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Articles

Comparison between Elisa and Nested PCR for Detection of *Toxoplasma Gondii* in Blood and Milk and Its Genotyping in Goats and Aborted Women in Iraq

Entesar Hussain Madi [a], Firas Rashad Al-Samarai [a, *], Yusuf Mohammad Attia Maaeni [b]

[a] College of Veterinary Medicine, University of Baghdad, Iraq
[b] Office of Agricultural Research, Ministry of Agriculture, Baghdad, Iraq

Abstract

The purpose of the study was to compare the immunological and nested PCR (nPCR) for detection of *Toxoplasma gondii* in blood and milk in a flock of goats (local and Shami) in the middle of Iraq, as well as to identify the genotyping of *Toxoplasma gondii* in goats and aborted women. 80 female goats' samples (blood and milk) were collected. Samples of blood and milk were tested using Elisa and nPCR. The results revealed that 23 (28.7 %) goats tested positive for Elisa, and 17 (21.2 %) also tested positive for nPCR. The Kappa values (0.14) showed slight agreement with nPCR and Elisa in blood, with sensitivity (58.82) and specificity (74.60). The comparison between nPCR in blood and nPCR in milk revealed positive results of 17 (21.2 %) and 23 (28.7 %) in the samples respectively. The results showed a fair agreement according to kappa (0.471), had a sensitivity of 70.59 and a specificity of 82.54, and had a 95 % confidence interval (0.252 to 0.689). Elisa had a positive 17 (21.2 %) milk result. A positive sample in the blood nPCR was 17 (21.2 %), with sensitivity (82.35) and specificity (22.22) and a 95 % (-0.254 to 0.162), the kappa values (-0.046) showed no agreement with Elisa in milk and nPCR in the blood. By the (SAG3) gene, DNA sequencing of *T. gondii* based on goat and human isolates was submitted to NCBI Genbank and assigned the accession numbers (OL792791, OL792792, OL792793, OL792794, OL792795) for goat isolates and (OL792796, OL792797, OL792798, OL792799, OL792800) for human isolates. The homology sequence identity between Toxoplasma gondii goats and human isolates with NCBI BLAST-related *Toxoplasma gondii* genotype I and III isolates showed genetic homology sequence identity ranging from (98.65-99,90 %). In conclusion, the similarity of *Toxoplasma gondii* in molecular detection of milk and blood and homology identity isolates humans and goats as a zoonotic disease.

Keywords: toxoplasmosis, prevalence, genotyping, nested PCR, Elisa.

1. Introduction

Toxoplasma gondii is an intracellular parasite that an obligated affects humans and animals and causes illness and zoonosis (Elgodwi, Mohamed, 2021). Around a third of the world's population was impacted (Djurković-Djaković et al., 2019). A parasitic coccidian that has cats as its main host although it infected any warm-blooded mammals, including humans as a result it is found all across the world and may cause both acute and chronic toxoplasmosis, infection can be

* Corresponding author
E-mail addresses: firasrashad@gmail.com (F. Rashad Al-Samarai)

