

Molecular detection and phylogenetic analyses of *Toxoplasma gondii* from naturally infected sheep in Northern and Central Tunisia

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Abstract

Toxoplasmosis is a worldwide zoonosis caused by the parasitic protozoan *Toxoplasma gondii*. It can infect all warm-blooded vertebrate species and causes abortions and birth defects in pregnant women and pregnant ewes. The objective of this study was to estimate the prevalence of infection with *T. gondii* in sheep meat in the region of Sidi Bouzid (central Tunisia) and Beja (northern Tunisia), the realization of a descriptive study of risk factors and the phylogenetic analyses of *T. gondii*. Neck muscle samples were obtained from 174 ewes and ewe lamb slaughtered in Sidi Bouzid and 150 lambs slaughtered in Beja. DNA was extracted from the samples using the Wizard[®] genomic DNA purification kit. A nested PCR using two pairs of primers (NN 1 and NN2, Tg-NP1 and Tg-NP2) were used to detect infection with *T. gondii*, which was then confirmed by sequencing. Eight *T. gondii* amplicons were sequenced (accession number KT896498) and deposited in GenBank. The *T. gondii* amplicons showed 97–100% identities with GenBank sequences. A phylogenetic tree was then constructed. The nested PCR detected *T. gondii* DNA in 31% of animals tested in Sidi Bouzid and 32% of lambs tested in Beja. No significant difference in the prevalence of *T. gondii* infection was established between the two tested regions. In both regions, no significant variation of the infection depending on age, breed and locality was found.

Keywords: Sheep, lamb, Sidi Bouzid, Beja, Tunisia, *Toxoplasma gondii*, PCR.

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Introduction

The total population of sheep in Tunisia is 6.5 million (Ben Salem *et al.* 2011). They are among the most economically important livestock species in Tunisia and play an important role in the livelihood of resource-poor farmers. Small ruminants face important health problems with high prevalence of several diseases such as brucellosis, foot and mouth disease, border disease and several parasitic infections like fasciolosis (Akkari *et al.* 2011), lungworms (Lahmar *et al.* 2012) and gastrointestinal helminths (Akkari *et al.* 2012). *Toxoplasma gondii* infection is also common in sheep in Tunisia. This parasite is distributed worldwide

infecting all warm-blooded vertebrate species, including humans. In humans, infection during pregnancy can cause congenital toxoplasmosis (Dunn *et al.* 1999). Horizontal transmission occurs frequently after ingestion of cysts in undercooked meat or by consumption of food contaminated with sporulated oocysts (Dubey *et al.* 2014). Recently, infection through goat and sheep raw milk has been confirmed (de Santana Rocha *et al.* 2015; Amairia *et al.* 2016). It has been shown that insemination, using experimentally contaminated fresh semen with different *T. gondii* tachyzoites concentrations was able to transmit infection to sheep, indicating the possibility of a sexual transmission route (Bezerra *et al.* 2014).

Toxoplasma gondii infection occurs asymptotically in most immunocompetent hosts (Długońska 2014). Most infected humans do not show clinical signs, but transmission of *T. gondii* to the foetus can cause serious neurological abnormalities and ultimately death (Flatt & Shetty 2013). In some cases, the infection remains unapparent until the second or third decade of life (Jones *et al.* 2001). A study conducted by World Health Organization ranked foodborne toxoplasmosis as the most important foodborne parasitic disease with 10.3 million cases between 2010 and 2015 (Torgerson *et al.* 2015). In 2001, the overall prevalence of toxoplasmosis in girls living in the North of Tunisia was 58.4%. It rose from 24.5% at 10 years age to 52.1% at 20 years (Bouratbine *et al.* 2001). Prevalence in pregnant women in other African countries varies from 85.4% in Ethiopia (Gelaye *et al.* 2015) to 23.7% in the Governorate of El Fayoum, Egypt (Ghoneim *et al.* 2010).

In sheep, primary infection during pregnancy can lead to foetal resorption during early pregnancy or abortion, causing high economic losses (Johnston 1988; Buxton *et al.* 1991). Infection between 50 and 120 days of gestation leads to abortion, mummified foetuses or the birth of stillborn and weak lambs. After 120 days of gestation, the infection generally leads to apparently normal lambs that can survive the first week, grow normally and are immune to reinfection (Buxton & Finlayson 1986). Sheep become infected by either consuming oocysts contaminated food or vertically. The presence of cats is also a risk factor for animals' infection.

