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SEROLOGICAL AND MOLECULAR DIAGNOSIS OF *Toxoplasma gondii*

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ABSTRACT

This study aimed to determine the immune status of pregnant women at the Central Hospital of Ain Naâdja (HCA) in Algeria. For this purpose, the identification of anti-*Toxoplasma* immunoglobulins IgG and IgM by ELFA test was carried out in 393 patients, as well as the comparison of the serological profile for the mother-child, serum-aqueous humor pairs by the Western Blot (WB) assay. Parasite search was done by standard PCR on placenta samples using JW62/JW63 primer. The results obtained from 393 serums collected showed that: 40.96% of the pregnant women having a serological profile in favor of an old Toxoplasmosis, and 59.03% were not immunized ($P<0.05$). Serological comparison by WB analysis of the pairs revealed that 90.1% of the couples possessing the same serological profile (negative), and 9.09% were positive (congenital toxoplasmosis) ($P<0.05$). To conclude, The PCR performed on placentas showed a high level of specificity for diagnosis. It allows early diagnosis and should be recommended for identification of congenital infection.

Keywords: *Toxoplasma gondii*; ELFA; western blolt; PCR; pregnant woman.

INTRODUCTION

Toxoplasmosis is a cosmopolitan zoonosis caused by an obligatory intracellular parasite, *Toxoplasma gondii* [1]. It is certainly the most widespread parasitic disease in the world, operating in all latitudes and able to infect all animal species. The definitive host is essentially represented by the cat and the intermediate hosts by all mammals and birds, including Human. The pathology qualifies as a One Health disease because it significantly affects the health of human, domestic animals, wildlife, and ecosystems, and is perceived as a threat by those who rely on animal resources [2].

This organism is obligatorily entered in all the cells of the body, preferably in the nerve, ophthalmic, digestive or muscular cells [3]. This pathology is classically benign and often latent in children and adults but is more frequent in the fetus, the

newborn and the immunosuppressed subject. Toxoplasma is a possible source of serious congenital diseases when it infects pregnant women (primary infection). The congenital form can be manifested by severe neurological malformations and retinal damage that may lead to blindness, and results from a toxoplasmic primo-infection that occurred during pregnancy [4,5].

In animals, the disease is similar to that of Humans; it is one of the major causes of abortion, embryonic resorption, stillbirth or birth of weak goats and lambs in ruminants. Sheep is the most affected species [4]. This disease affects one third of the world's population. According to the continents, 04 to 84% of the individuals are infected, with a very heterogeneous prevalence according to the countries and varies mainly according to the level of hygiene of the populations and the food habits [6,7].

In Algeria, the situation remains unclear. Seroprevalence would be around 50% through several studies carried out in the Center and the East of the country. There is a gradual decrease in the immunization rate of pregnant women reaching 54% in 1995 [8]. The serological diagnosis is based on the study of specific IgM and IgA, allowing an accurate diagnosis and determines the evolution of the disease during pregnancy. The first problem of the result interpretation arises sometimes in the presence of a very low level of specific IgG. This is why the present study aimed to determine the immune status of pregnant women by the search of specific immunoglobulins anti-toxoplasmic IgG, IgM by ELFA technique and by the comparison of the serological profiles of mother-child and serum-aqueous humor pairs by Western blot technique, performed to confirm congenital and ocular toxoplasmosis.

MATERIALS AND METHODS

Material for ELFA Analysis

In this study, 393 serums of patients were collected on agar tubes in the Parasitology-Mycology laboratory of HCA. The serums were recovered after centrifugation at 2500 rpm for 3 to 5 minutes and stored at + 4°C.

Material for Western-blot Analysis

The study focused on the analysis of 11 pairs of mother-child serum (these women experienced a seroconversion during their pregnancy) and 4 pairs of serum-aqueous humor (from patients with uveitis).

Material for PCR Analysis

Positive control

It was prepared from tachyzoites recovered from the ascites of BALBc mice infested with this form of parasite.

Samples

Placenta: 8 placentas were recovered from the Gynecology Department of HCA.

