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IMMUNOHISTOCHEMICAL INSIGHTS OF HUMAN PLACENTAL MICROENVIRONMENT OF ABORTED PATIENTS IN BAGHDAD FOR RATES OF HERPES VIRAL INFECTIONS TYPE 1, 2, 4 & 5

Saad H. Mohammed Ali[1], Basim S. Ahmed[2], Zainab A. Hamid[3]
[1]Communicable Disease Research Unite, Baghdad Medical College, Baghdad, Iraq.
[2]Department of Pathology, College of Medicine, AL-Mustansyria University, Baghdad, Iraq.
[3]Department of Microbiology, Baghdad Medical College, Baghdad, Iraq.

ABSTRACT
Among many viruses infections of placenta with Human Herpes viruses may be harmful in pregnancy leading to disorders in fetal growth, premature delivery, miscarriage, or major congenital abnormalities. This research work aimed to study the rates of occurrence of four herpetic viral infections, namely HSV-type 1, HSV-type 2, CMV, and EBV in placental tissues from patients with spontaneous abortion compared to normally delivered women. Immunohistochemistry technique was performed to detect Anti–HSV1, HSV2, CMV and EBV antibody. The study was designed as a retrospective one during the period September 2014 till October 2014. Forty paraffin embedded placental tissues were obtained from histopathological archives of Teaching Laboratories at AL-Yarmouk Teaching Hospital/Iraq, while another (40) placental tissues of normally-delivered women were enrolled as the control group which collected from obstetrics and gynecology wards in the same Hospital. The IHC- expressions of HSV-1, HSV-2, EBV and CMV proteins were detected in 4(10%), 15(37.5%), 9(22.5%) and15 (37.5%) in placental tissues from miscarriage women, respectively. The considerable proportion of HSV-1, HSV-2, EBV and CMV infections found in the placental tissues from miscarriage women could drag the attention of obstetricians to implicate these viruses as possible targets in the etiology of spontaneous abortion cases.

KEYWORDS: Spontaneous abortion, miscarriage, herpes viruses.

INTRODUCTION

The causes of abortions in many cases are still unknown (Oliver and Overton, 2014). However microbial Infections represent a major cause in abortion, of which viruses appear to be the most frequently involved pathogens (Khameneh *et al*., 2014). The placenta is a dynamic organ whose structure and function changing throughout pregnancy. Compelling evidence has notice pointing for an integral role the placenta in the vertical transmission of viruses from the mother to the fetus. Despite devastating sequelae of congenital viral infection, yet very little is known about the passage of viruses across the placenta and the pathologic consequences of placental viral infection (Turowski *et al*., 2014). Among many viruses, Human Herpes virus infections of placenta may be harmful in pregnancy leading to disorders in fetal growth, premature delivery, miscarriage, or major congenital abnormalities (Di Stefano *et al*., 2008), and some of them can produce chronic or recurrent maternal infection. In particular, CMV during pregnancy can reach the placenta by viremia, following both primary and recurrent infection, or by ascending route from the cervix, mostly following reactivation. Herpes simplex virus type 2, and less frequently type 1 cause recurrent infections of the genital tract, which can lead to abortion (Nigro *et al*., 2011).The virus with a least among herpesviruses, Epstein-Barr virus has been associated only with occasional abortions (Avgil and Ornoy, 2006). It has long been recognized that routine histological examination of the placenta during pregnancies has limitations, especially with regard to the diagnosis of infectious diseases or that may cause severe in utero fetal damage, However Immunohistochemical testing of the placenta following abortions can be very useful in terms of identifying the role of such infectious agents (Turowski *et al*., 2014). The aim of this study was to look for the rates of occurrence of different herpetic viral infections, namely HSV-type 1, HSV-type 2, CMV, and EBV in placental tissues from patients with spontaneous abortion compared to normally delivered women.