Prevalence of *T. gondii* infection in sheep has been estimated around the world. In North Africa, the lowest molecular prevalence was reported in Tunisia (1.8% in the Governorate of Siliana) (Gharbi *et al.* 2013) and the highest one in Egypt (98.4% in the Governorate of El Fayoum) (Ghoneim *et al.* 2010). In Ghana, 33.2% of the studied sheep were infected (Van der Puije *et al.* 2000), whereas in South Africa, the overall national seroprevalence in sheep was found to be 5.6% by IFAT and 4.3% by ELISA in five studied provinces (Samra *et al.* 2007). In Europe, the seroprevalence values varied between 27.8% and 97% (Opsteegh *et al.* 2010; Leblebici & Yildiz 2014). In Asia, prevalence varied between

17.8% in Kuwait (Alazemi 2014) and 69.9% in Bangladesh (Rahman *et al.* 2014). Toxoplasmosis has also been studied in several Latin American countries such as Colombia (58%), Brazil (53.3%) and Argentina (17.3%) (Perry *et al.* 1978; Hecker *et al.* 2013; Cosendey-KezenLeite *et al.* 2014).

The diagnosis of toxoplasmosis can be made using serological assays, isolation of the parasite in mice or amplification of parasite DNA by different molecular techniques, namely PCR, qPCR and loop-mediated isothermal amplification (LAMP). Although the LAMP technique is a sensitive, easy and least time-consuming method (Wang *et al.* 2013), PCR was used in this trial because the price of LAMP reagents is high.

In Tunisia, despite the importance of toxoplasmosis, few studies have been devoted to this parasite in sheep. The aim of this work was to estimate the molecular prevalence of *T. gondii* in two different regions of Tunisia (North and Central) to analyse the risk factors and to characterize this parasite genetically.

Material and methods

Study area

This survey was carried out in two slaughterhouses located in two Tunisian governorates Sidi Bouzid and Beja (Fig. 1). This choice of the geographical areas was based on the previous study by Gharbi *et al.* (2013) targeting regions with the highest and the lowest seroprevalence rates, namely the Centre and the North of the country, respectively. The district of Sidi Bouzid has a latitude of 35°02' N, a longitude of 9°29' E and a mean altitude of 332 m (Dateandtime.info). Sidi Bouzid is an arid area located in Central Tunisia with a mean annual rainfall of 223 mm. The mean minimal temperature is 8.8°C in January and the mean maximum temperature is 27.6°C in July (Climate data.org). Sidi Bouzid is known as an important sheep production area with 635 000 head in 2013–2014 (Ministry of Agriculture 2014) under semi-intensive conditions relying on grazing in degraded rangeland and the main breeds are the hardy Barbarine and Queue Fine de l'Ouest.

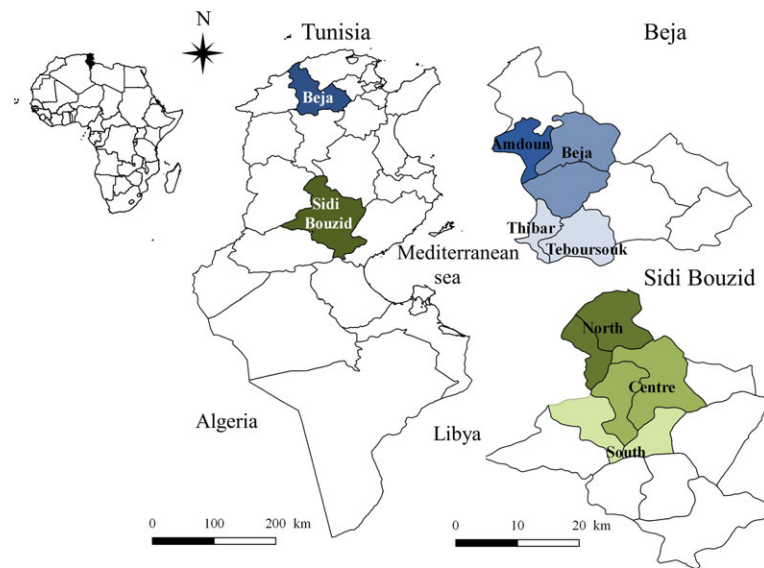


Fig. 1. Study area of *Toxoplasma gondii* in sheep: Northern (Beja) and Central (Sidi Bouzid) Tunisia (North Africa).