contracted by ingesting tissue cysts from infected intermediate hosts' meat or oocysts from cat feces through polluted water or food (Freppel et al., 2019). This parasite is caused by an opportunistic member of the phylum Apicomplexa which is a protozoan (Mancianti et al., 2013). Toxoplasmosis in goats and sheep is very important because it leads to many economic and production losses, consequently transmitted to humans (Camossi et al., 2011). In addition, goats are regarded to be more susceptible to toxoplasmosis than sheep because of their higher activity and mobility, which increases the chances of coming into contact with polluted sources (Abu-Dalbouh et al., 2012). The acute phase in immunocompetent individuals causes transient low or mild symptoms that go unrecognized *T.gondii* has been a life-threatening opportunistic infection that could result from the reactivation of silent infection or primary infection in immunocompromised people (Manuel et al., 2020). Furthermore, congenital toxoplasmosis occurs when tachyzoites migrate via the placenta into the baby during a pregnant primary infection, could lead to miscarriage, stillbirth, ocular, or and neurologic illness, and neurocognitive defect in the newborns, there are still cases of congenital toxoplasmosis (Manuel et al., 2020). Goats infected with toxoplasmosis are a major source of human infection due to the consumption of meat and milk of infected animals (Dubey, 2004). Goats excrete tachyzoites in their milk (Spišák et al., 2010; Bezerra et al., 2013), and are resistant to processing in fresh cheeses (Dubey et al., 2014). There are many different diagnostic methods for *T. gondii* diagnosis, such as serological detection enzyme-linked immunosorbent assay (ELISA) and latex agglutination test (LAT), cell line culture, bioassays, and molecular techniques (Switaj et al., 2005). Amplification-based assays including PCR, nPCR, and real-time PCR have been widely developed for quick, definite, and accurate detection of toxoplasmosis among the many detection techniques (Bretagne, 2003; Al-Samarai and Al-Kazazm 2015 ;Liu et al., 2015). Many stage-specific surface antigens (SAG) have been discovered, and many of them are divided into two groups: SAG1 and SAG2. So SAG3, bradyzoite specific recombinant (BSR) 4 SAG-related sequences (SRS) 1–4 proteins, SAG5, SAG5.1, and SAG5.2 are all members of the SAG1 family (Parigi, 2014).

SAG1 and SRS1–SRS3 are exclusively found on tachyzoites, whereas BSR4 is only found on bradyzoites and SAG3 is found on both stages. As SAG1 and SAG3 have shown, this family of proteins is likely to have a function in the attachment process prior to parasite invasion. SAG2A (formerly SAG2) and SAG2B–SAG2D are four related proteins in the SAG2 family. SAG2A and SAG2B are only expressed by tachyzoites, whereas SAG2C and SAG2D are exclusively expressed by bradyzoites (Parigi, 2014; Lyons et al., 2002).

2. Materials and methods

Samples collection

The research was conducted in Iraq at the Ruminant Research Station of the General Authority for Agricultural Research/Ministry of Agriculture/Baghdad. And in AL-Dibuni Research Station for Researches/Wasit. Blood (10 ml) was drawn from the jugular veins of 80 female lactating goats using disposable needles and simple vacutainer tubes (gel tubes) and then brought to the laboratory on ice in a cooler box. Serum samples were extracted using a 2,000 g centrifuge for 10 minutes and kept at – 20 °C in labeled Eppendorf tubes until ELISA testing. Samples were obtained from the jugular vein using a medical syringe with a capacity of 10 ml (Vacum Tube Needle) with EDTA (Ethylene Diamine Tetra Acetic Acid) samples are kept at - 20 °C for the DNA extraction process G-spin TM Total DNA Extraction Kit iNtRON Korea and is based on B1 gene by nPCR. Milk samples were taken from 80 female animals using plastic tubes with a capacity of 100 ml for Elisa IgG (Toxo-IgG) ELISA Kit Goat *Toxoplasma gondii* SunLong Biotech Co., LTD China, and DNA extraction. In aborted women, results showed that out of (30), the positive cases were 15 in ELISA and 10 in nPCR.

Nested PCR

This technique was performed for direct detection of *Toxoplasma gondii* based on B1 gene from animal blood, milk and human blood samples. This method was carried out according to method described by Halleyantoro et al. (2019) as following steps: Primers and Primer3 plus. These primers were provided by (Scientific Researchers. Co. Ltd/Iraq) as following table:

Table 1. B1 gene PCR primer and B1 gene Nested primer

Primers		Sequence 5'-3'	PCR product size
B1 gene PCR primer	F	GGAACTGCATCCGTTCATGAG	160 bp
	R	GGCGACCAATCTGCGAATACACC	
B1 gene Nested primer	F	TGCATAGGTTGCAGTCACTG	131 bp
	R	TCTTTAAAGCGTTCGTGGTC	

Polymerase chain reaction (PCR) for Sag3 gene

The PCR technique was performed for detection Sag3 gene in *Toxoplasma gondii* human blood goats' samples. These genes were used DNA sequencing analysis for genetic variation genotyping analysis. This method was carried out according to method described by (Vitale et al., 2008).