METHODOLOGY

This study was designed as a retrospective research work that enrolled paraffin embedded placental tissues which were collected from histopathological archives of Teaching Laboratories at AL-Yarmouk Teaching Hospital /Iraq and belonging to (40) female patients with miscarriage as patients' group, were their ages were ranged between 19 to 43 years), while the (40) placental tissues of the control group were collected from obstetrics and gynecology wards of the same Hospital . Expose Mouse and Rabbit Specific HRP \DAB Detection IHC Kit ab80436 (2013) Abcam, used for detection of HSV1-HSV2- CMV - EBV– specific primary antibodies. Statistical analysis: Analysis of data was carried out using the available statistical package of SPSS-22 (Statistical Packages for Social Sciences- version 22).The significance of difference percentages (qualitative data) was tested

using Pearson Chi-square test (χ^2-test) with application of Yate's correction or Fisher Exact test whenever applicable.

RESULTS

The IHC- expressions of the proteins of any of the tested markers were detected as a brownish discoloration or signal at cytoplasmic localizations. The HSV-1 protein was detected in 4 out of 40 (10 %) placental tissues from miscarriage women , while the control placental tissue have showed IHC expression for HSV-1 antigen in 5% (2 out of 40) of these cases . As a reference for viral dispersion in placental tissues, three quarter 75% (3 out of 4) of miscarriage placental tissues have revealed an IHC signal with high score (score III), while the all the 4-positive HSV-1-IHC reactions of the miscarriage placental tissues have moderate signal intensity . In the placental control tissues, the only two tissues that revealed positive IHC reaction for HSV-type 1 antigen were of low signal score (score I)and each positive tissues showed either low or moderate signal intensity . Statistically, no significant differences (p >0.05) could be found between placental tissue in the miscarriage and control groups (Table 1).

TABLE 1: Signal scoring and Signal intensity stratification of immunohistochemical reactions for HSV1 protein in the studied groups

HSV-1 IHC Signal Score& Signal intensity		Miscarriage Group		Control Group		P value
		No	%	No	%	
HSV-1 IHC Score	Negative	36	90.0	38	95.0	0.396
	Positive	4	10.0	2	5.0	
	Score I	-	-	2	100	-
	Score II	1	25.0	-	-	
	Score III	3	75.0	-	-	
HSV-1 IHC Intensity	weak / I	-	-	1	50.0	-
	Moderate / II	4	100	1	50.0	
	strong / III	-	-	-	-	

*Significant difference between proportions using Pearson Chi-square test at 0.05 level

TABLE 2: Frequencies distribution of immunohistochemical reaction results of HSV-2 protein according to their signal scoring &signal intensity

HSV-2 IHC signal Score & signal intensity		Miscarriage Group		Control Group		P value
		No	%	No	%	
HSV-2 IHC Score	Negative	25	62.5	37	92.5	0.001*
	Positive	15	37.5	3	7.5	
	Score I	3	20.0	3	100	-
	Score II	6	40.0	-	-	
	Score III	6	40.0	-	-	
HSV-2 IHC Intensity	weak / I	3	20.0	2	66.7	0.252
	Moderate / II	11	73.3	1	33.3	
	strong / III	1	6.7	-	-	

*Significant difference between proportions using Pearson Chi-square test at 0.05 level

Herpes simplex virus type-2 antigen was noticed in 15 out of 40 (37.5%) of IHC reactions in the miscarriage placental tissues group, the highest percentage for signal intensities with the moderate intensity (73.3%). All the 3 positive placental tissues in the control group(2.5 %) showed low score (score I) and predominantly (66.7%) revealed weak signal intensity. Statistically, the overall HSV-2–IHC scoring results of miscarriage placental tissues on comparing to control placental tissues group showed significant differences ($p<0.001$) (Table 2). Expression of Epstein Barr - viral capsid antigen IHC Signal was detected as a brownish discoloration at nuclear localization (Figure 3). The placental tissue samples of aborted women showed 22.5% (9 out of 40), while none of healthy control placental tissues showed EBV - VCA antigen expression. The highest percentage of EBV-IHC reactions have revealed moderate signal intensity (66. 7 %: 6 out of 9) (table 3).

The overall expression of CMV protein at nuclear localization was detected by in 37.5% of the placental tissues of miscarriage group, and in 5% cases of healthy placental tissues .A high percentage 60.0% among placental tissues in the miscarriage group have weak score (score I). The highest positive CMV-IHC reactions in those with miscarriage group were showed strong signal intensity 66.7%. The two placental tissues in control group were found to have weak score (score I) , with weak signal intensity. Statistically, significant differences ($p <0.05$) were found when comparing the infection rate between miscarriage and control group (Table 4).