The district of Beja has a latitude of $36^{\circ}43' N$, a longitude of $9^{\circ}10' E$ and a mean altitude of 248 m (Dateandtime.info). It is a temperate area located in North Tunisia with a mean annual rainfall of 662 mm. The mean minimal temperature is $9.3^{\circ}C$ in January and the mean maximum temperature is $27.3^{\circ}C$ in August (Climate data.org). The district is also known for semi-intensive sheep production under integrated crop–forage systems with a population of 369 580 heads mainly represented by the dairy Sicilo Sarde, the northern type of the Barbarine breed and several crosses between the autochthonous breeds (Ministry of Agriculture 2014). In both districts, the locality where the animal originated was noted.

Sample collection and preparation

During the 2013 summer season, 174 ewes and ewe lambs from the slaughterhouse of Sidi Bouzid (103 ewes and 71 ewe lambs) and 150 ewe lambs from the slaughterhouse of Beja were sampled for *Toxoplasma gondii* infection. Animals in the slaughterhouses came from different regions of Sidi Bouzid and Beja, and were chosen randomly. Both ewes and ewe lambs were available at this time because it was the culling period. Sheep from Sidi Bouzid were divided into four age groups: less than 1 year,

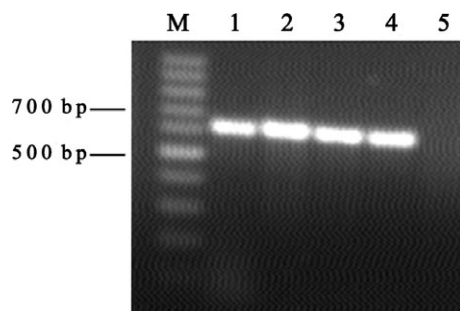
between 1 and 3 years, between 4 and 5 years and finally between 6 and 10 years. Sheep from Beja were divided into four age groups: 5, 6, 7 and between 8 and 12 months. A piece of meat weighing approximately 10 g was cut from the neck of each animal using a disposable scalpel blade, placed in sterile bags and brought in a cooling box to the laboratory of parasitology of the National School of Veterinary Medicine for further processing, storage and analyses. Samples were stored at $-20^{\circ}C$ at maximum for 4 months before being used.

DNA extraction and polymerase chain reactions

DNA was extracted from 0.5 g of each meat sample using Wizard[®] genomic DNA purification kit (Promega, Madison, WI) according to the manufacturer's instructions and then stored at $-20^{\circ}C$ until used. A PCR, targeting the 18S rRNA, was carried out in a mixture consisting of $1\times$ PCR buffer, 2 mmol/L $MgCl_2$, 10 μ mol/L of each primer (1A and 564R), 0.2 mmol/L of each dNTP, 2U Taq polymerase (Vivantis, Chino, CA), 1.5 μ L of DNA template and distilled water to a total volume of 25 μ L (Wang *et al.* 2014) (Table 1). The DNA was amplified using the following program: 5 min denaturation at $94^{\circ}C$, followed by 25 cycles ($94^{\circ}C$ for 50 s and $58^{\circ}C$ for

Table 1. Summary of markers used for universal and *Toxoplasma gondii* PCR

Primer specificity	Target gene	Name	Type	Primers 5'—3'	Product size (bp)	Reference
Universal PCR	18S rRNA	1A	Forward primer	AACCTGGTTGATCCTGCCAGT	–	Wang <i>et al.</i> (2014)
		564R	Reverse primer	GGCACCAGACTTGCCCTC		
<i>T. gondii</i>	ITS1 rDNA	MN1	External forward primer	CCTTTGAATCCCAAGCAAAACATGAG	227	Hurtado <i>et al.</i> (2001)
		MN2	External reverse primer	GCGAGCCAAGACATCCATTGCTGA		
		Tg-NP1	Internal forward primer	GTGATAGTATCGAAAGGTAT		
		Tg-NP2	Internal reverse primer	ACTCTCTCTCAAATGTTCTT		

**Fig. 2.** Agarose gel electrophoresis of universal DNA. M: 100 bp ladder; 1: positive control; 2, 3, 4: positive PCR products.