Statistical analysis

A total of 80 blood and milk samples were collected and subjected to Elisa test and nPCR. To evaluate the Elisa test, a comparison was done between the results to obtain sensitivity, specificity, and Kappa coefficient. The odds ratios were estimated to identify the risk of some factors. Two types of odds ratios were estimated; crude odds ratio and the adjusted odds ratio by using logistic regression.

3. Results and discussion

Serological test and molecular test in blood and milk

The ELISA and nPCR tests were used for the detection of *Toxoplasma gondii* in the blood and milk of goats and women aborted. Several studies have been carried out in goats to study the prevalence of toxoplasmosis; various laboratory approaches were utilized. *T.gondii* seroprevalence was discovered by researchers. (Agglutination test modified, ELISA, Immunosorbent agglutination assay, indirect fluorescent antibody agglutination assay, Hemagglutination assays indirect, and Dye test) (Subedi et al., 2018). In the current study, we decided to employ an ELISA for this investigation approach (Iovu et al., 2012; Asal, 2016) because it provides pleasing, quick, and sensitive results. In general, many studies have been conducted using serological and molecular diagnosis of *T. gondii*, (Burg et al., 1989; Al-Sanjary et al., 2012; El-Madawy and Metawea, 2013; Al-Abady et al., 2014; Hade et al., 2015), and all of these studies agree with our conclusion that molecular (PCR) is a more sensitive and specific tool than serological tools for the detection of *T. gondii*. The sensitivity (95.45 %) and specificity (97.29 %) of PCR for identifying toxoplasmosis were determined using an ELISA test as a standard (Mikaeel et al., 2015).

Total infection by indirect ELISA and nPCR

Milk and blood

Out of 80 female lactating goats' blood and milk were examined by using indirect IgG Elisa and nPCR. In blood, Elisa was positive 23 (28.7 %), blood nPCR, positive sample 17 (21.2 %). The Kappa results (0.14.) indicated slight agreement with Elisa in blood and nPCR in blood with sensitivity (58.82) and specificity (74.60), 95 % CI (-0.0886 to 0.368), Table 2.

Table 2. Comparison between infection rate using ELISA and PCR for blood

	Blood-nPCR		
Serum-Elisa	-	+	
-	47	10	57 (71.2 %)
+	16	7	23 (28.7 %)
	63 (78.7 %)	17(21.2 %)	80
Weighted Kappa[a]			0.140
Standard error			0.117

95 % Cl	-0.0886 to 0.368
Sensitivity	58.82
Specificity	74.60

The presence of the parasite in a lower amount in the blood by the nPCR assay compared to the antibodies may be due to *T. gondii* being more tenacious in tissues than in blood after infection. Consequently, the umbilical cord, heart, placenta, and brain are appropriate samples for PCR analysis (Kijlstra, Jongert, 2009). *Toxoplasma. gondii* has been found in sheep and processed meat using PCR (Mason et al., 2010; Duncanson et al., 2001). In their study, (Nematollahi et al., 2014) found that the prevalence of antibodies against *T. gondii* was 34.6 % in aborted fetal tissue and 56.6 % when the prevalence was assessed by nested-PCR. These findings corroborate one another. The following explains the existence of sero-negative and positive samples using nPCR: no serologic test is sure in the diagnosis of toxoplasmosis, according to (Dubey, Frenkel, 1998) this parasite's seroconversion is complicated, and numerous variables might be at play. The comparison of nPCR in blood and milk, with positive 17 (21.2 %) and 23 (28.7 %) in blood and milk respectively, with fair agreement by results kappa (0.471) with sensitivity (70.59) and specificity (82.54), 95 % CI (0.252 to 0.689), Table 3.

