Molecular and immunological detection of Toxoplasma gondii and Cytomegalovirus in Aborted Women with Covid -19

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Abstract

Toxoplasma gondii and Cytomegalovirus infections are the most common causes of congenital infections worldwide in addition the coronavirus disease is a serious public health emergency and a previous report said pregnant women usually developed a mild form of COVID-19 despite a few cases for acute form with maternal morbidity and mortality . So, this study aimed to molecular and immunological detection of Toxoplasma gondii and Cytomegalovirus in aborted Women with Covid -19. This study involved 719 samples taken from women with recurrent pregnancy loss who attending the Maternity and Children Hospital in addition to some medical laboratories in Al-Diwaniyah Governorate during the period from February 2021 to September 2022, their ages ranged between (18-47) years. For the purpose of diagnosing infection with Human cytomegalovirus and Toxoplasma gondii, where IgG and IgM were evaluated for both using immunodiagnosis by rabid test and ELISA, and then the results were confirmed using RT-PCR. Also, their was relationship to previous infection with Covid-19 in a studied aborted women. The results obtained by rabid test and ELISA were 45 for HCMV, 40 for T. gondii, and 11 for HCMV and T. gondii together in the same patient, and when confirming the results of the diagnosis by RT-PCR, it showed less positive results, amounting to 28 for HCMV and 22 for T. gondii. Also, it was found that there was a relationship between previous infection with Covid-19 and the activation of latent chronic infection of HCMV and T. gondii in aborted women.

Keyword: *T.gondii, HCMV, RT-PCR, ELISA, Covid19.*

INTRODUCTION

Toxoplasmosis is a zoonotic parasitic disease caused by Toxoplasma gondii (T.gondii) and it is endemic worldwide and all people may be at risk of infection by the parasite and toxoplasmosis is one of the parasitic parasites that live inside cells (Khalil et al., 2018).

Toxoplasmosis can have more serious complications and consequences such as birth defects, eye infections, or fatal toxoplasma encephalitis in immunocompromised individuals (Ozgonul and Besirli, 2017). Cats and other members of the subfamily Felidae are considered definitive hosts for the parasite while humans and other livestock are

intermediate hosts for T. gondii (Pal et al., 2014). The infection is transmitted to the fetus of a mother infected with toxoplasmosis for the first time during pregnancy and the most serious type of infection is congenital infection (Adeniyi et al., 2018). Humans are usually infected by eating raw or undercooked meat containing cysts of T. gondii or through vertical transmission(Sukemori et al., 2016). Toxoplasmosis is usually asymptomatic in infected humans. However, some infected groups such as immunocompromised patients can suffer fatal and life-threatening outcomes such as encephalitis, pneumonia, retinitis, epilepsy, and other neurological complications (Ngoungou et al., 2015; Wang et al., 2017).

Human cytomegalovirus (HCMV) is an opportunistic pathogen consisting of linear single- and double-stranded DNA particles of about 235 kbp in size capable of causing a wide range of diseases in humans (Beam and Razonable, 2012). HCMV remains a common virus that affects people of all ages. HCMV has ubiquitous distribution; the range is between 40-100% of all adults who carry the virus worldwide (Ebina et al., 2015). Infectious agents can impair human functions, including reproduction. The viruses were able to interfere with reproductive function in both sexes. CMV is transmitted by close contact between infected persons, such as blood or its derivatives, congenital, sexual intercourse (Vora et al., 2018). It is mostly noted that congenital HCMV infection is a cause of hearing loss and mental retardation; Latent CMV infection can be reactivated in immunocompromised patients, for example with immunosuppressive receptor transplants or AIDS (El-Hajjar and Mousavi, 2020).

HCMV infects the placenta through the transportation of the virus across the placental barriers which may occur before fetus infection (Maidji et al., 2007). However; this infection may be restricted to the placenta (Iwasenko et al., 2011).These evidences gave

the important idea that HCMV may contribute to unlike pregnancy results (Maidji et al.,2010).

Serological tests are the mainstay of diagnosis(Sen et al.,2012). The diagnosis of acute infection in pregnant women is usually established by demonstration levels of specific IgM antibodies that usually decrease after one to six months of infection and become undetectable within seven months. IgG may be detected one to three weeks after the initial rise in IgM level. IgG synthesis reaches a plateau within two or three months and then decreases more or less rapidly and persists lifelong at residual titers(Robert-Gangneux et al.,2012). A 4 to 8 fold rise in IgG titre in the serum samples taken two weeks apart also indicate a recent infection. A positive IgG titre alone is enough to prove a previous infection (Razzak et al.,2005 and Sarkar et al.,2012).

