 454 bp PCR product from Herpes Simplex Virus I. 1: Human DNA. 2: Mouse DNA. 3: Cytomegalovirus DNA. 4: Staphylococcus aureus DNA. 5: Adenovirus DNA. 6: Hepatitis B virus DNA. 7: Saccharomyces cerevisiae DNA. C-: negative control.

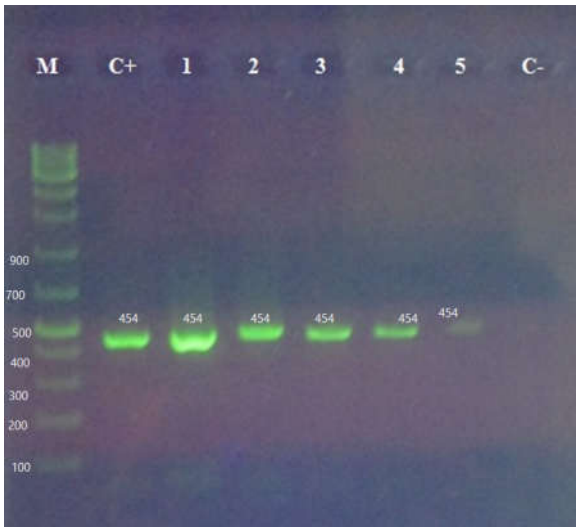

Figure 3: detection limit of the optimized PCR test. M: DNA Ladder (bioflux) marker size 1Kb. C+: (positive control) 454 bp PCR product from Herpes Simplex Virus I. 1: 500,000 DNA/Reaction. 2: 50000 DNA/Reaction. 3: 5000 DNA/Reaction. 4: 500 DNA/Reaction. 5: 50 DNA/Reaction. C-: negative control.

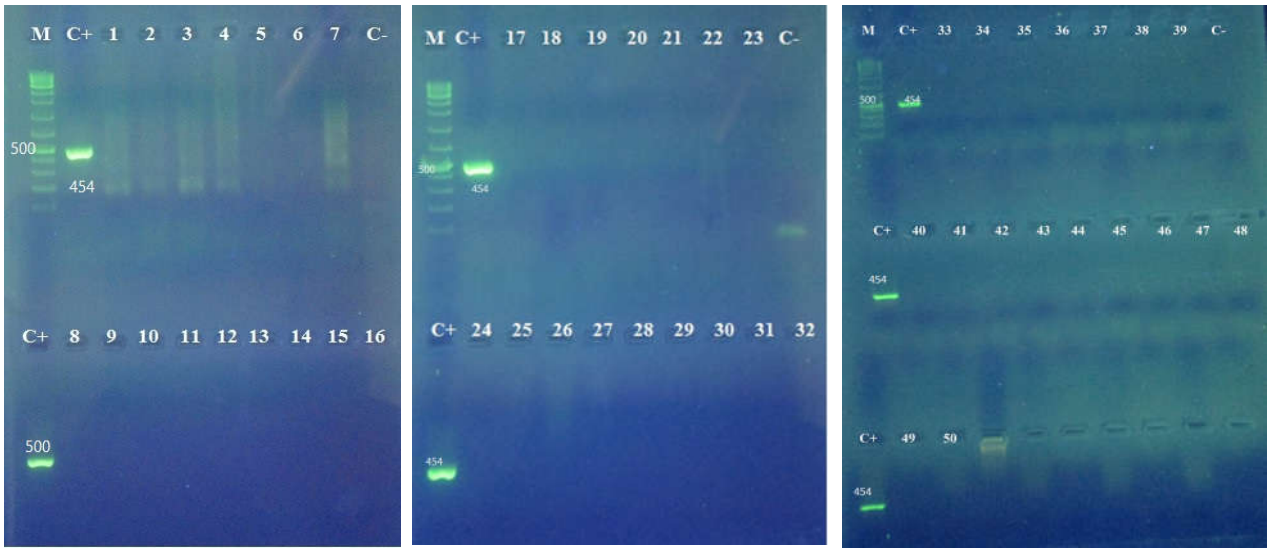

Figure 4: 8. Negative results of PCR test in all of the test samples. M: Kb DNA Ladder (bioflux) marker size 1 C+: (positive control) 454 bp PCR product from Herpes Simplex Virus. 1-50: Negative serum samples of the participants. C-: negative control

4. Discussion

Despite the controversial hypothesis of linkage between viral infections and type 2 diabetes, this study does not show any connection between them. However, that must be mentioned the aim of this research was just to evaluate the rate of this association. Because approving or disapproving of this idea needs much more retrospective or prospective studies that consider many related variables.