Table 3. Comparison between infection rate using PCR in milk and PCR in blood

	Blood_nPCR		
Milk_nPCR	-	+	
-	52	5	57 (71.2%)
+	11	12	23 (28.7%)
	63 (78.7 %)	17 (21.2 %)	80
Weighted Kappa[a]			0.471
Standard error			0.111
95 % CI			0.252 to 0.689
Sensitivity			70.59
Specificity			82.54

These results are in agreement with Mancianti et al. (2013) who found a perfect agreement between nPCR blood and nPCR milk in 100 % of the cases (n = 10) goats that tested positive to n-PCR on blood samples also tested positive to n-PCR on milk samples. As a result, it's possible to claim that a certain n-PCR result on blood samples proves a goat is shedding *Toxoplasma* DNA into its milk. Also, Camossi et al. (2011) and Silva et al. (2010) they claimed that weakened immunity in sheep through the peripartum period may favor the revitalization of cystic forms of *T. gondii*, resulting in the tachyzoite being eliminated from milk. Because not all positive animals remove the parasite in their milk, the serology results do not always match the PCR results. This elimination is dependent on the animal's infection stage as well as their immunity.

In milk Elisa was positive 17 (21.2 %), blood nPCR, positive sample 17 (21.2 %). The Kappa results (-0.046) which indicated no agreement with Elisa in milk and nPCR in blood with sensitivity (82.35) and specificity (22.22), 95 % CI (-0.254 to 0.162), Table 4.

Table 4. Comparison between infection rate using ELISA in milk and nPCR in blood

	Blood nPCR		
Milk Elisa	-	+	
-	49	14	63 (78.7 %)
+	14	3	17 (21.2 %)
	63 (78.7 %)	17 (21.2 %)	80
Weighted Kappa[a]	-0.046		

Standard error	0.106
95 % CI	-0.254 to 0.162
sensitivity	82.35
specificity	22.22

The results of this study are comparable to those of de Santana *Rocha et al.* (2015), who discovered that anti-*T. gondii* antibodies were present in 41.5 % of the studied animals (114/275). However, there was no association between parasite excretion in milk and the presence of IgG in 38.9 % of the studied animals (7/18), Because of the high seropositivity and the detection of parasite DNA in the milk, it was determined that the sheep population in southern and southwestern Bahia is infected with *T. gondii*. A very low value of kappa presented that there was no agreement between seroprevalence and prevalence parasite in milk. The animals who tested positive for milk in the PCR but negative in the serology might be in the early stages of disease, with inadequate antibodies to be identified in the serology so the presence of *T. gondii* DNA in goat milk does not imply that the parasite is still alive (Bezerra et al., 2013). Because of IgGs are detectable within 1-2 weeks of infection and reach their peak between 1-2 months later; they are present for the rest of one's life at levels that progressively drop (Montoya, 2002). Disagree with results were reported by Amairia et al. (2016) in Northwest Tunisia were Elisa (31.2 %) while nPCR (7.8 %) According to Saad and Ewida (2018), the low incidence of *T. gondii* observed by qPCR is due to the late expansion of IgG antibodies, and the parasite is limited to organs and tissues rather than being dispersed in the circulation, thus it does not enter the milk.

Nested PCR product analysis

Samples were obtained from human blood and goat's milk, Lanes (1-15) were showed some *Toxoplasma gondii* were showed at (131bp) Nested PCR product and N: non-DNA template negative control samples. Based B1 gene, were subjected to nested PCR based SAG3 for sequence, Figures 1, 2.