50 s each) and a final extension at 72°C for 10 min (Fig. 2). Three microlitres of each positive sample were amplified by a nested PCR detecting *T. gondii* DNA (Hurtado *et al.* 2001). Two PCR reactions were performed in two tubes with two programs using successively a set of two primers: external primers (NN1 and NN2) hybridizing with a *T. gondii* and *Neospora caninum* region of the *ITS1* gene and internal primers (Tg-NP1 and Tg-NP2) amplifying a region of 227 bp of *ITS1* gene of *T. gondii* (Hurtado *et al.* 2001) (Table 1) (Fig. 3). The two amplification rounds were carried out in 25 µL of mixture consisting of 1× PCR buffer, 200 µmol/L of each dNTP, 2 mmol/L of MgCl₂, 0.5 U of Taq and 0.1 µmol/L of each external and internal primer, respectively. The DNA was amplified using the following program: 3 min denaturation at 94°C, followed by 30 cycles

(94°C for 30 s, 67°C for 45 s and 72°C for 1 min for the first round and 94°C for 30 s, 53°C for 30 s and 72°C for 30 s for the second round) and a final extension at 72°C for 5 min. PCR products were examined by electrophoresis on 1.5% agarose gel stained with ethidium bromide and visualized under ultraviolet light.

DNA sequencing and phylogenetic analysis

Four *T. gondii* amplicons from animals from Sidi Bouzid and four amplicon from Beja were randomly chosen for genetic analysis. They were purified with the ExoASP-IT[®] (usb) according to the manufacturer's instructions and sequenced in both directions using an ABI 3730xl DNA Analyser (96 capillary type). Sequencing reactions were performed in the DNA Engine Tetrad 2 Peltier Thermal Cycler (BIO-RAD) using the ABI BigDye[®] Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using internal primers (Tg-NP1 and Tg-NP2). The fluorescent-labelled fragments were purified from the unincorporated terminators with the Big Dye XTerminator[®] Purification Kit (Applied Biosystems). An electrophoresis was performed in an ABI 3730xl DNA Analyser (Applied Biosystems).

The chromatograms were evaluated with ChromasPro software (version 1.7.4). MEGA 5.1 software was used to perform multiple sequence alignments

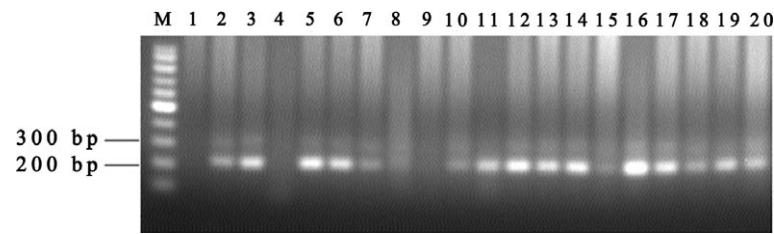


Fig. 3. Agarose gel electrophoresis of *Toxoplasma gondii* infection PCR. M: 100 bp ladder; 1: negative control; 20: positive control; 2, 3, 5, 6, 7, 10, 11, 12, 13, 14, 16, 17, 18, 19: positive PCR products; 4, 8, 9, 15: negative PCR products.

(Tamura *et al.* 2011), and the sequences were compared with the GenBank database by nucleotide sequence homology search made at the network server of the National Centre for Biotechnology Information (NCBI) with BLAST. A phylogenetic tree of *T. gondii* was constructed from the *ITS1* rDNA gene sequences of our amplicons and those available in GenBank.

Statistical analysis

The infection prevalence percentages were compared using Epi Info 6 (Dean *et al.* 2011). A chi-square Mantel–Haenszel test was performed. A probability less than 0.05 was used as a threshold for statistical significance (Schwartz 1993).

Results

One hundred and seventy-four animals from Sidi Bouzid belonged to two breeds, namely Barbarine (56.3%) and Queue Fine de l'Ouest (QFO) (43.7%), and were a mixture of lambs and ewe lambs. One hundred and twenty lambs from Beja sheep were Barbarine (75.3%) and cross-breed (24.7%).

Molecular prevalence of *T. gondii*

The overall prevalence of infection was 31% (54/174) and 32% (48/150) for animals from Sidi Bouzid and Beja, respectively.