Aqueous Humor: 4 aqueous humors were recovered from patients in the Ophthalmic Department of HCA.

Umbilical cord blood: A blood sample was collected from a newborn on EDTA tube at the Gynecology Department of HCA.

Peripheral blood: Two blood samples were collected from patients with ocular toxoplasmosis on EDTA tubes at the Parasitology-Mycology Laboratory of HCA.

Methods

In this study, we used two serological techniques, ELFA, Western-blot and PCR.

Identification of Anti-toxoplasma antibodies by the Enzyme Linked Fluorescence Assay (ELFA)

TOXO IgG II is an automated qualitative test on a VIDAS instrument, mini Vidas® (BioMérieux, France), allowing quantitative measurement of anti-toxoplasmic IgM and IgG in Human serum or plasma (lithium heparin or EDTA) by ELFA. All the steps and the results were performed automatically. The threshold of positivity was 08 IU / ml. Women who presented serology: IgG + and IgM - were considered immunized, and those with serology: IgG + and IgM + were considered non-immune. If the result remained ambiguous, the test was repeated with a new sample.

Identification of Anti-*T. gondii* antibodies by Western-Blot test (LDBIO Diagnostics, France)

The antigens of *Toxoplasma gondii* after electrophoretic separation on polyacrilamide gel were electro-transferred onto the surface of nitrocellulose strips. The strips were supplied ready-to-use, numbered and pre-cut.

For congenital toxoplasmosis, the presence of bands in the newborn that were absent in the mother results in a proper synthesis in favor of a fetal infection.

For ocular toxoplasmosis, the presence of additional bands in the aqueous humor relative to serum reflects a local production of IgG in favor of ocular toxoplasmosis.

Detection of the Parasitic DNA of *Toxoplasma gondii* by PCR

DNA extraction of *Toxoplasma gondii*

It was extracted by three methods depending on the type of sample: Qiagen QIAamp DNA Mini Kit for tissues (Placenta), Qiagen stools kit for stool specimens (QUIAGEN ®, USA) and the SaMag 12 for blood (Sacace™ Toxoplasma gondii Real-TM, USA).

Amplification of extracted DNA

The primer used was: JW62/JW63.

JW62:5'-
TTCTCGCCTCATTTCTGGGTCTAC-3'
JW63:5'-
GCACCTTTCGGACCTCAACAACCG-3'

This sequence targets the B1 gene that is most commonly amplified. It is repeated 35 times in the genome of the parasite. This gene is found in all *T.gondii* strains [9].

According to the protocol reported by Pelloux et al. [10], the results of amplification showed nonspecific and specific bands whose size in 286 bp. To avoid the problem of nonspecific bands, the same amplification conditions were performed while changing the protocol using the Master Mix.

Preparation of the MIX

In a tube of 1.5 ml, mix the reagents in order. Start with the water, the buffer, the MgCl2, the primers and lastly the Taq polymerase. Divide the total volume of the mix into 0.5 ml Eppendorf tubes and add the extracted DNA volume to each of the Eppendorf's except for the negative control. Introduce the Eppendorfs containing the

amplification components into the thermocycler.

Revelation by agarose gel electrophoresis

The revelation by electrophoresis was carried out on a 2% agarose gel in TAE (Tris-Acetic Acid-EDTA). 20 µl of ethidium bromide (ETB) was added to the agarose solution to visualize the DNA strands. The electrophoretic migration was performed at 90V for 45 min. The revelation of the PCR data was done on a UV Trans-illuminator. Finally, bromophenol blue staining was performed.

Statistical Analyses

Statistical analyses were conducted using SPSS (SPSS. IBM Corp Ver. 20.0). The comparison of the serological profile of pregnant women by ELFA test, the serological profile of the 11 pairs of mother-child serum obtained by WB as well as the PCR results and the direct parasitic examination data were performed using Student's t-test. A P-value less than 0.05 was considered statistically significant.