PCR techniques have advanced further with development of quantitative real – time PCR (qRT-PCR). This technique is more sensitive than conventional PCR and enables the number of RNA or DNA copies in sample to be measured(Ntziora et al.,2013). In general real – time PCR assay for the detection and quantification of viral DNA or RNA are sensitive, specific, reproducible and significantly reduce the time necessary to report results that may have impact on the care and management of patients (Ono et al.,2008). The coronavirus disease (COVID-19) is a serious public health emergency and global crisis. It was declared a pandemic in the world by the World Health Organization (WHO) in March 2020. It transmits through respiratory droplets mainly and perinatal transmission is not well understood(Alzamora et al.,2020; Blumberg et al.,2020 and Richtmann et al.,2020) . However, a previous report said pregnant women usually developed a mild form of COVID-19 despite a few cases for acute form with maternal morbidity and mortality (Alzamora et al., 2020; Dong et

al.,2020 ; Yang and Liu, 2020) . A pregnant woman is immunologically weak and has a unique immunologic state where the maternal immune system tolerates paternally derived fetal antigens that leads to increase susceptibility to infection especially pneumonia (Richtmann et al.,2020 and Zeng et al.,2020). Early pregnancy is a challenging and vulnerable period and affects embryogenesis and fetal organ development. At present, there are limited resources for the guidance of the critical care management of pregnant patients with COVID-19 infection. Also, the miscarriages or fetal demises due to COVID-19 is not established yet due to very few reports on this particular issue (Zeng et al.,2020; Zaigham and Andersson,2020 and Juan et al.,2020). So, this study aimed to molecular and immunological detection of Toxoplasma gondii and Cytomegalovirus in aborted Women with Covid -19.

Materials and methods

Source of Samples

Complete samples were taken from the Maternity and Children Hospital in addition to some medical laboratories in Al-Diwaniyah Governorate during the period from February 2021 to September 2022. The total number of study samples was 719 from women who suffered from recurrent pregnancy loss , their ages ranged between (16-48) years.

Sample Collection

Blood samples from aborted mother (5 ml) were collected, 2 ml of the 5 ml were collected by EDTA tubes for genetic study and the rest 3 ml were put in the tubes made of plastic or (gel tube), and centrifugation of blood samples for 5 minutes at 4,000 rpm, collection of the serum in plane tubes for ELISA test. Serum remains stable for 4 hours at 20 ± 50 °C.

Questioner performance: A questionnaire on personal information was prepared, asking the patients and controls about their name, age, residence, in addition to the question whether they had previously been infected with Covid19.

Ethical criteria

Assignments were made and all samples were obtained from abortion and pregnant women, and the doctors after they had received their consent.

Diagnosis of the HCMV and T.gondii in aborted Women by Rabid test :

A 917 samples from aborted women with an age range between (16-48) years were diagnosed by adding 25μl of a serum sample to the hole cassette rapid test kit (CTK Biotech's /USA) and adding 80μl of buffer directly to each well for diagnosis of HCMV and T.gondii antibodies. The findings were read after 15 minutes of the test procedure.

Detection of Cytomegalovirus (CMV) and Toxoplasma gondii IgM and IgG by ELISA:

All serum samples were tested for the levels of IgG & IgM antibodies concentration according to the manufacturer's instructions of enzyme immunoassay test kit(DRG Instruments GmbH / Germany) and reading of the optical density at 450 nm within 15 min by using ELISA reader(Bio metra /USA).

Molecular study: The DNA of was extracted from the whole blood samples by using ExiPrepTM Plus Genomic DNA Kit (BioNeer /Korea). The extraction was performed according to the manufacturer's specifications. The primers and probe that used for direct detection of Human Cytomegalovirus by Real-Time PCR were designed in this study by using NCBI-Genbank Human cytomegalovirus UL83 gene sequence (KJ743149.1) and primer3 plus and provided by (Scientific Researcher Co. Ltd, Iraq) as following tables:

Table (1): Contents of the Reaction Mixture

PCR Master mix	Volume
DNA template	5µL
Forward primer (10pmol)	1µL
Reverse primer (10pmol)	1µL
qPCR master mix	10 µL
qPCR water	3 µL
Total volume	20µL

Table (2): Contents of the Reaction Mixture

PCR Master mix	Volume
DNA template	5µL
B1 gene Forward primer	1µL
B1 gene Reverse primer	1µL
B1 gene probe (20pmol)	1µL
qPCR master mix	10 µL
qPCR water	2 µL
Total volume	20µL

Table (3): Primers and Probe for Human cytomegalovirus:

Primer		Sequence (5'-3')	Product Size
CMV-primer	F	AGCTCTTTATGCACGTCACG	111bp
	R	ACAACACCGTAAAGCCGTTG	
CMV-probe		FAM-CCTTCATGCGCCCCCACGAG-BHQ1	

Table (4): qPCR Thermocycler conditions

Step	Condition	Cycle
Pre-Denaturation	95 °C 5 min	1
Denaturation	95 °C 20 sec	40
Annealing/Extension	60 °C 30 sec	
Detection (Scan)		
Melt cycle	65-90°C	

Amplification and detection of T.gondii DNA by Real- Time Polymerase chain reaction by using AccuPower® TG Real -Time PCR Kit (BioNeer /Korea).