There were no statistically significant differences in infection prevalence between the three localities of Sidi Bouzid (Sidi Bouzid North, Centre and South) and between the four age groups

($P > 0.05$). In Beja region, no significant differences were recorded between animals from different breeds, localities and age groups ($P > 0.05$) (Table 2).

Phylogenetic analyses of *T. gondii*

The BLAST comparison of the partial sequences of the *ITS1* rDNA gene (260 bp length) revealed 100% homology between the eight genotypes (GenBank accession no: KT896498). Amplicons from Sidi Bouzid and Beja shared 99.2% homology with the recently reported sequences for the *ITS1* rDNA gene of *T. gondii* from America (KM527503, AY488166, AF252408 and KP999999), Brazil (JF810959, FJ966049 and FJ176233), Norway (KM657806), China (JX456457 and AJ628251), Portugal (AY582110) and Germany (EU025025). They shared 98.5% and 97% homology with two Brazilian isolates (JF810935 and GQ160472, respectively). A phylogenetic tree of *T. gondii* was constructed from the *ITS1* rDNA gene sequences of our amplicons and those available in GenBank (Fig. 4).

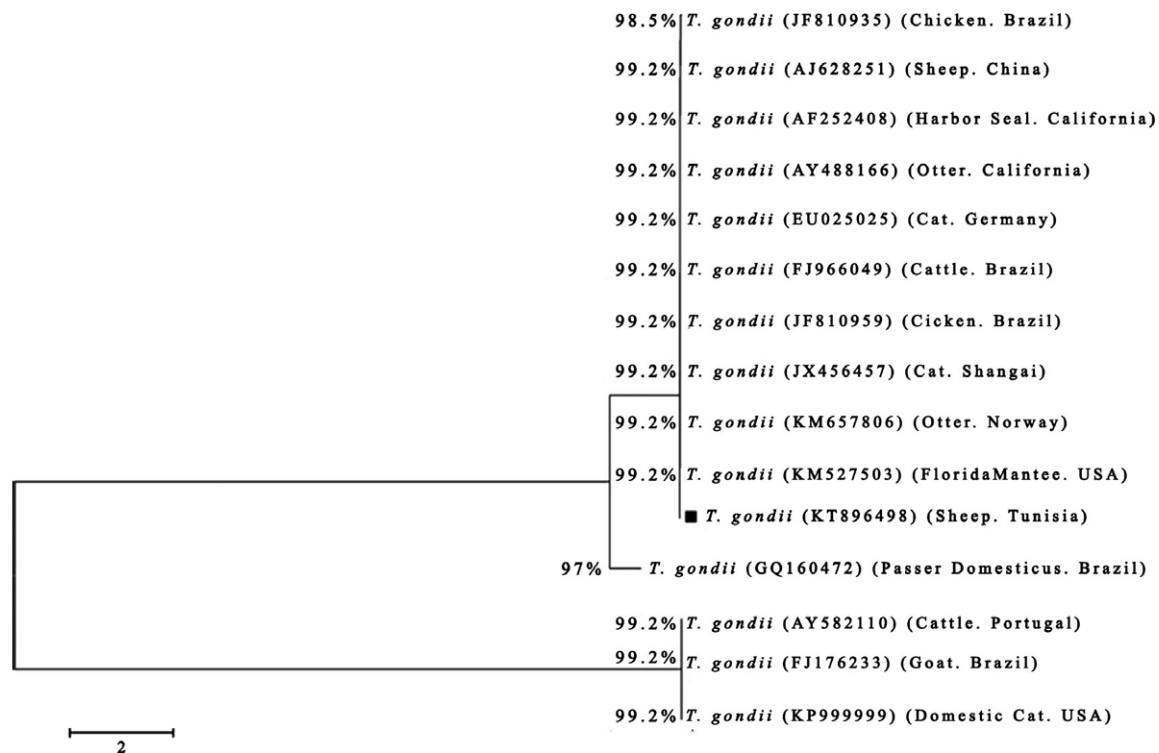
Discussion and conclusion

Toxoplasma gondii is a worldwide-distributed parasite that infects all warm-blooded vertebrate species, including human. Acquired human infection is caused by ingestion of either sporulated oocysts from the environment, water or vegetables or bradyzoites in undercooked meat products. Cats are the key in the circle of *T. gondii* because they are the only domestic hosts that can excrete the environmentally resistant oocysts in their faeces (Dubey 2010).