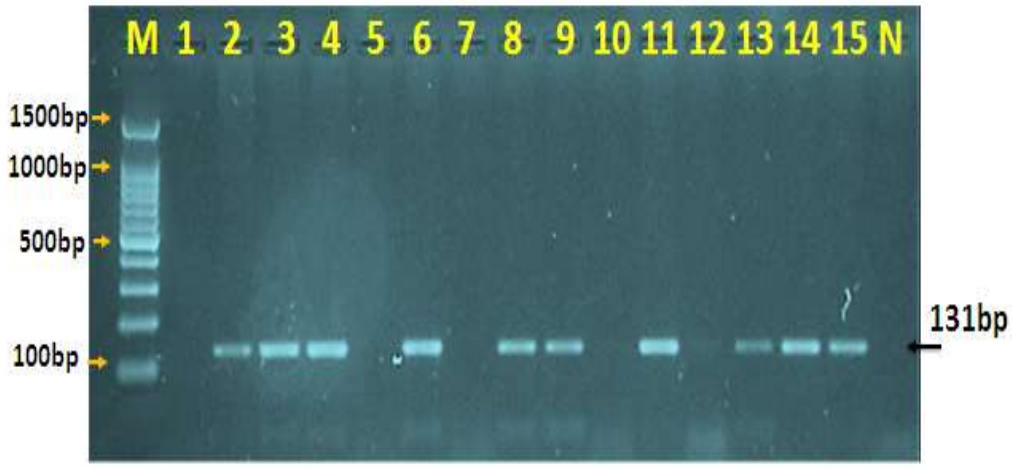

Fig. 1. Agarose gel (1 %) electrophoresis image that showed the Nested PCR product analysis of B1 gene in *Toxoplasma gondii* from human blood samples. Where M: marker (1500-100 bp) Lanes (1-15) were showed some *Toxoplasma gondii* were showed at (131 bp) Nested PCR product and N: non-DNA template negative control samples

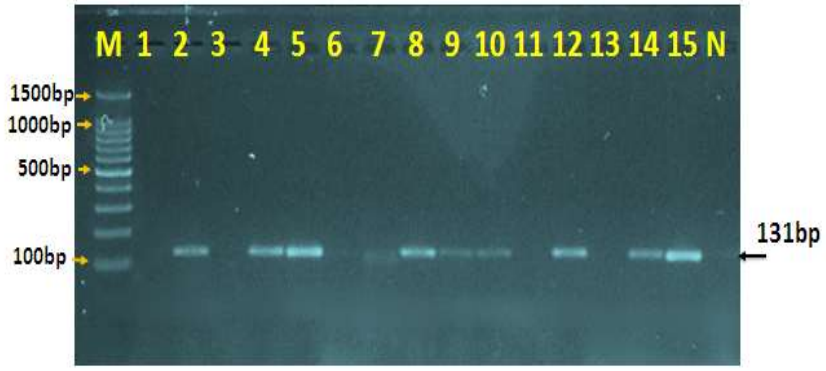

Fig. 2. Agarose gel electrophoresis image that showed the Nested PCR product analysis of B1 gene in *Toxoplasma gondii* from milk samples. Where M: marker (1500-100bp) Lanes (1-15) were showed some *Toxoplasma gondii* were showed at (131bp) Nested PCR product and N: non-DNA template negative control samples

DNA Sequence results

The DNA sequencing method was carried out to *Toxoplasma gondii* genotyping analysis based on surface antigen (SAG3) gene complete nucleotide sequence in local *Toxoplasma gondii* goats and human isolates with related NCBI-Blast related *Toxoplasma gondii* clonal lineages genotype I, II, and III isolates. The phylogenetic tree genetic relationship analysis was showed that the local *Toxoplasma gondii* goats and human isolates (Tg-IQ-Goat.1, Tg-IQ-Goat.2, and Tg-IQ-Goat.4) and The *Toxoplasma gondii* Human isolates (Tg-IQ-human.3, Tg-IQ-human.4, and Tg-IQ-human.5) were showed closed genetic related into *Toxoplasma gondii* genotype III (AF340229.1). The *Toxoplasma gondii* goats' isolates (Tg-IQ-Goat.3 and Tg-IQ-Goat.5) and the *Toxoplasma gondii* human isolates (Tg-IQ-human.1 and Tg-IQ-human.2) were showed closed genetic related into *Toxoplasma gondii* genotype I (AF340227.1). And there are no *Toxoplasma gondii* isolates were related as genotype II (AF340228.1). At total genetic change (0.01) as showed in Figure 3.