The primers and probe that used for direct detection of Toxoplasma gondii and Human Cytomegalovirus by Real-Time PCR were according (Mei-Huiln et al., 2000) and provided by (Scientific Researcher Co. Ltd, Iraq) as following tables:

Table (5): Primers and Probe for Toxoplasma gondii:

Primer		Sequence (5'-3')	Product Size
Toxo- primer	F	TCCCCTCTGCTGGCGAAAAGT	111bp
	R	AGCGTTCGTGGTCAACTATCGATTG	
Toxo-probe		FAM-TCTGTGCAACTTTGGTGTATTCGCAG-TAMRA	

Table (6): qPCR thermocycler conditions

Step	Condition	Cycle
Pre-Denaturation	95 °C 5 min	1
Denaturation	95 °C 20 sec	40
Annealing/Extension	60 °C 30 sec	
Detection (Scan)		
Melt cycle	65-90°C	

Quantification of genomic HCMV and T.gondii DNA was determined using a spectrophotometrically (Nano- drop/ Thermo Scientific /USA) instrument to measure the optical density (O.D) (Rosline et al.,2015)

Statistical Analysis

The resulted data were analyzed using the computer Statistical analysis system (SPSS), The Chi-square test was performed for assessing the statistical significance of the data values where the descriptive statistics is used., the level of probability at P values below of < 0.05 was used to identify the significant difference (Urdan , 2005).

Results and Discussion:

1. Detection of Cytomegalovirus and Toxoplasma gondii by rapid test

The present study enrolled randomly 719 suspected patients to surveillance Cytomegalovirus and Toxoplasma gondii infection by monitored the rapid test findings and the results were showed in table (7). The present results show only 45 (6.25) of suspected patients have active Cytomegalovirus infection by finding positive results of rapid test. Whereas T. gondii findings show only 40 (5.56) of suspected patients have Toxoplsmosis infection. But it has been found only 11 (1.52) of suspected patients have mixed infection.The results of the diagnosis by Rabid test were identical in number to the results of the diagnosis by ELISA, and these results are consistent with a study by (Abdulkhaliq et al., 2017) where this study was conducted for 210 women (180 women suffered from recurrent miscarriage and 30 women with normal healthy pregnancy as a control group) were diagnosed by Rabid test and ELISA for the purpose of detecting their infection with TORCH, so the seropositivity by Rabid test was 70 (38.9%) out of 180 abortion cases, while 86 (47.7%) were recorded by ELISA, which indicated that the results of the diagnosis by Rabid test, an approach to diagnostic results by an ELISA test for the purpose of immunological detection of TORCH infection. Also, our results were similar to the results in a study by (Al-Marsoomy and Al-Omer, 2021), which

used a rapid latex test to diagnose T.gondii in aborted women in the city of Nineveh in Iraq, where the test showed high levels of infection with T.gondii, 62 were seropositive by latex. While by ELISA technique ,there was 44 seropostive samples for IgG and 6 for IgM and 32 positive samples by RT-PCR. This study confirmed that the accuracy and effectiveness of the diagnosis depends on the quality, efficiency and quality of the kit used and according to the manufacturers.

Table (7) Number and percentage of infected patients with CMV and T. gondii diagnosed by rapid test

Total samples examined	CMV	*T.gondii*	Mixed infection
719	45 (6.25)	40 (5.56)	11 (1.52)
Calculated X^2	22.04		
Calculated P value	0*		

* Significant difference at P<0.05

2. Detection of CMW and T.gondii IgM and IgG in patients group by ELISA

The present study enrolled 56 suspected patients with CMV infection to surveillance infection by monitored IgG and IgM findings. The results show 26 (46.42) of suspected patients have active Cytomegalovirus infection by finding positive results for IgM. Whereas the IgG findings show 48 (85.71) of suspected patients have previous Cytomegalovirus infection. Also the present study enrolled 51 suspected patients with T. gondii infection by monitored IgG and IgM findings. The results show 16 (31.37) of suspected patients have active T. gondii infection by finding positive results of IgM. Whereas the IgG findings show 50 (98.03) of suspected patients have positive results which mean have a previous infection, as shown in table (8). This result was similar to previous studies presented by (Yasir et al., 2020) in Al-

Najaf city that used the minividas method and the same result appear in a study of (Al-dorri, 2018) in the city of Salah al-Din using the ELISA method. Also, a study by (Dhami,2020) in Nasiriyah, showed that the highest seropositive rates for IgG were recorded in HCMV-infected women, indicating the presence of an old or latent infection. On the other hand, the results of this study differ from the previous study presented by (Anwar and Al-Bayati, 2018) in the city of Samarra, where the results reported a higher rate for IgM than IgG in women infected with CMV using the ELISA method. The presence of IgM antibodies indicated the presence of a new infection with HCMV or re-infection with a new strain of the virus. And that association between the rates of seroprevalence of anti-Igm and IgG anti-HCMV with recurrent miscarriage represents a real risk and based on pregnancy complications. As HCMV is the most common source of congenital infectious diseases, more than 70% during the first trimester of pregnancy may lead to miscarriage (Vasani and Kumar, 2019). As for infection with T. gondii, the results of our study were consistent with previous studies of T. gondii and (Ekanem et al., 2018) and (Modrek et al., 2015) as well as (Oz, 2014) whose research indicates that the persistence of IgG is one of the most important basic components of the humoral immune response in controlling the parasite and limiting its spread, and these antibodies work On the teaching of rapid prophase differentiation by receptors of type Fraction crystallizable phagocytes that lead to the process of opsonization and normal phagocytosis of the rapidly proliferating phase covered with antibodies. As for the low level of IgM, it begins to appear after about 1-2 weeks of infection and reaches its peak in the fourth week. The IgM level decreases after about 4-8 weeks, and its presence continues for six months, and sometimes it remains for more than a year (Al-samarraiy, 2021(. Through our