TABLE 3: Immunohistochemical scoring results of Epstein Barr viral capsid antigen detection in tissues from miscarriage and successfully delivered women

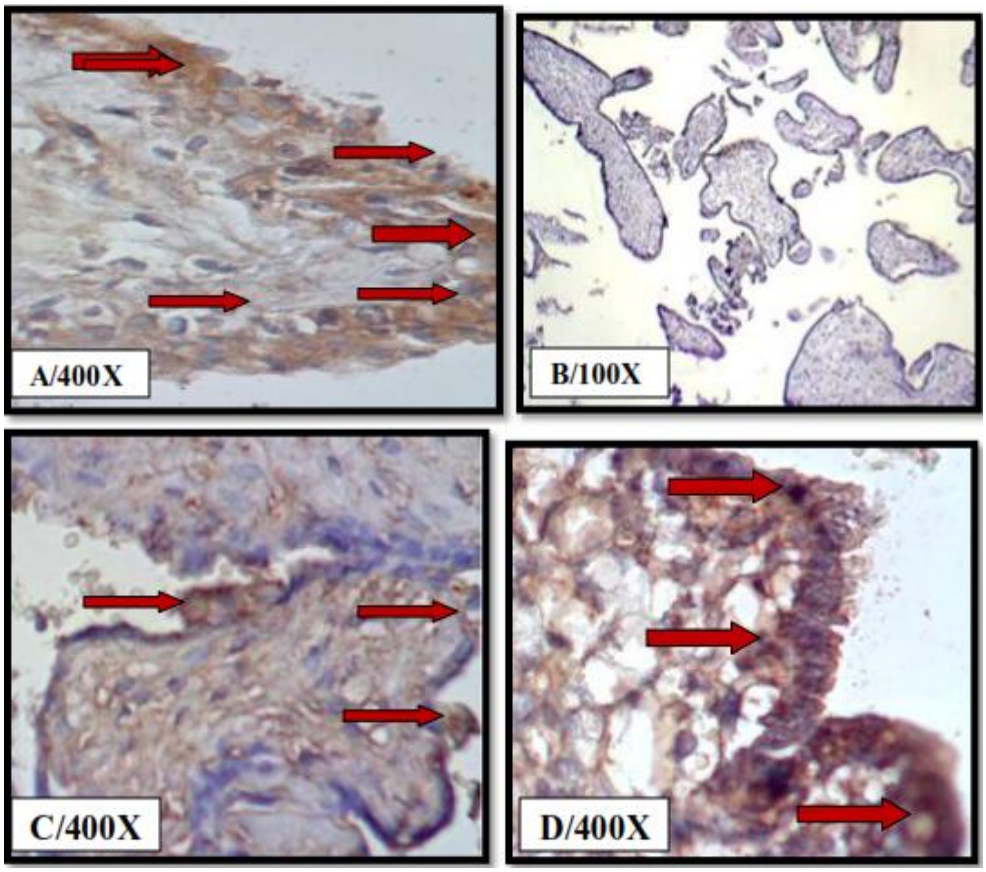
EBV IHC signal Score & signal intensity		Miscarriage Group		Control Group		P value
		No	%	No	%	
EBV IHC Positive Score	Negative	31	77.5	40	100	-
	Positive	9	22.5	-	-	
	Score I	7	77.8	-	-	-
	Score II	2	22.2	-	-	
	Score III	-	-	-	-	
EBV IHC Positive Intensit	weak / I	3	33.3	-	-	-
	Moderate / II	6	66.7	-	-	
	strong / III	-	-	-	-	

*Significant difference between proportions using Pearson Chi-square test at 0.05 level

TABLE 4: Results of HCMV-protein signal scoring & signal intensity of IHC in placental tissues of the two study Groups

CMV IHC signal Score & Signal intensity		Miscarriage Group		Control Group		P value
		No	%	No	%	
	Negative	25	62.5	38	95.0	0.0001*
	Positive	15	37.5	2	5.0	
CMV IHC Positive Score	Score I	9	60.0	2	100	-
	Score II	1	6.7	-	-	
	Score III	5	33.3	-	-	
CMV IHC Positive Intensity	weak / I	1	6.7	2	100	-
	Moderate / II	4	26.7	-	-	
	strong / III	10	66.7	-	-	

