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Detection of Cytomegalovirus and Epstein Barr Virus in Placental Tissues of Aborted Women

ALI HATTEM BAYATI[1], YASMEEN J.AL-BAYAA[2], SEVAN NAJEM ALWAN[2] and IBRAHIM ISAM AL-KARKHI[3]

[1]Community Health Department, Technical College of Health, Sulaimani Polytechnic University, Sulaimani city, Kurdistan Region/Iraq.
[2]Department of Microbiology, Baghdad Medical College, Baghdad, Iraq.
[3]Memorial University of Newfoundland, NL, Canada.

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ABSTRACT

Among many viral causes of miscarriage, maternal infections caused by Cytomegalovirus and Epstein-Barr virus infections are important causes. The aim of this study was to detect the possible occurrence of Cytomegalovirus and Epstein-Barr virus infections in placental tissues from patients with spontaneous abortion using immunohistochemistry and in situ hybridization techniques. Immunohistochemistry technique and chromogenic in situ hybridization assay was used to detect placental infection with Cytomegalovirus and Epstein-Barr virus in 40 women with spontaneous miscarriage and in 40 healthy delivery n Baghdad/Iraq. An equal detection rates of Epstein-Barr virus in placental tissues by either CISH or IHC were (22.5%), yet the validity results of Epstein-Barr virus - VCA by IHC as compared to Epstein-Barr virus - EBER by CISH have showed a sensitivity and specificity of 44.4% and 83.9%, respectively. The detection rates of Cytomegalovirus -DNA by CISH and Cytomegalovirus -protein by IHC were (30%), (37.5 %), respectively. The results of Cytomegalovirus -DNA -ISH as compared to this Cytomegalovirus - IHC-protein had revealed a sensitivity and specificity of 41.7% and 64.3%, respectively. Cytomegalovirus and Epstein-Barr virus are important causes of placental infections among miscarriage females in Baghdad, and Cytomegalovirus might be detected in placenta of normal delivery. Although *CISH* technique considered as the gold standard method *for* detecting of latent Epstein-Barr virus and /or Cytomegalovirus infection were IHC has showed a compatibility to that technique and might reach rates of high sensitivity and specificity similar to it.

Keywords: Cytomegalovirus; Epstein-Barr virus, Immunohistochemistry, chromogenic in situ hybridization, Miscarriage, Pregnancy, Placenta.

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).

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. The virus with a least among herpes viruses, Epstein-Barr virus has been associated only with occasional abortions (Avgil and Ornoy, 2006).

The aim of this study was to investigate the differences in the occurrence of two herpetic viral infections in placental tissues from patients with spontaneous abortion through determination of infections with CMV, and EBV involving the placenta as a possible causes for subsequent abortion by using the following techniques: Immunohistochemistry and In situ hybridization; as well as comparing the two techniques for detection of CMV, and EBV.

METHOD

This retrospective study made the use of 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, their ages were ranged between 19 to 43 years, and 40 placental tissues of normal delivery as a control group. Expose Mouse and Rabbit Specific HRP \DAB Detection IHC Kit ab80436 (2013) Abcam was used for detection of CMV –protein and Epstein Barr - viral capsid antigen (EBV –VCA) specific primary antibodies . CISH Implementation AP-NBT kit for detection of EBV (EBER – RNA) and CMV (DNA) for chromogenic in situ hybridization (CISH) using biotin – labeled Zyto Fast CISH probe was purchased from ZytoFast/Germany Cat. Numbers (T-1070-40 - 2011). Statistical analysis**:** Analysis of data was carried out using the available statistical package of SPSS-22 (Statistical Packages for Social Sciences- version 22).

RESULTS

For IHC technique, the expression of EBV –VCA IHC signal was detected as a brownish discoloration at nuclear localization (Figure1, A). 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 1).

The overall expression of CMV protein at nuclear localization (Figure1, B) was detected in 37.5% of the placental tissues of miscarriage group and in 5% of healthy placental tissues. A high percentage (60.0%) among placental tissues in the miscarriage group had weak score (score I). The highest positive CMV-IHC reactions in those with miscarriage group were showed strong signal intensity 66.7% .Statistically, significant differences ($p < 0.05$) were found when comparing the infection rate between miscarriage and control group (Table 2).