results, it is clear that the cases of HCMV infection are higher than the cases of toxoplasmosis, and these results are consistent with a study by (Al-Jbouri, 2020). Which showed that the most effective factor for recurrent miscarriage is HCMV followed by T. gondii in Babylon city. The reason may be attributed to the absence of any symptoms in acute infection, as it is possible to stay in the body for a long time, transmission through the placenta, feeding the child and transmission, or due to transmission of the disease through contaminated food and water, sexual intercourse and the placenta. If a pregnant woman becomes infected in the first months of pregnancy, it causes congenital malformations or miscarriages in the last stage of pregnancy, as well as an increased need for blood transfusions during surgeries or during pregnancy and childbirth, among the common reasons for the increase in the prevalence of HCMV infection (Jassim, 2017), where infection is linked HCMV significantly causes miscarriages in women in the first trimester of pregnancy (Abdul-Aziz, 2015). Also, another reason for its positive serotypes is different and similar to HCMV and T. gondii infection in pregnant women from one region to another due to healthy habits. and cultural differences related to dietary habits, education level, primary health care program, and early infection diagnosis (Kakayi, 2021) while (Pribakovic et al., 2019) found that T.gondii seropositivity increased with age, which may be due to lifestyle and increased consumption of frozen meat. While our results differed from a study by (Anwar and Al-Bayati, 2017), which showed that antibodies were higher for T.gondii by 31.2% of aborted women, while it was 13.3% for HCMV and 4.1% for women infected with HCMV and T.gondii.

Table (8) Number and percentage of cases with CMV and T. gondii infection according to IgG and IgM positive result

dis. condition	Total No.	IgG positive	IgM positive	P value
CMV	56	48 (85.71)	26 (46.42)	0*
T.gondii	51	50 (98.03)	16 (31.37)	0*
P value		0.022*	0.111	

* Significant difference at P<0.05

3. Frequency distribution of patient groups according to age groups

Regarding to diagnostic tests, the present study enrolled 45 patients with Cytomegalovirus infection, 40 patients with T. gondii infection and 11 patients with mixed infection. The frequency distribution of patients with Cytomegalovirus infection according to age group included 25 cases with 16-26 age group, 14 cases within 27-37 age group and only 6 cases from 38-48 age group, and the difference was significant (P = 0.005). While the frequency distribution of patients with T. gondii infection according to age group included 22 cases with 16-26 age group, 17 cases within 27-37 age group and only 1 case from 38-48 age group, and the difference was significant (P = 0.001). But the patients with mixed infection show non-significant differences according to the frequency distribution of age groups ,theses results clarified in Table (9).

Table (9) Number and percentage of CMV and T. gondii according to the age interval by ELISA test

Age	Total No. examined	CMV	*T.gondii*	Mixed infection	Calculated X^2	Calculated P value
16-26	250	25(10)	22(8.8)	4(1.6)	16.28	0*
27-37	256	14(5.46)	17(6.64)	5(1.95)	6.82	0.033*
38-48	213	6(2.81)	1(0.46)	2(0.93)	4.73	0.094
Calculated X^2		10.53	16.07	0.806		
Calculated P value		0.005*	0*	0.668		

* Significant difference at P<0.05

As the reason for the incidence of abortion in the age group (16-26) more than the rest of the other age groups is that this group is the optimal period for marriage and the incidence of pregnancy in it is very large, and these results are consistent with many studies, as these results agreed with what (Singh and Pathak , 2015) indicated in his study that was conducted on women in India to detect toxoplasmosis and its social effects, where the ages in that study ranged between 18-45 years, and the age group 18-25 years was the highest in infection rates with a rate of 48.3%, followed by the age group 30-56 years, with a lower rate of 28.2%, and the age group 31-35 years, with a rate of 3.6%, as the study confirmed that the period from 18-45 years is the period in which marriage is frequent. Also, in a study by (Coskun et al.,2020) in Turkey, it was found that the highest rate of abortion was among women in their twenties, because it is the period in which pregnancy occurs, most frequently for aborted women with TORCH infection. Also, it is consistent with the results presented by (Kadhim et al., 2020), which found that the rate of miscarriage increased in the group of patients infected with HCMV with young age groups ranging between (16-25) more than patients with large age groups.While the results of our study differed from a previous study by (Sahu et al., 2019), which indicated that the infection rate of

HCMV was highest in the age group over 31 years. It also disagreed with the results of a study by (Pribakovic et al., 2019) who found that T. gondii seropositivity increased with age.