*Significant difference between proportions using Pearson Chi-square test at 0.05 level



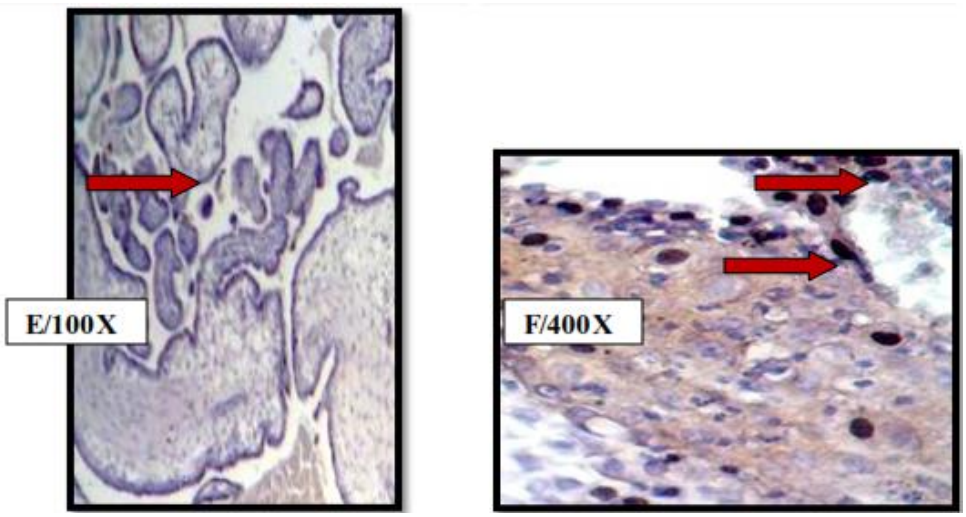


FIGURE 1: Microphotograph of IHC staining of trophoblastic placental tissues from miscarriage patients (red arrow): A- HSV1 in cell cytoplasm showed score 3 and moderate intensity. B- Negative IHC-signal staining for HSV1. C- HSV2 in cell cytoplasm showed score2 and strong intensity. D- IHC staining for EBV in in cell nucleus showed score 1 and moderate intensity .E- Negative IHC staining for EBV n F- IHC staining for CMV in cell nucleus showed score 2 and moderate intensity.

DISCUSSION

The most frequently involved pathogens during pregnancy appear to be viruses. Herpes simplex virus type 2, and less frequently herpes simplex virus type 1, and occasionally EBV, can cause recurrent infections of the genital tract, so as involve the feto-placental tissue units (Giovanni *et al*., 2011). Since HSV-1 has emerged as a principle causative agent of genital herpetic infection (Anzivino *et al*., 2009), this research work was designed to study the frequency rate as well as the possible role of HSV-1 infection in the placental tissues among a group of women with spontaneous abortion. HSV-1 protein positivity was detected in 10% of miscarriage placentae while the placental tissue of control group showed the expression of HSV-1protein in 5%. A controversy was noticed regarding the frequency of HSV-1 infection among women with abortion since previous studies showed no relation or low relation between HSV-1 infection and spontaneous abortion. Our results disagree with earlier reports regarding this subject; here in a study done by Robb and coworker (1986) for detection of HSV antigen, he found that placental HSV positivity (39%) was significantly correlated with spontaneous abortions. The infrequency with which congenital HSV-1 infection occurs may be attributed to several factors, the prevalence of protective antibodies against HSV1 in reproductive -aged women, and the possibility that the placenta functions to preventing vertical transmission of HSV from the maternal to the fetal circulation as a physical barrier. Our results are a bit higher than another earlier study done by Sifakis, and associates (1998), who examined women with spontaneous abortion by PCR and serological assays and found HSV1/2 genome in 3 cases (2.9%), out of total cases. Although HSV-1 infection was not found commonly, and there is no significant difference in the detection of the virus between spontaneous abortion cases and control group, this study and the result of, HSV-1 infection rate was many times more than these results that such results should be considered carefully at this time, yet of a lower infection rate of HSV in our study population. HSV-1was well known to infect both or facial areas, yet in some developed countries herpes simplex virus type 1 has yet emerged as the prominent causative agent in genital lesions. Changes in sexual behaviors of young adults may partly explain its incidence in the genital tract (Gupta, and Warren, 2007, Paz-Bailey, 2007). Villous trophoblast cells were found resistant to infection by HSV-1 and may serve as a barrier that prevents vertical transmission of HSV-1 although these villous syncytiotrophoblast is in direct contact with maternal blood and forms a continuous layer between the maternal and fetal circulation. However, in a previous study done by Hideki Koi, *et al*. (2002), they found that primary maternal infections with HSV-1 were common during pregnancy and associated with an increased risk of miscarriage, perhaps as a result of placental dysfunction.