Regarding chromogenic in situ hybridization (CISH) technique, the total percentage of positive CISH of EBERS among placental tissues in the miscarriage group was 22.5%, while none of the 40 control placental tissues have revealed positive CISH- signals for this EBV marker (figure 1, D). The scoring of EBER RNA- CISH reactions was in its highest percentage 66.7% of all positive cases in the low score (score I), while the their signal intensity of the color development where the percentage of EBER- RNA –CISH reactions that showed weak, moderate, and strong intensities were 4(44.5%), 3(33.3%), and 2(22.2%) respectively (Table 3).

CMV DNA was detected in (30%) of examined placental tissues in the miscarriage group, whereas placental tissues of healthy delivered women have revealed in (15%). Regarding the signal scoring, the most frequently detected CMV – positive placental tissue in the miscarriage group were observed to have the *lower score (score I),* (50%), 58.3 % revealed weak signal intensity ,Statistically, no significant differences ($p > 0.05$) could be observed between the study groups, (Table 4).

The Validity of CISH and IHC techniques for diagnosing placental tissues of miscarriage patient infected with EBV showed that of the total 40 placental tissues in the miscarriage group, nine have been identified to express CISH reaction for EBERs and IHC reaction VCA. There was no statistically significant difference between these results of the two detection methods ($P<0.005$). The statistical observed sensitivity and specificity were 44.4% and 83.9%, for CISH and IHC for detection of EBV in placental tissues of miscarriage patients respectively (Table 5), while the validity of CISH and IHC techniques in diagnosis CMV placental

infections showed that the percentage of positive – reaction results of CMV –DNA -CISH in placental tissues of miscarriage patients was 30% while CMV-IHC protein expression in placental tissues of miscarriage group was 37.5% . The number of cells in serial sections that showed positive IHC was slightly greater than those have showed CISH positive reaction**.** Labeling results of both in situ hybridization and immunohistochemistry were showed in five cases. Statistically the observed sensitivity and specificity were 41.7% and 64.3%, for CISH and IHC for detection of CMV in placental tissues of miscarriage patients, respectively (Table 6).

DISCUSSION

EBV can cause infection of placenta in pregnancy, with consequent complications to the fetus. Primary and secondary EBV infections may both occur during pregnancy. However, primary infections with EBV with apparent transplacental transmission are rare, while secondary maternal EBV infections are not uncommon (Avgil and Ornog 2006).

Most of laboratory tests that were performed to diagnose EBV infections (including infections during pregnancy) use molecular

Table 1: Immunohistochemical signal scoring & signal intensity results of EBV –VCA detection in tissues from miscarriage and successfully delivered women.

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

Table 2: CMV-protein signal scoring & signal intensity of IHC in placental tissues of the two study groups

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

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

techniques (like PCR) and serological tests for EBV antigens or antibodies specific to EBV by ELISA, immunofluorescent technique, or rapid tests *(*Christian *et al.,* 2012). In this study, 9 out of 40 (22.5%) of the examined placental tissues were positive for EBV (EBER) by CISH technique. Also the expression of EBV-VCA was detected at nuclear localization in 22.5% (9 out of 40). In the current study which is presented here, a semi quantitative CISH technique was used for scoring of color development , according to the number of placental cells infected with EBV and the results showed that most of infections have of score I, and this might represents the relative mild to moderate EBV infections of placental tissues during pregnancy as well as these findings were supported by measurement of color intensity of positive cases were most of the examined slides were having mild signal intensity.

In the present study, the positive placental tissues of miscarriage group may indicate recently infected, or lytically reactivated EBV infection, however, the presence of EBER as detected by CISH, does not always correspond with the presence of VCA by IHC**.**

Immunohistochemistry was applied for detection VCA of EBV in placental tissue samples and it showed compatibility of those obtained by ISH and this might indicate a high sensitivity and specificity of the IHC-VCA test, since *the EBER in situ* hybridization *for* detecting of latent EBV infection is considered the gold standard method and as

Table 3: Distribution of signal scoring and of signal intensity of CISH reactions for EBER RNA- in placental tissues of miscarriage and control group