4. Frequency distribution of patient groups according to residency

Regarding to diagnostic tests, the frequency distribution of patients with Cytomegalovirus infection according to residency included 19 cases from urban areas and 26 cases from rural areas, and the difference was significant (P = 0.018). While the frequency distribution of patients with T. gondii infection according to residency was 13 cases from urban areas and 27 cases from rural areas, and the difference was significant (P = 0.001). But the patients with mixed infection show non-significant differences according to the frequency distribution of residency, (P= 0.334). These results are more clarified in table (10) .Perhaps the reason for the spread of infectious agents in the countryside more than in the city is due to the lack of sterile drinking water, and there is poverty about health preventive measures, in addition to livestock breeding, poor hygiene, low social and economic level, and crowded living conditions, especially that the

lifestyle of families who live in large numbers is common in society in which our study was conducted, asocial and cultural customs. These results are consistent with a study by (Al-Jbouri et al., 2020) who found that the prevalence of HCMV and T.gondii infection is higher in rural than urban areas for aborted women. While it was differed with the results of the scientist (AL-Taei et al., 2015) which confirmed that there is an important correlation between increased rates of infection with T. gondii and residence in cities. And it also differs with a study by (Kulf et al., 2012) in a study conducted in 2012 of 180 samples from aborted women, and it was found that the highest percentage (58.8%) of miscarriages was in the city compared to the rural population, and 41.2% for the city and the countryside. The reason for the increase in infection rates in the countryside in our results compared to the city population is the spread of mixing with animals, birds and cats that carry the parasite and the possibility of transmission of the disease increases, while the spread of the virus may be due to the poor level of hygiene and health awareness among the rural population, which helps to transmit the disease through contaminated food and water.

Table (10). Number and percentage of CMV and T. gondii infection according to the residency by ELISA test.

Age	Total No. examined	CMV	*T.gondii*	Mixed infection	Calculated X^2	Calculated P value
Urban	369	19(5.14)	13(3.52)	5(1.35)	8.69	0.013*
Rural	350	26(7.42)	27(7.71)	6(1.71)	15.49	0*
Calculated X^2		5.61	13.05	0.932		
Calculated P value		0.018*	0*	0.334		

* Significant difference at P<0.05

5. Frequency distribution of patient groups according to Covid-19 infection   The frequency distribution of patients groups according to the results of Covid-19 infection is showed in table (11). The present study

show 23 patients with Cytomegalovirus infection have Covid-19 infection, 21 patients with Toxoplasma gondii infection have Covid-19 infection and 7 cases with mixed infection showed Covid-19 infection, but there

was non-significant differences according to results of Covid-19 infection, (P= 0.753). According to the results of our current study, infection with Covid-19 can lead to reactivation of HCMV in pregnant women, and thus HCMV contributes to pregnancy loss in some cases. Perhaps the reason for reactivation of latent HCMV occurs in patients due to immune pressure or due to the use of corticosteroids as a treatment for infection with Covid-19. This is consistent with a study by (Grand et al., 2022) that confirmed that asymptomatic infection with HCMV occurs in a high percentage of patients diagnosed with covid19 as a result of reactivation of HCMV due to the use of high doses of corticosteroids that may have a direct effect on reactivation of HCMV .In a study by (Pande et al., 2023),the serological examinations showed strong positive maternal IgG and high activity of HCMV, as high concentrations of maternal CMV IgG indicate a possible activation of HCMV after SARS-Cov-2 infection, and this study did not exclude the contribution of other diseases caused by TORCH in pregnancy loss. These reasons may also be an important reason that contributed to the high concentrations of IgG and IgM that were recorded for our current study samples infected with Covid-19 and HCMV, as the IgG reached 56 compared to the IgM, which reached 26, as shown in Table (11). In the case of patients infected with T. gondii who had a previous infection with Covid 19, they had high concentrations of IgG, which amounted to 50 and 16 of IgM, as shown in Table (11). IgG for T.gondii in patients may due to the subsequent apoptosis of memory lymphocytes, this can lead to reactivation of latent infection. This is consistent with a study by (Sharaf-El-Deen et al., 2021) that showed this association clearly when comparing IgG levels for T.gondii that increased in Covid-19 patients until it was statistically higher in severe cases that was due to immune exhaustion caused by infection with Covid-19, which promoted the reactivation of chronic toxoplasmosis. Also, In a study by (Geraili et al., 2022) it was found that patients infected with Covid-19 were positive for IgG and IgM for T. gondii, as the study revealed a relatively high prevalence of latent toxoplasmosis in these patients. Also, these results are consistent with other documented studies such as (Ghaffari et al., 2021) study, which indicate that there is an important relationship between infection with toxoplasmosis and infection with Covid-19, as the results of this study showed that latent T. gondii infection is widespread among patients with Covid-19, and it did not find a very significant association. Therefore, our results also indicate that further studies with larger sample sizes and case-control design should be conducted for any association between T. gondii and COVID-19. It was confirmed in a study by (Sharaf-El-Deen et al., 2021), that both Covid-19 and T. gondii share a common point of immune interaction, which is the depletion of lymphocytes, as chronic infection of T. gondii is associated with a gradual increase in lymphatic expression of PD-1, which it leads to post apoptosis of memory T lymphocytes and this can lead to reactivation of latent infection (Khan et al., 2019). While the results of our study differed with a study by (Mohammed and Abbas, 2021), which indicated that there was no actual participation among pregnant women infected with Covid-19, HCMV and T.gondii, which led to the death of the fetus inside the mother's uterus.