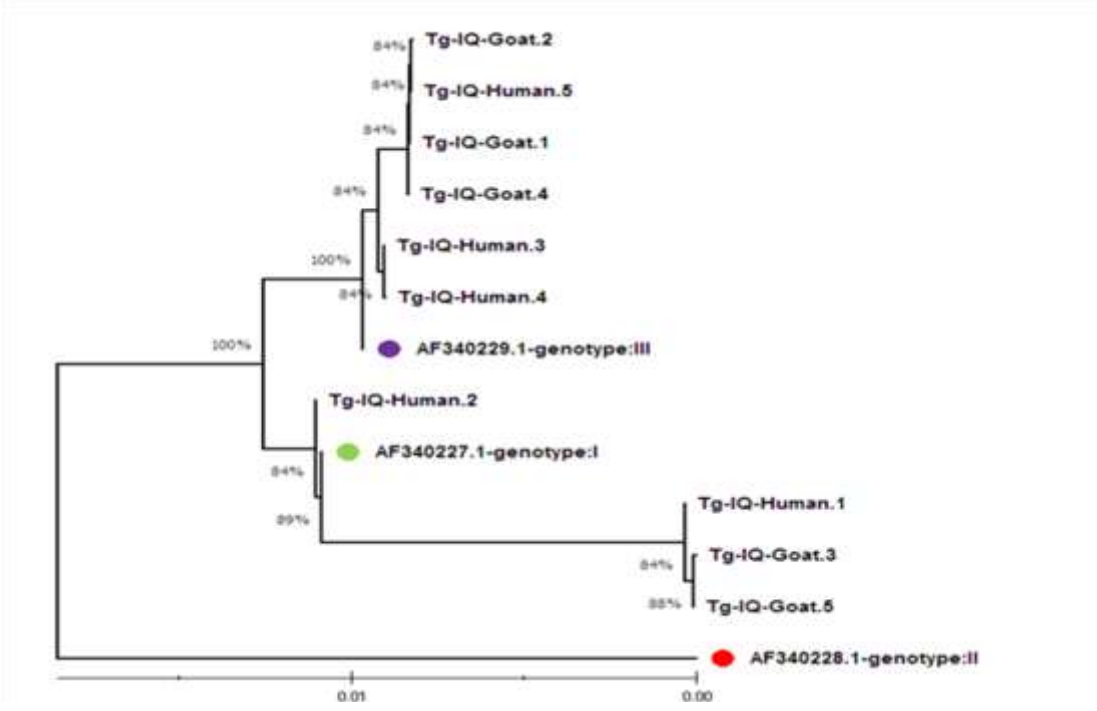

Fig. 3. Phylogenetic tree analysis based on the complete sequence of surface antigen (SAG3) gene in local *Toxoplasma gondii* goats and human isolates that used for genotyping analysis. The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in

(MEGA 6.0 version). The *Toxoplasma gondii* goats isolates (Tg-IQ-Goat.1, Tg-IQ-Goat.2, and Tg-IQ-Goat.4) and The *Toxoplasma gondii* Human isolates (Tg-IQ-human.3, Tg-IQ-human.4, and Tg-IQ-human.5) were showed closed genetic related into *Toxoplasma gondii* genotype III (AF340229.1).The *Toxoplasma gondii* goats' isolates (Tg-IQ-Goat.3 and Tg-IQ-Goat.5) and The *Toxoplasma gondii* Human isolates (Tg-IQ-human.1 and Tg-IQ-human.2) were showed closed genetic related into *Toxoplasma gondii* genotype I (AF340227.1). And there are no *Toxoplasma gondii* isolates were related as genotype II (AF340228.1). At total genetic change (0.01).

The homology sequence identity between *Toxoplasma gondii* goats and Human isolates with NCBI BLAST related *Toxoplasma gondii* genotype I and III isolates were showed genetic homology sequence identity ranged from (98,65-99,9 %). Finally, the local *T. gondii* goats and human isolates were submitted into NCBI Genbank and identified by accession numbers (OL792791, OL792792, OL792793, OL792794, OL792795) for goat and accession numbers (OL792796, OL792797, OL792798, OL792799, OL792800) for human isolates. As showed in Table 5.