EBV CISH signal Score & signal intensity		Miscarriage Group No	%	Control Group No	%	P value
EBV CISH	Negative	31	77.5	40	100	-
Positive Score	Positive	9	22.5	-	-	
	Score I	6	66.7	-	-	
	Score II	3	33.3	-	-	
	Score III	-	-	-	-	
EBV CISH	weak / I	4	44.4	-	-	-
Positive Intensity	Moderate /II	3	33.3	-	-	
	strong / III	2	22.2	-	-	-

Table 4: Distribution of HCMV DNA CISH result of the examined placental tissues of miscarriage and their counterpart control tissues according to their signal scoring and signal intensity

CMV CISH signal Score & signal intensity		Miscarriage Group No	%	Control Group No	%	P value
CMV CISH	Negative	28	70.0	34	85.0	0.108
Positive Score	Positive	12	30.0	6	15.0	
CMV CISH	Score I	6	50.0	5	83.3	0.308
Positive Intensity	Score II	3	25.0	1	16.7	
	Score III	3	25.0	-	-	
	weak / I	7	58.3	6	100	-
	Moderate /II	5	41.7	-	-	
	strong / III	-	-	-	-	

Table 5: Validity of EBV- CISH and EBV- IHC among placental tissues in the miscarriage group

EBV IHC Score	Miscarriage Group EBV CISH			
	Negative (n=31)		Positive (n=9)	
	No	%	No	%
Negative	26	83.9	5	55.6
Positive	5	16.1	4	44.4
P value		0.073		

Table 6: Validation of CMV for DNA CISH and for CMV protein IHC among the placental miscarriage tissues

CMV IHC Score	Miscarriage Group EBV CISH			
	Negative (n=28)		Positive (n=12)	
	No	%	No	%
Negative	18	64.3	7	58.3
Positive	10	35.7	5	41.7
P value		0.722		

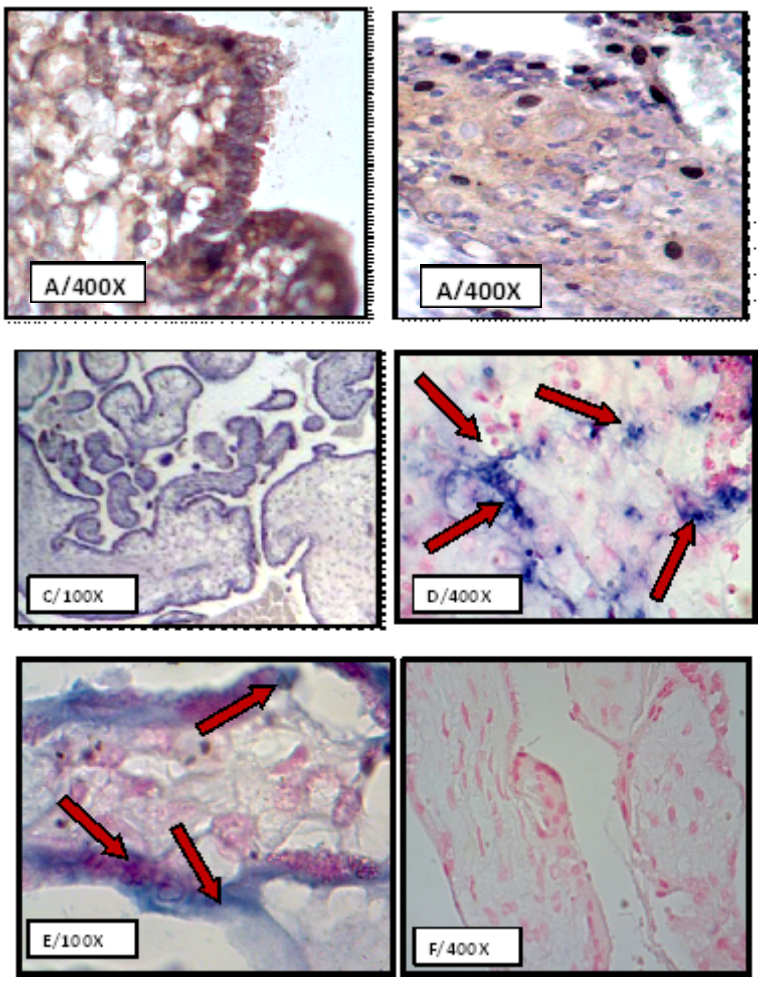

Fig. 1: Microphotograph staining of trophoblastic placental tissues from miscarriage patients (red arrow): A- EBV IHC staining in cell nucleus intensity. B- IHC staining for CMV at cell nucleus. C- Negative IHC staining for EBV. D- EBV-ERER CISH positive signals in the cell nucleus of trophoblastic placental tissues showed score 2 and moderate intensity. E-CMV -DNA staining CISH of trophoblastic placental tissues in the cell nucleus score 1 and weak intensity. F- Negative CISH staining for EBV