Table (11) Number and percentage of Covid-19 infection associated with CMV and T. gondii infection

Infection	Total No.	Covid-19 Infection	
		Positive No.	%
CMV	45	23	51.11
Toxoplasma	40	21	52.5
Mixed infection	11	7	63.63
Calculated X^2	0.568		
P value	0.753*		

* No significant difference at P<0.05

6. Diagnosis of Cytomegalovirus and T. gondii by Molecular tests

The RT-PCR test was also used in the current study to evaluate the accuracy of ELISA tests compared to its results in the blood of aborted women for the diagnosis of HCMV and T. gondii infection. The positive result of Real Time PCR for capsid protein gene was 28 (50.0 %), while negative results was 28 (50.0%), as shown in figure (1) and table (12). Also the present results showed 22 (43.1%) samples were positive for B1 gene of Toxoplasma gondii, while negative results was 29 (56.9%), as shown in figure (2) and table (12). These results are consistent with a study by (Alsaide et al., 2019) conducted on 400 women who suffered from recurrent miscarriages, whose ages ranged between 15-45 years, in which IgG and IgM antibodies to T. gondii were detected. By using ELISA, 176 out of 400 cases were positive for IgG and 44 were positive for IgM, and the positive samples by RT-PCR were only 32 samples. Also, our results agreed with a study of (Awad, 2020) that was conducted for 60 aborted women with a bad birth history; 25% for IgM and 65% for IgG. As for the diagnosis by RT-PCR, it was 10%. Also, with a study of (Najm, 2020) conducted for 68 women (34 aborted women and 34 women with normal pregnancy as a control group), where the

samples were tested for each of T. gondii and Rubella virus, and the results of the diagnosis by ELISA were 19 positive samples for T. gondii. When diagnosed using RT-PCR, 14 samples were positive for T. gondii . Also, in a study by (Flegr, 2011), a few of the results obtained by using the ELISA were confirmed by RT-PCR. The results of our study agreed with the study of (AL-Hajjar, 2021), which included 90 samples from women who suffered from recurrent miscarriage (70 miscarriages and 20 cases without a history of miscarriage as a control group), the results of ELLISA diagnosis were 62 (89%) for positive HCMV IgG While 65 (93%) of the patients were positive for HCMV IgM from 70 aborted women, while the molecular detection results showed that a small number of samples only 13 (19%) were positive and HCMV DNA could be detected in the blood of aborted women. And in a study by (Ali,2019) of 420 samples from pregnant and aborted women from different hospitals in the city of Baghdad. The ELISA diagnosis revealed 81 samples out of 420 were infected with HCMV, while the RT-PCR diagnosis found that 64 samples had positive results for HCMV DNA, where the RT-PCR reaction was a more reliable method. Sensitivity and reliability in the diagnosis of HCMV in pregnant women in this comparative study.

Table (12): Real Time PCR results for capsid protein gene of CMV and B1 gene of T. gondii.

Characteristic	Capsid protein gene n = 56	B1 gene n= 51	P
Positive, *n* (%)	28 (50.0 %)	22 (43.1 %)	0.477 ¥ NS
Negative, *n* (%)	28 (50.0%)	29 (56.9%)	
Total	56 (100.0 %)	51 (100.0%)	

n: number of cases; ¥: Chi-square test; NS: non- significant at P < 0.05

Figure (1): Real Time PCR amplification plots for capsid protein gene in CMV patients' blood samples. Where the positive amplification were showed in threshold cycles ranged 18-38 Ct values.
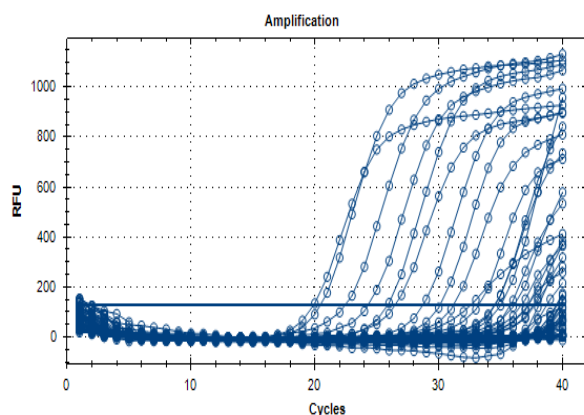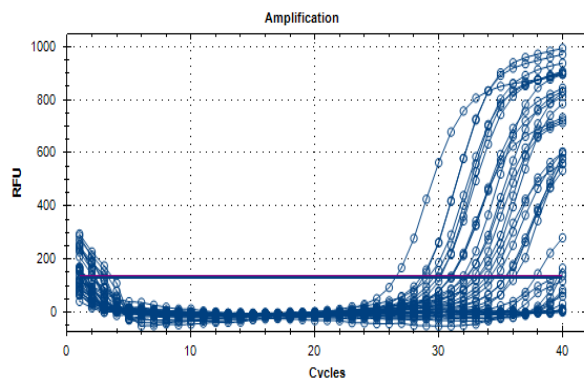

Figure (2): Real Time PCR amplification plots for B1 gene in T. gondii patients' blood samples. Where the positive amplification were showed in threshold cycles ranged 25-35 Ct values.