RESULTS OF THE SEROLOGICAL STUDY

Serological Results Obtained by the VIDAS Automaton

The analysis of the 393 serums taken from pregnant women showed that 40.97% of the cases (161/393) presented a serological profile in favor of an old toxoplasmosis with IgG positive and absence of IgM. Of the 161 patients (2.8% or 11/161) who seroconverted with IgG and IgM positive, 59.03% of the patients were negative (IgG- and IgM-) (Table 1).

Table 1. The results of the serological profile of pregnant women by ELFA test

	1st control	**2nd control**	**3rd control**	**VIDAS**	**Number of cases**	**%**
1st case	IgG-/IgM-	IgG+/IgM+	IgG+/IgM+	seroconversion	11	2.80%
2nd case	IgG+/IgM-	IgG+/IgM-	IgG+/IgM-	Old immunity	150	38.17%
3rd case	IgG-/IgM-	IgG-/IgM-	IgG-/IgM-	Non imune-patient	232	59.03%***

**** : P<0.0001*

Table 2. The results of the serological profile of the 11 pairs of mother-child serum obtained by Western-blot test

Period	**Western-blot**				
	IgG		**IgM**		
	Mother	**Child**	**Mother**	**Child**	
Day 0	+	+	+	-	90.91%,
Day 10	+	+	+	-	Same profile as the mother, so no
Day 90	+	+	+	-	infection
Day 0	+	+	+	+	9.09%***, congenital
Day 10	+	+	+	+	toxoplasmosis
Day 90	+	+	+	+	

**** : P<0.0001*

Serological monitoring using the ELFA technique revealed a seroconversion of 11 pregnant women during the first two trimesters; the application of the Western blot in the follow-up of the newborn appears necessary. This test allowed us to identify the antibodies neo-synthesized by the fetus.

Serological Results Obtained by Western-blot

Results of congenital toxoplasmosis

The sera of the 11 women who showed seroconversion and the sera of their children were analyzed by Western-blot.

The serological analysis of the 11 pairs of mother-child serum revealed that the serology of 10 newborns (90.91%) presented the same profile as their mother. This result indicated the absence of fetal infection. However, only 1 pair was positive where the newborn's profile was different from that of his mother (Table 2, Figs. 1 and 2).

Results of Ocular Toxoplasmosis

Our study focused on 4 couples serum / aqueous humor, whose patients presented uveitis. The serological results revealed by Western-blot are shown in Table 3.

The comparative study between the anti-toxoplasmic IgG and IgM profile of the 4 pairs serum / aqueous humor, taken from patients with uveitis showed three positive pairs, with 75% of cases which had 4 additional bands in the aqueous humor relative to the serum. Only 1 negative case that revealed the absence of IgG and IgM in the aqueous humor (Figs. 3 and 4).

Results of Molecular Diagnosis of *Toxoplasma gondii* by PCR

PCR results from placentas

Our study focused on PCR analysis of 8 placentas received at the Parasitology-Mycology laboratory of HCA. Amplification of *T. gondii* DNA extracted from placentas using JW62 / JW63 primer showed that 4 placentas were positive with 2 cases corresponding to the patients who underwent a therapeutic abortion at 250 days and 282 days of pregnancy, respectively and the two others did not know a serological follow-up during their pregnancy (Figs. 5 and 6).

PCR Data Carried Out with the DNA Extracted from *T. gondii* (from biological fluids)

Analysis of 4 aqueous humor samples by conventional PCR revealed 4 negative results, as well as umbilical and peripheral blood samples from patients with ocular toxoplasmosis.

Table 3. Profile of serum / aqueous humor (AH) couple obtained by Western-blot test

	Western-blot				Interpretation
	IgG		IgM		
	AH	Serum	AH	Serum	
Patient 1	+	+	-	+	Positive: presence of an extra-band In AH
Patient 2	+	+	-	+	Positive : presence of 3 an extra-band in AH
Patient 3	+	+	-	+	Positive: more than 4+ additional bands in AH
Patient 4	-	-	-	+	Negative : no infection