supported by the finding of Truong *et. al*., 2009. IHC has also been used where in this regard , Xiao and his worker, 2014 have showed also strong agreement for both techniques which were used for detection EBV in patients with lupus nephritis.

The present result are supported by Xuan-Hong (2011) who suggested that the possibility of the EBV virus to transmission from the uterus to the fetus , which were resulting in stillbirth , abortion , or congenital defects.

The current study provide a novel information about the histological localization of EBV nucleic acid in placental tissues using CISH, were the overall frequency of EBV infection is 22.5 % of the examined placentae, the present result is higher than those reported by other abroad studies such as that done by Gervasi and his colleagues (Gervasi *et al*.,2012) who found viral genomic sequences of EBV (0.1%) of their examined cases of the amniotic fluid (in the mid trimester of pregnancy) by using quantitative real-time PCR for the presence of EBV-genome sequences. Recent studies done by Song and his Colleagues in 2015, used ISH for detection EBV in lympho-proliferative disorders, nasopharyngeal carcinoma and related malignancies with final color or fluorescence detection however, there is no reported data on detection of EBV by ISH during pregnancy and this signify a novelty for this study(Song, 2015).

Although the EBV infections is still as debatable as a cause of spontaneous abortions, One explanation for the conflicting high rate of EBV infection of placental tissues in the current study as compared with the previous studies could be related to geographical variation in EBV prevalence which may play a significant role in these differences in the detection rates.

Human cytomegalovirus is an important etiological agent of intrauterine infection, which may lead to some serious results in pregnant women such as miscarriage, stillbirth, cerebellar malformation and fetus development retardation, (Staar and Israa, 2012).

In the present study, in situ hybridization using CMV specific probe has detected CMV DNA as nuclear inclusions in 30% of placental tissues of the examined miscarriage group, whereas counterpart control placental tissues have reveled HCMV in only 6 (15%) cases. In addition to CMV ISH, IHC was also performed and the expression of CMV protein was detected as a brownish discoloration at nuclear localization. According to our results the frequency of placental CMV infections was detected in higher percentage by IHC technique than *in situ* hybridization. The prevalence rates for CMV in placental tissue of miscarriage group as well as in the control placental tissues indicate that most of the women during pregnancy and /or - before childbearing age were exposed to this virus because CMV infection found be easily acquired through contact with the saliva or urine of young children, other modes of transmission include person to person through close contact with body fluids, such as saliva, blood, vaginal fluid, semen, blood ,tears and breast milk . Once one infected, the person may never be rid of the virus, although if the immune status is satisfactory, the viral replication may be suppressed, and this leading to a latent state with the ability of this virus to reactivate from latency. Reactivation of infection occurs during immunosuppression that associated with many complex factors such as, the stress, an unbalanced and inadequate diet, 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 to discover the association of TORCH infections in women with spontaneous abortions , CMV Ab prevalence of positive cytomegalovirus CMV(38.5%) in 2009 and , the lowest percentage were observed only (29.1%) of cases in 2010. This could suggest that the exposure to CMV infection was declined over this period in Iraq.

Another study carried out in Baghdad done by Maysara and their colleaque (2012) to evaluate the prevalence of seropositivity of specific IgM antibody for CMV by ELISA, CMV specific IgM antibody was detected in 15.7% of the 108 women with history of abortion.

Jenna and here colleagues (2015) in Australia found that the overall CMV DNA was detected in 5% of placenta tissues of miscarriage women along with the infections that were confirmed by using the immunohistochemical assay and viral proteins.

Another seroepidemiological study that done by Aimée and co worker (2015) in Havana maternity hospital by using commercial ELISA kits has shown that the prevalence of active cytomegalovirus infection was detected in 16.7%, with cytomegalovirus in a population of mothers.