7. The frequency distribution of Real Time PCR results according to age groups.

The frequency distribution of Real Time PCR results according to age groups of patients were shown in table (13). Where the results of the study indicated that there were no significant differences between the age groups of aborted women at P > 0.05, where the frequency distribution of patients infected with HCMV included 17 (60.7%) cases in the age group (16-26) and 7 (25%) in the age group (27-37) and 4 (14.3%) in the age group (38-48), while the frequency distribution of patients infected with T. gondii included 10 (45.5%) in the age group (16-26) and 11 (50.0%) in the age group (27-37) and 1 (4.5%) in the age group (48-38). These results are consistent with the study of (Kalaf and Jameel, 2023) which confirmed that women between the ages of 21 and 30 years had a higher incidence of HCMV infection. Also, our results are consistent with a study by (Naame et al., 2021) where the study was conducted on 120 aborted women, whose ages ranged from 15-40 years in the city of Basra, to be diagnosed with HCMV, herpes simplex virus, and T.gondii. The highest rate of infection was recorded in the age groups from 30-21, since this is the appropriate age for pregnancy. It also agreed with the results of a study by (Najm et al., 2020), where the highest rate of diagnosis of T.gondii was recorded in women whose ages ranged between (40- 20) years.

Table (13): Frequency distribution of Real Time PCR results according to age groups.

Characteristic	Capsid protein gene $n = 28$	B1 gene $n= 22$	P value
Age (years)			
16-26, *n* (%)	17 (60.7 %)	10 (45.5 %)	
27-37, *n* (%)	7 (25.0%)	11 (50.0%)	0.147 ¥ NS
38-48, *n* (%)	4 (14.3%)	1 (4.5%)	
Total	28 (100.0 %)	22 (100.0)	

n: number of cases; ¥: Chi-square test; NS: not significant at P > 0.05

8. The frequency distribution of Real Time PCR results according to residency.

As for the RT-PCR test, the frequency distribution of patients infected with HCMV by residence included 14 cases in both urban and rural areas, and the difference was non-significant at P > 0.05, while the frequency distribution of patients with T. gondii according to residence included 10 cases in the rural regions and 12 cases in urban areas, and the difference was not significant at P > 0.05

due to the small size of the sample that appeared positive for the RT-PCR test. The sensitivity of the RT-PCR test is significantly more sensitive than the serological test (Najm et al., 2020), and this result is consistent with a study by (Ali et al., 2019), which confirmed that HCMV DNA was detected only in a small number of samples of aborted women compared to healthy pregnant women. The frequency distribution of Real Time PCR results according to residency of patients were shown in table (14).

Table (14): Frequency distribution of Real Time PCR results according to residency.

Characteristic	Capsid protein gene $n = 28$	B1 gene $n= 22$	P value
Residence			
Rural, n (%)	14 (50.0 %)	10 (45.5 %)	0.749 ¥ NS
Urban, n (%)	14 (50.0%)	12 (54.5%)	
Total	28 (100.0 %)	22 (100.0)	

n: number of cases; ¥: Chi-square test; NS: not significant at P > 0.05

9. The frequency distribution of Real Time PCR results according to Covid-19 infection.

Table (15) shows the frequency distribution of the patient groups according to the results of infection with the Covid-19 virus. The current study showed that 20 patients infected with HCMV had COVID-19 infection, 12 patients infected with T. gondii had COVID-19 infection, but there were non-significant differences according to the results of COVID-19 at P > 0.05. As we explained earlier in our current study, infection with Covid-19 can lead to reactivation of latent HCMV infection in pregnant women, and thus HCMV causes abortion in some cases. Reactivation of latent infection in aborted women occurs due to immune stress or due to the use of high doses of corticosteroids as a treatment for Covid-19 infection, and this is consistent with a study by (Granda et al., 2022), which confirmed that accidental infection with HCMV occurs at a high rate in

patients diagnosed with Covid-19 infection due to reactivation of latent HCMV infection as a result for the use of high doses of corticosteroids, while in the case of patients infected with T. gondii with a previous infection with Covid-19, the receiving of immunosuppressive therapy by Covid-19 patients stimulates the rise of IgG of T.gondii (Sharaf-El-Deen et al., 2021). The results of our study agreed with the results of a study by (Geraili et al., 2022) that found that patients with Covid-19 were positive for anti-T.gondii IgG, where the study revealed a relatively high prevalence of latent toxoplasmosis in these patients, as there was a study by (Sharaf-El-Deen et al., 2021) that confirmed that both COVID-19 and T. gondii share a common point of immune interaction, which is lymphocyte depletion; chronic infection with T.gondii is associated with progressively increased lymphatic expression of PD-1 leading to subsequent apoptosis of memory T lymphocytes. This could lead to reactivation

of latent infection of T.gondii (Khan et al., 2019). While the results of our study differed with a study by (Muhammad and Abbas, 2021), which indicated that there was no actual participation among pregnant women with Covid-19, T. gondii, and HCMV, which caused the killing of the fetus inside the mother's uterus.

Table (15): Frequency distribution of Real Time PCR results according to Covid-19 infection