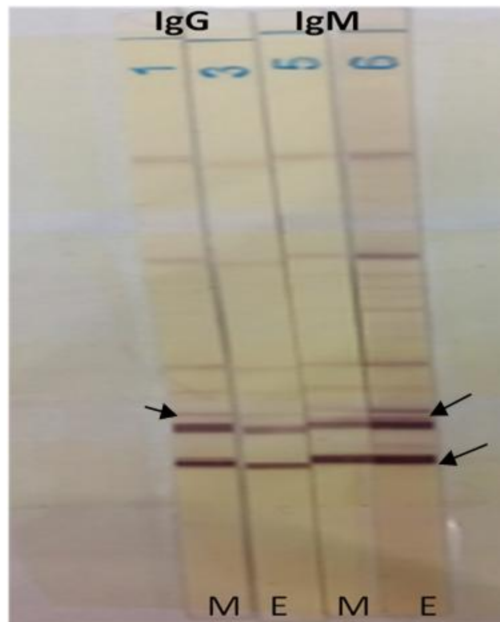

Fig. 1. Positive profile of IgG-IgM in the mother (M) and the same profile in the child (E) at day 90 (so no fetal infection)

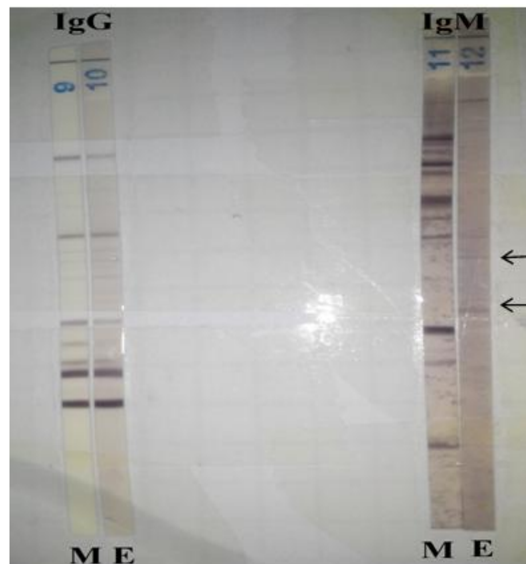

Fig. 2. IgG profile of the child (E) similar to that of his mother (M), and presence of neo-synthesized IgM in the Child

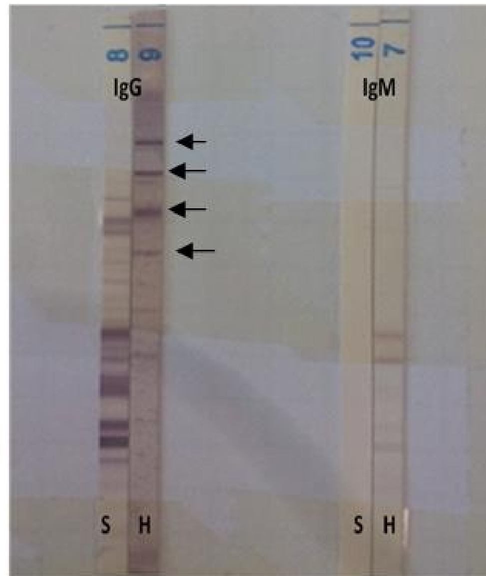

Fig. 3. Analysis of positive profile IgG-IgM anti-*Toxoplasma gondii.* Serum (s) and aqueous humor (H)

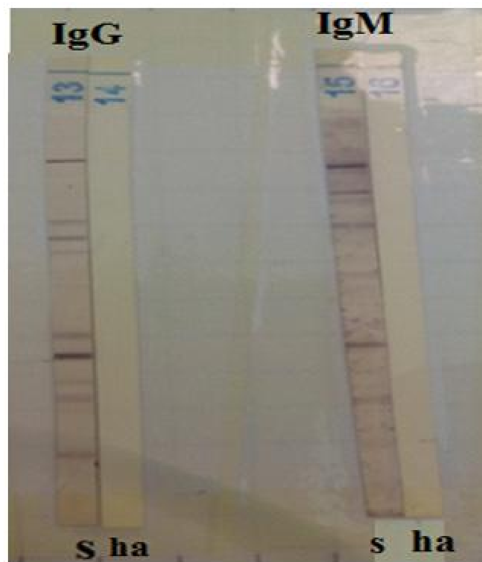

Fig. 4. Analysis of negative profile IgG-IgM anti-*Toxoplasma gondii.* Serum (s) and aqueous humor (ha)