The literature available in this field can be categorized into two lines. The first line believes in the probable relationship between viral infections and developing diabetes. For instance Ghane (2018) using PCR and ELISA checked the serum samples of 180 diabetic patients and compared the results with187 healthy controls. Their results showed that only 6.1% of the test

group were positive for DNA of HSV and this rate was 2.7% for the healthy control group. However, 65% of the test group were positive for anti-HSV IgG and this was 57.75% for the non-diabetic control group. They concluded that there is a meaningful relationship between the frequency of HSV infection and type 2 diabetes (Ghane, 2018). Similarly, Woelfle et al. (2021) in a cohort study, examined 1275 people with normal blood sugar at the beginning of the test and then over a 7-year period time were confronted with the risk for (pre)diabetes. The HbA1c indexes and antibodies for herpes simplex virus (HSV) 1 and 2, varicella-zoster virus, Epstein–Barr virus, cytomegalovirus (CMV), and human herpesvirus 6 and 7 were measured in this group. After adjusting the demographic variables such as sex, age, BMI, education, smoking, physical activity, parental diabetes, hypertension, lipid levels, insulin resistance, and fasting glucose, their results showed that HSV2 and CMV were connected with (pre)diabetes incidence. They also found that the presence of both viruses has a direct relationship with HbA1C index. Their results demonstrated that seropositivity for other viruses was more common in pre diabetes participants. They concluded the presence of HSV2 and CMV antibodies in the serum of these persons may come up with unbalanced metabolism of glocose. However these results had been affected by confounder variables (Woelfle et al., 2022).

The second line of this literatures, less confirms the effects of infections in developing diabetes. For example, findings of Lee et.al (2013) and Lutsey et.al (2009) dosenot show a relevant stream between presence of viral infection and diabetes (Lee et al., 2013; Lustey et al., 2009). Any way, the lack of consistency between obtained results from different studies, should be justified.

Although inflammation caused by infections and some other virulent mechanisms of microorganisms may lead to impaired glucose metabolism (yoo et al., 2019) it seems that the idea of considering infections as a risk factor for diabetes is still arguable. The results of the present study also show that the genome of HSV 1 or 2 was not detected in the any of serum samples belonging to the test or control group.

To describe the outcomes of this research, some technical and demographic factors should be considered. These include the employed technique, the scale of the study group, the A1C index in this group, and finally sex and age of the participants. In the matter of technique, PCR has been used in this project. Articles that have methods comparing trend, explains that finding the nucleic acids in the serum samples means the active and chronic infections that normally leads to chronic inflammation too. But, serological techniques like ELISA, are more proper to show former confronting with the viral agent (yoo et al., 2019; Sanousi et al., 2016; L akzayi et., 2020). The next affecting factor, which may also seem as a limitation, is the small scale of the study group. Because as opposed to, some studies that have more than 100 participants, this study just has 50 participants in each group. Here that should be reminded again that the aim of this study is just assessing the robustness of the association between HSV and diabetes. Conclusively, if this association rate would be so high, that must be also observed even in small population size. The difference in sexuality is another item in this regard, that seems to not have any significant dissimilarities between the two sexes. As the fourth factor A1C index can be mentioned. Refrring to the demographic tables, only around 40% of the population has an A1C index of less than 7, and the blood sugar in the rest of the population had not been properly controlled chronically. Despite the impaired balance of blood glucose levels, no serum sample contained the HSV genome. Ultimately, the age of the participants should be considered. This item looks like the most important one. According to Lutsey et.al (2009) among 1000 Diabetic type 2 participants with an age range between 45-84, not any sort of viral infection was observed. That resulted to be concluded no relation is there between type 2 diabetes and infection by the authors. However, another study with the same goal in 2012, showed that in 17% of the participants there was a meaningful association between viral infection and type 2 diabetes. The most notable difference between these two studies was the participant's age. In the latter study, all the study population was above 85, and the researchers claimed that viral infection can be a potential risk factor for type 2 diabetes. Apparently, the point is that age is an affecting factor. Because chronic inflammation is a necessary condition for infections to cause diabetes (Lutsey et al., 2009; Chen et al., 2012)

and this condition is available in the second study with an older study group. Backward to the present study confirms this idea. As it can be seen the participants in this study are to some extent young and they are in the range of 20-80. Supposedly viral infection in the first steps of developing diabetes at a young age population, can not be a strong risk factor, but if appearing diabetes in the elder maybe in some way be related to chronic infection and inflammation.

Taking into account, the result of this project, the connection rate between both types of HSV and diabetes mellitus can not be very bold and highlighted. That should be said infection with HSV 1 and HSV2 does not locate in the category of primary risk factors, and for evaluating its probable effect in higher age groups, more studies still should be done. Actually for managing or preventing diabetes well established risk factors like unhealthy nutrition are more affecting and to assess the power of other risk factors such as infection, studies should be designed that eliminate the role of other demographic or environmental factors.

Refereces

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