Characteristic	Capsid protein gene *n = 28*	B1 gene *n= 22*	P value
Covid-19 infection			
Positive, *n* (%)	20 (71.4 %)	12 (54.5%)	0.217 ¥ NS
Negative, *n* (%)	8 (28.6%)	10 (45.5 %)	
Total	28 (100.0 %)	22 (100.0)	

n: number of cases; ¥: Chi-square test; NS: not significant at $P > 0.05$

10. Results of IgM, IgG and PCR testing for study groups

10.1. Results of IgM, IgG and PCR testing for diagnosis of HCMV

The results showed in Table (16) that the number of samples that were positive for the PCR test and ELISA was 18 samples out of a total of 28 with a percentage of 72%, while 3 samples were positive for the PCR test and IgG whereas negative for IgM ,and 7 samples positive for PCR were negative for IgG and positive for IgM, and no PCR-positive sample were negative for both IgG and IgM. As for PCR-negative samples, there was one sample positive for IgG and IgM, 27 samples were negative for PCR, positive for IgG and negative for IgM, and no negative sample for PCR and negative for each of IgG and IgM was recorded. As well as no one reported to positive IgM and negative for IgG. Also, there were 3 aborted women infected with HCMV who had positive results with IgG for HCMV only, and they also had positive results when diagnosed by RT-PCR. It is attributed to the susceptibility of sero-negative women in pregnancy to primary infection with HCMV or to reactivation as a result of weak immunity (Jassim, 2017).This also agrees with a study by (AL-Hajjar and Al-Mousawi, 2021) which indicated that an elevation in the IgM titer occurs before the elevation in the IgG titre, with prognosis dependent on both the patients and the sensitivity of the antibody assay. And in the study of(El Sanousi et al., 2016) it was confirmed that the RT-PCR reaction depends on the early direct major gene, which appears only for a short period of time during the infection cycle, while there may be false negative results for IgM due to the high abundance of IgG where it can mislead the level of IgM is undetectable due to late seroconversion as a result of immunosuppressive factors, while IgM antibodies can also persist long after infection in some healthy individuals. Finally, the RT-PCR test is a quick, accurate and practical way to identify the active pathogen and track the treatment response more than the ELISA test, because it is an accurate and sensitive diagnostic tool to detect acute and chronic stages of infection in clinical samples without any false positive results that may help in targeted treatment. This method may also be suitable for examining T. gondii infection in special cases such as immunocompromised groups that usually fail to produce a specific IgM or have an increased titer of IgG. It is the best method for detecting recent, reactive, and latent infection by T.gondii (Awad and AL-muffti, 2020). And the fact that the results of serological tests by ELISA are specific and

may give false positive results can be explained by the chances of infection with other microorganisms, and this is confirmed by the results, as a small number of the results obtained by PCR were confirmed (Najm et al., 2020).

Table (16) Results of IgM, IgG and PCR testing for diagnosis of CMV

PCR Results	Serological Results				
	IgG+ IgM+	IgG+ IgM-	IgG- IgM+	IgG- IgM-	Total
PCR(+)	18(72)	3(10.71)	7(25)	0(0)	28(50)
PCR(-)	1(3.57)	27(96.42)	0(0)	0(0)	28(50)
Total	26	30	0	0	56

10.2. Results of IgM, IgG and PCR for diagnosis of T. gondii

The results in Table (17) showed that the number of samples that were positive for the PCR test and ELISA was 15 samples out of a total of 22 with a percentage of 68.18%, while 6 samples were positive for the PCR test, negative for IgM and positive for IgG ,and 1 sample positive for PCR was negative for IgG and positive for IgM, and no PCR-positive sample was recorded negative for IgG and IgM. As for PCR-negative samples ,there was no sample positive for IgG and IgM , and 29 samples were negative for PCR, positive for IgG and negative for IgM, and no negative for PCR and negative for both IgG and IgM were recorded, there was no sample for IgM positive and IgG negative. It is interesting that 6 aborted women had positive results for IgG of T. gondii only and they also had positive results by diagnosing RT-PCR. The reason for this is that IgM begins to appear after about 1-2 weeks of infection and reaches its peak in the fourth week and decreases. The level of IgM after about 4-8 weeks and its presence continues for six months and sometimes it remains for more than a year at a very low level in aborted women infected with T.gondii (Al-samarraiy, 2021). In a study by (Abd al-ameer, 2021) ,it was confirmed Serological tests are one of the most common methods for diagnosing toxoplasmosis that cannot distinguish between distinct toxo-antigens IgG and IgM during chronic and acute T. gondii infection, due to the fact that these antibodies are formed after a period of infection with the parasite in the blood for several weeks. Also, the variation of the test performance is mostly associated with target genes and primer composition. Most investigators have used the B1 or RE gene for detection (Cassaing et al., 2006 and Dadimoghaddam et al., 2014).

Table (17). Results of IgM, IgG and PCR for diagnosis of T.gondii

PCR Results	Serological Results				
	IgG+ IgM+	IgG+ IgM-	IgG-IgM+	IgG-IgM-	Total
PCR(+)	15(68.18)	6(27.27)	1(4.54)	0(0)	22(43.13)
PCR(-)	0(20.68)	29(58.62)	0(0)	0(0)	29(56.86)
total	13	32	1	0	51

Conclusion:

The age group (16-26) is more infected than the rest of the other age groups because this group is the optimal period for marriage and the incidence of pregnancy is very high. The prevalence of HCMV and T. gondii infection is higher in rural areas than in urban areas, and the reason in the countryside there is spread of mixing with animals, birds, and cats carrying the parasite, while the spread of the virus may be due to poor hygiene, low social and economic level, and crowded living conditions, social and cultural habits. Covid-19 infection can lead to reactivation of HCMV and toxoplasmosis in pregnant women, and thus they contributes to pregnancy loss in some cases. Finally, the RT-pCR test is a

quick, accurate and practical way to identify the active pathogen and track the treatment response more than the ELISA test.

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