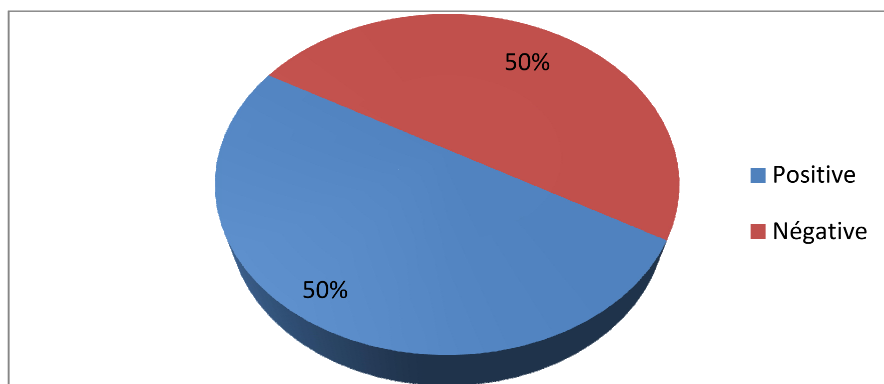

Fig. 5. PCR data from DNA extracted from the placenta

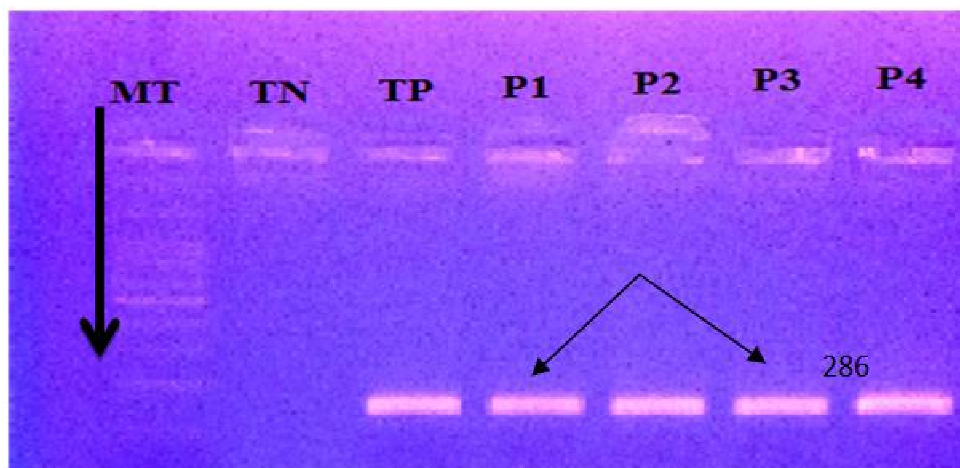

Fig. 6. Results of amplification of parasite DNA extracted from placentas, by JW62 / JW63 primer

(MT: size marker; TN: negative control; TP: positive controls extracted from tachyzoites; P1, P2, P3, P4: T. gondii DNA extracted from placentas)

DISCUSSION

Toxoplasma gondii is a protozoan parasite of mammals and birds that is responsible for toxoplasmosis, the latter is often asymptomatic but it has serious consequences in pregnant women and immunodepressed patients [1]. It is a cosmopolitan parasite; its frequency depends on diet and lifestyle [11].

Congenital toxoplasmosis is currently one of the main conditions that can compromise the development of a pregnancy [5].

In the current work, the analysis of 393 sera collected from pregnant women in the Parasitology-Mycology laboratory of HCA showed that 40.97% of the cases (161/393) presented a serological profile in favor of an

old toxoplasmosis with IgG positive and absence of IgM. These results were in accordance to those reported by Montoya et al. 1999. The seroprevalence of toxoplasmosis has been studied by several authors. It has been reported in pregnant women a seroprevalence of 33.33% in Marrakech (Morocco) [12], 57% in Tunisia [13] and 54% in France in 1995 [14]. The study conducted by Makuwa et al. [15] in the Congo showed 40% of the negative and 60% of the positive cases. The seroprevalence of *Toxoplasma gondii* in humans is very heterogeneous and varies greatly from one country to another, but also from one region to another in the same country and between different ethnic groups living in the same region [16,7].