Table 5. Homology sequence identity between local *Toxoplasma gondii* gaots and Humans isolates with related to NCBI BLAST *Toxoplasma gondii* genotypes isolates

Toxoplasma gondii isolate No.	Genbank accession number	Homology sequence identity		
		Genotypes	Genbank accession number	Identity, %
Tg-IQ-Goat.1	OL792791	Genotype: III	AF340229.1	99,90
Tg-IQ-Goat.2	OL792792	Genotype: III	AF340229.1	99,90
Tg-IQ-Goat.3	OL792793	Genotype: I	AF340227.1	99,31
Tg-IQ-Goat.4	OL792794	Genotype: III	AF340229.1	99,90
Tg-IQ-Goat.5	OL792795	Genotype: I	AF340227.1	986
Tg-IQ-human.1	OL792796	Genotype: I	AF340227.1	99,28
Tg-IQ-human.2	OL792797	Genotype: I	AF340227.1	99,90
Tg-IQ-human.3	OL792798	Genotype: III	AF340229.1	99,90
Tg-IQ-human.4	OL792799	Genotype: III	AF340229.1	99,90
Tg-IQ-human.5	OL792800	Genotype: III	AF340229.1	99,90

Genotyping is critical for studying the parasite's epidemiology and demographic genetics since various strains of *T. gondii* generate distinct clinical signs in humans and animals (Grigg et al., 2001; Wang et al., 2013). In spite of the majority of strains *T. gondii* detected in animals and humans in Europe belong to one of three clonal lineages (I, II, or III), atypical strains have also been discovered (Ajzenberg, 2010). Type II is more prone to cause persistent illnesses, but type III is more likely to affect domestic animals (Sroka et al., 2017). In France, toxoplasmosis type II was discovered in over 90 % of human congenital cases and also in animal isolates (Ajzenberg et al., 2002; Dardé, 2008).

4. Conclusion

Type III or type I may be more prevalent in Portugal, Spain, and the Slovak Republic, according to research (Fuentes et al., 2001; de Sousa et al., 2006; Turčeková et al., 2013). Genetic characterization confirmed the presence of genotype III in seven samples, genotype I in one

sample, and atypical genotypes in two samples, according to Mancianti et al. (2013). Only type III lineage is distinguished in 14 samples by de Sousa et al. (2006), eleven samples exhibited alleles type III at the 5'SAG2 locus, four samples had SAG3 alleles, and seven samples had GRA6 alleles, seven samples showed allele types I/III at the 3'SAG2 gene, whereas one had allele types I/III at the BTUB locus. Two samples have alleles II/III in the SAG1 gene. Goat muscle with the phylogenetic tree's major branch, sequence KC607824.1, at 100 % identity (Altamemy, Khiry, 2016). This finding was identical to that of Bezerra et al. (2013) who used PCR to match 5 positive samples of goat milk with (DQ779196.1) at 99.9 % identity in North Eastern Brazil. Conferring to the (Mohammed et al., 2015), that genotyping of *Toxoplasma* Type II 9(15.25 %), Type I, and Type III 2(3.38 %) has grown substantially, the genotype I the most fatal type, Type II was the most widespread genotype, type III, an entero-zoonotic parasite discovered for the first time in Iraq in aborted women. There is very little information on the genetic diversity of *T. gondii* in humans in Iraq (A'aiz, 2016a). In the current study, no *Toxoplasma gondii* isolates were related genotype II. This is in disagreement with (A'aiz 2016a; A'aiz 2016b), in Iraq in the Wasit province aborted women and sheep who found in aborted women the genotyping assay revealed that 6.6 % (1/15), 13.3 % (2/15), and 80 % (12/15) of the examined isolates represent the genotypes of I, III, and II respectively. Additionally, a sheep genotyping assay showed that 60 % (6/10), 30 % (3/10), and 10 % (1/10) of the isolates under examination correspond to genotypes II, III, and I, respectively, whereas, the type II looked to be the predominant image of both humans and sheep. These outcomes may be based on nested PCR-RFLP of the SAG2 gene or they could be based on genetic characterizations.

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