According to our findings, HSV-1 infections may be acquired before the pregnancy so associated with low risk of occurrence of spontaneous abortions, thus, there was no statistically significant difference in the occurrence of the SA between the "HSV-1 positive miscarriage group and the control group. Our results are consistent to these done by Syridou *et al*. (2008) where by using nested PCR they found 6% of specimens in SA group were HSV1/2 positive. Similar results 2.8% (1/35) of samples were found in study done by (Matia *et al*., 2011) in Iran using PCR. More recently a study has investigated HSV1/2 in women with spontaneous miscarriage in the first trimester of pregnancy has found HSV-1/2 DNA using (Real-time PCR), in (7) out of the (1,716) by serology in 39.7% for HSV IgG, The lack of virus DNA in the majority of cases indicates that HSV-1/2 infection is not commonly associated with spontaneous abortion (Zhouy *et al*., 2015). Since HSV-2 infection is ubiquitous, the risk of fetal transmission makes this virus as global public health issue, particularly because HSV-2 infection is most common among women of childbearing age (Fabiana, 2013). Although some studies showed inconclusive evidence of

primary HSV-2 infection with abortions (Haider *et al*., 2011), our study showed significant correlation between HSV infection and abortions. The present results are consistent with Kapranos and Kotronios (2009) results where by using nested PCR, they detected HSV-2 in (43.2%) of those with early pregnancy loss. This could be related to that herpesvirus reactivation which could frequently occur during the first months of pregnancy which in turn could be related to the progesterone-linked immunodepression (Burlingham, 2009).The present results were higher than the results of other Iraqi serological study done in Baghdad and by using (ELISA) they found (4.76%) of their sera were positive for HSV2 IgM in recurrent spontaneous abortion cases (Basim, 2014). This could be explained in that these infectious agents might induced a shift of immune response during pregnancy from Th2 to Th1 and consequently rejection as an abortion process and as supported by the results of Haddow *et al*., 2007. Studies in Brazil by using (PCR) showed a prevalence of 9.0% of HSV-2 in the maternal-side placental tissue (decidua) samples where as only (3.5%) were positive for HSV-2 on the fetal side. (Fabiana *et al.*, 2013). Previous studies presumed that HSV-2 prevalence in miscarriage cases were 3.3 to 9.0% [McDonagh *et al.*, 2004; Syridou *et al*., 2008; Al-Buhtori *et al*., 2011]. However, based on our study and the results in the study by (Kapranos and Kotronias, 2009) HSV-2 infection is likely to be an important cause of fetal death, with a great deal of variations of infections within regions or even variation of the individual immune states. One could proposed explanations for the higher rates of HSV-2 that such infections varied in relation to the characteristics at individual-level, including, age, sexual activity level, socioeconomic status, education, and race (Xu *et al*., 2006). The present results have been supported by the results of (EI Kalu *et al*., 2015), who had found that first episode HSV-2 infection among pregnant women in Benin, Nigeria is associated with an increased risk of spontaneous abortion occurrence. However, comparison with other studies (Satosar *et al*., 2004; Syridou *et al*., 2008; Al-Buhtori *et al*., 2011), (prevalence rates of 2.6–6.8%), the prevalence in the current study was higher , and such finding may be related to the possible effects of immunohistochemical technique used in the present study which was accomplished with antibodies which were highly specific that recognized the target protein that will bind only to the protein of interest in the tissue sections and also suggested by (David *et al*., 2013). Primary infections with EBV with apparent transplacental transmission are rare, while secondary maternal EBV infections are not uncommon (Avgil, 2006). In this study, the expression of EBV viral capsid antigen was detected at nuclear localization in 22.5%. Semi quantitative IHC technique used for scoring of color development, revealed that most of IHC reaction have score I ,which in turn represents a relative mild to moderate EBV infections of placental tissues during pregnancy and that all the EBV infected placental tissue in the miscarriage group have been under conditions that allow for EBV from latency EBV-infected uterine tissues to be actively replicated and transmitted to placental tissues, while the detection of EBV infection during pregnancy or after miscarriage is usually achieved serologically. The use of biopsy from placenta after abortion as a specimen, although is uncommon yet it gives direct evidence of