Of the 161 patients, 2.8% (11/161) were seroconverted with positive IgG and IgM. The prevalence of congenital toxoplasmosis seroconversions worldwide was estimated to be between 0.68 in England and 40 cases per 1000 women seronegative in the United Arab Emirates [17]. In the Center of Algeria, the incidence of toxoplasmosis in pregnant women was 0.98% according to Schneider [18] and rose to 3.5% [19] with 2.5% of congenital toxoplasmosis. In the East of the country, it was 1.1% [8].

In this study, the prevalence of non-immunized women was 59.03% of the cases with negative IgG and IgM. These rates may be related to cultural and culinary habits, the consumption of bleeding meat, the ingestion of unwashed fruits and vegetables contaminated with oocysts and the presence of a cat in the home. It would seem, however, that the direct role of the cat is less determinative than the other factors [20,7]. Some studies also suggested that the high relative humidity that typifies Northern Algeria (Center, Eastern and Western) enhances oocyst viability [7].

The sera of the 11 women who showed seroconversion and the sera of their children were analyzed by Western-blot. Serological analysis of the 11 pairs of mother-child serum revealed the absence of fetal infection in 90.91%. However, only 01 pair (9.09%) was positive after a check. A prevalence of 18.3% of congenital toxoplasmosis in the analysis of 126 pairs of sera (newborn-mother), ie 23 newborns infected during pregnancy was reported [21].

Simultaneous comparison of the profiles from different compartments: maternal blood / newborn blood, demonstrated the presence of additional bands in the newborn, proving that the fetus, via its antibodies, recognizes parasite antigens different from those of the mother, and thus asserts the diagnosis of congenital toxoplasmosis [22,5].

Congenital toxoplasmosis is the consequence of a primary infection during pregnancy. After contamination of the mother, the parasite passes into the bloodstream. This maternal infestation is early and transient (of the order of 10 to 15 days) and prior to the appearance of antibodies. This explains the rarity of congenital toxoplasmosis following an infection of the mother contracted before pregnancy [23,5].

In the case of a maternal infection near to conception, parasite transmission to the fetus is low: less than 2%. When maternal infection is close to the end of pregnancy, transmission of toxoplasma to the fetus is around 90%. At this stage, fetal contamination is generally contemporary with maternal contamination. Indeed, the

fetal immune system is in place and will be secondarily reinforced by the passive immunity of the mother [24].

The use of the Western-blot test in the follow-up of the newborn until the age of 3 months appears necessary, in particular in the seroconversions of the first two trimesters. For this purpose, it is necessary to insist on the interest of repeating the test every month in case of early seroconversion of the mothers, because of the lack of maturation of the fetal immune system before the 20th week, or on the contrary in case of very late seroconversion (there was not enough time between infection and birth to observe antibodies synthesis) [25]. It is clearly demonstrated that the value of serological diagnosis at the end of pregnancy resides in the diagnosis of asymptomatic cases of congenital toxoplasmosis following seroconversions at the end of pregnancy when the risk of fetal contamination is high, estimated at 90% for infections during the last 2-3 weeks of gestation. The majority of infected newborns are asymptomatic at birth hence the need for routine screening because if they are not diagnosed and treated, they will develop sequelae during childhood and adulthood. The consequences of congenital toxoplasmosis in newborns are the appearance of occular toxoplasmosis, which results in uveitis or chorioretinal scar [24,5].

The comparative study between the anti-toxoplasmic IgG and IgM profile of 4 couples serum / aqueous humor, taken from patients with uveitis by the Western blot showed 75% of the positive cases and a single negative pair (25%). The sensitivity of Westen-blot in our study is of the order of 75%. This result is close to that found by Thelliez [26] which revealed the presence of local IgG in 56% of cases. Bastien [27] reported percentages ranging between 15 and 53%. Desmonts 1973 recommended repeating the analysis with a new eye sample in case of negative results. For some authors, the date of realisation of the ocular puncture seems to be an essential factor that can modify the results. Indeed, at the beginning of the disease, the local synthesis of antibodies has not yet started; the maximum production of antibodies is around the 4th week from the beginning of clinical signs [28]. A puncture performed 3 weeks after the onset of symptoms makes it possible to find local antibody production in 57% of the cases [29].