Our results disagree with a previous results done by Sharief,(2005) , who showed an absolute positive results of aborted women with a primary abortion i.e reveled highest percentage (100%) which was considered to carry a high titer of IgG. In developed countries such as United States it was stated that HCMV infection at rate of 80% was observed at age 35-40 years old women who have anti- HCMV IgG in compartion to 50% -80% HCMV infection in the younger age women (Gold, and Nankervis, 2007). the hematogenous route of CMV transmission in the placenta could , explain the focal infection of this virus in the floating villi .

The current study revealed that sensitivity of IHC is slightly higher than that of ISH for histological detection of CMV, this might be due to CMV protein over expression (which was studie here) can be detected by IHC but not by ISH, similar result were also obtained by LU DY and his colleagues (2009) found similar findings that the result of IHC is higher than those of ISH; in our opinion the CISH is more difficult to done and this might slightly affect the sensitivity of the procedure.

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. While all of the control tissues were negative by immunohistochemical staining. These findings indicate that HCMV, in the infected placental tissues undergoes a full replication cycle. While in the current study prevalent rate of control group (6 out of 40 by CISH) and (2 out of 40 by IHC), this reflect that the CMV positivity in those placental tissue in control group might reflect the need for 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 of placental CMV infection during pregnancy 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 by recording as well as the difficult interpretation of CMV infection in the control placental tissues group.

In study in Iraq , Alwan and Sera (2011) have indicated that CMV infection rate was (46.6%),These results were higher than our results , this different in the CMV prevalence rate may be due to different in the sample size included in the study group as well as may be due to substantial diffusion of infection in local population , perhaps that CMV is endemic in Iraq during that period , or may be CMV infections are un common in a particular region .

The current results obtained from both techniques for EBV have revealed that among Forty placental tissues from miscarriage patients , EBER-CISH and VCA-IHC have been found in 22.5% . While the detection rates of EBV-CISH and EBV-IHC in placental tissue sections were equivalent yet the validity results EBER by ISH where compared to VCA by IHC had showed a sensitivity and specificity of 44.4% and 83.9%, respectively,

In situ hybridization is a technique to determine and localize target nucleic acids in morphologically preserved tissue sections. Recent advances in methods have greatly increased the sensitivity of the technique, as the gold standard.

Also in this study we evaluated the efficacy of these two methods but for detection the presence of CMV infection in placental tissues from miscarriage patients. Both CISH and IHC for diagnosing nuclear inclusions of this virus in

placental tissues have showed that , CMV-DNA-CISH and CMV-protein -IHC were found in 30% , 37.5 % respectivly . The comparism of the results of both technique revealed a sensitivity and specificity of 41.7% and 64.3%, respectively. The numbers of infected cells scored were slightly higher with IHC than with in CISH technique. These findings clearly indicate that the use of both in CISH and IHC for EBERs and the EBV- VCA as well as CMV –DNA and CMV proteins are both specific, as well as sensitive methods.

The current result of this study are matching with Niedobitek *et al*.,(1988) result . They used ISH, IHC, and morphological analysis of tissue from patients with AIDS who have widespread CMV infections. They showed that the evaluation of the results in ISH and IHC were considerably more sensitive than the morphological analysis. It was further shown that IHC has detected a higher CMV infected cells than in CISH. They concluded that IHC as technique appeared to be more suitable to be used.

Several authors have denoted that ISH is the method of choice for detection of the EBERs – EBV in tissue sections and this is due to the large numbers of copies of EBERs present in latently infected cells where these studies have showed Positive staining in the nuclei of the EBV-infected cells (Weiss and Chen , 2013; Hänel et.al., 2001; Margaret and Weihua , 2008).

In addition our data were match with the fact that ISH was considered as the gold standard for precisely identifing the sites of expression of EBV as it and was highly sensitive and specific; however, some authors denoted that ISH is expensive (Lerner *et al*. ,1981). The results of the current study showed that the two detection techniques had strong agreement, which in turn indicated that the results of both were reliable.

In conclusion CMV and EBV are important causes of placental infections among miscarriage females in Baghdad, and CMV might be detected in placenta of normal delivery. Although *CISH* technique considered as the gold standard method *for* detecting of latent EBV and /or CMV infection were IHC has showed a compatibility to that technique and might reach rates of high sensitivity and specificity similar to it .

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