placental EBV infection (Meytal *et al*., 2008). Another study by Al-Buhtori (2011) failed to detect viral DNA in any of the placental samples even though they used PCR. Although the EBV infections is still debatable as a cause of spontaneous abortions, one explanation for the conflicting high rates of EBV infection of placental tissues in the current study as compared with the previous studies could be related to environmental condition and geographical variations in EBV prevalence which might play a significant role in these differences of detection rates . Human cytomegalovirus is an important etiological agent of intrauterine infection, with a serious results in pregnant women (miscarriage, stillbirth, cerebellar malformation and fetus development retardation), (Staar and Israa, 2012). The overall expression of CMV protein was detected in 37.5% of placental tissues of miscarriage group, while in only 5% in healthy placental tissue was detected. Accordingly these results indicate that most of the women during pregnancy and /or - before childbearing age could be exposed to this virus because CMV infection was found be acquired through contact with the saliva or urine of young children, which is the major cause of this virus infection among pregnant women, in addition to the different transmission from person to person through close contact with body fluids. Once one infected, and the immune status is satisfactory, the viral replication may be suppressed, and this leading to a latent state. Reactivation of infection occurs during immunosuppression that associated with many complex factors such as, stress; an unbalanced and inadequate diet (vitamin-poor, un sufficient protein); iron-deficiency; anemia; and weakening of the central nervous system (Aziza, 2011). The present outcomes of results were in line with a serological study done in Iraq by Majeed (2011) in women with spontaneous abortions, positive CMV (38.5%) in 2009 and declined to (29.1%) in 2010. suggesting declination in the exposure to infection in Iraq. Another study carried out in Baghdad done by Maysara and their colleaque (2012) concluded that the higher seropositive rates for (CMV) in women with spontaneous abortion could played a significant role in their abortions. Primary infection during pregnancy would be a source of fetal infection. Jenna and here worker (2015) in Australia, found that the overall, CMV DNA was detected in (5%) of placenta tissues of miscarriage. Another seroepidemiological study that done by Aimée and co worker (2015) in Havana by using commercial ELISA has detected CMV in 16.7%, with cytomegalovirus in a population of mothers. On comparison with results of other studies (Aysun *et al*., 2011) in Turkey the total HCMV-IgG rate was 69.99 % while HCMV-IgM rate was 16.36%. Since CMV infections are highly associated with poor hygienic conditions, close contact with day care units and communal life style as well as, past, recurrent and reinfections. A recent study, in Nepal revealed seropositivity for CMV in 64.2%, (Dhruba *et al*., 2014). Another recent study done by Hussein *et al.* (2014) using ELISA reported rate of 85% and 10% among aborted women for IgG and IgM respectively. The hematogenous

route of CMV transmission in the placenta could, explain the focal infection of this virus in the floating villi. Our results reveled low prevalence of infection as compare to serological study methods and this is due to immunohistochemistry can clarify the accurate diagnosis. These methods enable the diagnosis even when there is no evidence of placentitis as was observed by (Ozono *et al*., 1997), since it deals with a localized infection. Immunohistochemical analysis done by Yiska and co worker (2011) of the infected sections of maternal decidua revealed the expression of CMV immediate-early and pp65 early-late viral genes as well as gB that expressed late after infection. Indicating that HCMV, in the infected placental tissues underwent a full replication cycle. In the current study, the rate in control group (2 out of 40 by IHC), reflects that the CMV positivity in those placental tissues of control group might necessity and follow up study of the consequences of these infection of these pregnancies both on infants and mother after the delivery since abortion is not the only consequence and as such postnatal consequences should be considered. Since this study has followed the inclusion criteria which involved to study those patients with abortions, while we excluded the other outcomes of infected pregnancy like fetal congenital anomalies and diseases , this represented a limitation to this study which was manifested as difficulty in the interpretation of CMV result in the control placental tissues group.

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