The molecular diagnosis of parasitic diseases consists in identifying the DNA of the parasite in various samples. Among parasitic diseases, the diagnosis of toxoplasmosis benefits largely from the contribution of molecular tools. In the present study, we were interested in the extraction of the parasitic DNA of *Toxoplasma gondii* from placentas, aqueous humors and peripheral blood and also from umbilical cord blood.

For the DNA extraction from placentas, the Qiagen Kit method was combined with a tissue lysis step. The second step was to perform amplification of *Toxoplasma gondii* DNA by the primer pair: JW62 / JW63 of 286 bp. It is clearly demonstrated that several factors influence the specificity and efficacy of PCR, the concentration of Taq polymerase, the concentration of primers and the hybridization temperature [30].

The analysis of 8 placentas recovered from the Gynecology department of HCA for a neonatal diagnosis by PCR revealed that; the two cases that experienced prenatal serologic follow-up by serological tests (ELFA and WB), showed maternal

seroconversion but no fetal infection. Molecular diagnosis by PCR also revealed the absence of *Toxoplasma gondii* DNA in the placenta and cord blood, so no toxoplasmic infection. For the patient who had prenatal follow-up and had seroconversion and no neonatal follow-up with WB, the PCR result was negative; similarly, for the patient who had no prenatal serologic follow-up during pregnancy. And among these placentas, 02 cases that were not followed during pregnancy but revealed by PCR the presence of toxoplasmic DNA in the placenta. Lastly, in 02 cases whose immune status was not known during pregnancy or after birth, their neonatal PCR diagnosis of placentas revealed the presence of toxoplasmic DNA; these two cases have undergone a therapeutic abortion or fetal death at 30 AS and 5J and at 45 AS and 2J according to their gynecological follow-up, therefore fetal death can be linked to a toxoplasmic infection.

The PCR performed on placentas showed a high level of specificity for diagnosis. However, negative results cannot rule out a fetal infection. Neonatal and postnatal screenings will identify these cases [10].

In our study, the direct search of the parasite by PCR was carried out also on 4 samples of aqueous humor. In the 4 cases studied, the PCR was negative despite the presence of specific antibodies in the aqueous humor which was demonstrated by the WB technique.

According to some authors, the presence of antibodies would be responsible for the neutralization of Toxoplasma [31,32,29]. In addition, the distance between the anterior chamber and the retinal site of the lesion may be another explanation for the high percentage of negative cases [31].

CONCLUSION

In summary, Toxoplasma is a possible source of many congenital anomalies. Serological tests must be carried out early or during the declaration of the pregnancy, in order to avoid a possible seroconversion as well as a fetal transmission which is responsible for heavy consequences (abortion, prematurity, malformation, and uveitis). To avoid the risks of congenital toxoplasmosis, the seronegative pregnant women must follow some recommendations in order to protect their fetuses. From our study, it appears that the determination of the avidity of anti-toxoplasmic IgG and IgM by the ELFA method with the use of VIDAS automaton is reliable and easy to use. The analysis of the aqueous humor by WB does not reveal specific antibodies; it is useful to carry out a PCR. Two eventualities are to be considered: the PCR is positive, it is indeed a toxoplasmic chorioretinitis; the PCR is negative, two solutions are possible, the initiation of a preventive treatment or the realization of a second puncture of the anterior chamber. The PCR performed on placentas showed a high level of specificity for diagnosis. It allows early diagnosis and should be recommended for identification of congenital infection.

AUTHORS' CONTRIBUTIONS

Authors AK, MN and SN designed the study. Author BR performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors BS and HAH managed the analyses of the study. Author KR managed the literature searches. All authors read and approved the final manuscript.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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