Table 2. Association between the *Toxoplasma gondii* infection in sheep and different parameters based on PCR

Parameter	Sidi Bouzid		Parameter	Beja	
	Positive/ examined (%)	P value		Positive/ examined (%)	P value
Breed	Barbarine	25/98 (25.5)	Breed	Barbarine	38/113 (33.6)
	QFO	29/76 (38.2)		Cross-breed	10/37 (27.0)
Locality	North Sidi Bouzid	10/34 (29.4)	Locality	Amdoun	22/60 (36.6)
	Centre Sidi Bouzid	40/118 (33.9)		Beja	20/63 (31.7)
	South Sidi Bouzid	4/22 (18.2)		Thibar and Teboursouk	6/27 (22.2)
Age group (years)	≤1	14/44 (31.8)	Age group (months)	5	13/33 (39.4)
	1–3	12/37 (32.4)		6	12/48 (25.0)
	4–5	16/46 (34.8)		7	10/29 (34.5)
	6–10	12/47 (25.5)		8–12	13/40 (32.5)
Overall		54/174 (31.0)			48/150 (32.0)

QFO, Queue Fine de l'Ouest.

**Fig. 4.** Phylogenetic relationships based on the partial nucleotide sequences (260 bp) of *ITS1* rDNA gene for *Toxoplasma gondii* detected in Northern (Beja) and Central (Sidi Bouzid) Tunisia (North Africa) with other isolates deposited in GenBank. The host, the country of origin and the GenBank accession numbers are given in parentheses. Sequence obtained in the present study is indicated with a black square.

The molecular detection of *T. gondii* DNA showed a high prevalence (31% and 32% in Sidi Bouzid and Beja, respectively). These results are

different from those previously reported by Gharbi *et al.* (2013) who investigated three age groups (lambs, yearlings and rams). The *T. gondii* molecular

prevalence in Sidi Bouzid estimated here (31%) was similar to that reported in the same governorate by Gharbi *et al.* (2013) (25.5%; $P = 0.32$). In the northern region, our results showed higher molecular prevalence (32%) compared to the 12.7% found by Gharbi *et al.* (2013) ($P = 0.002$). Higher molecular prevalence was obtained in ewes' meat in Tunis City (50%) (Boughattas *et al.* 2014). A low-molecular prevalence rate of only 5.7% was reported for *T. gondii* in the hearts' apexes of 70 sheep slaughtered in households of Central East Tunisia during the Muslim Feast of Sacrifice (Khayeche *et al.*, 2014). This low prevalence found by Khayeche *et al.* (2014) may be due to the fact that 92.9% of the animals included in the study were aged under 1 year. Results of the current study do not lend support to this hypothesis as we were unable to show differences in prevalence between the different age groups.

On the one hand, molecular prevalence values obtained in some countries were close to results obtained in this trial. In fact, Asgari *et al.* (2010) showed that the prevalence of *T. gondii* infection was 37.5% in sheep from Fars province, Southern Iran. Azizi *et al.* (2014) showed that 38% of sheep in Southwest of Iran were infected with *T. gondii*. In Morocco, the DNA of *T. gondii* was identified by nested PCR in 38% of studied sheep (Azizi *et al.* 2014).

On the other hand, many studies showed higher or lower prevalence values of the infection. For example, in Ireland, the presence of *T. gondii* DNA was detected in diaphragm samples from 3.6% (3/83) sheep (Halová *et al.* 2013), and higher prevalence values (67.7%) were found in Egypt (Ghoneim *et al.* 2010).

In the present study, only 0.5 g meat samples were analysed which could induce an under-estimation of *T. gondii* infection prevalence, the ability of gene amplification by PCR allowed detection of small amounts of DNA. This suggests that PCR is a sensitive method for the diagnosis of toxoplasmosis (Wastling *et al.* 1993). Because of high contamination risks of the two-step nested PCR, drastic measures to prevent contamination were observed along all the PCR runs.

No significant differences were found between animals from different breeds, localities within district and age groups. This result is in concordance with the result obtained by Gharbi *et al.* (2013). This should be due to the presence of the same risk factors in the studied animal population. In Bangladesh, authors found that the age was not a significant risk factor in *T. gondii* infection (Rahman *et al.* 2014). However, Boughattas *et al.* (2014) found a difference in prevalence values between ages. Hecker *et al.* (2013) found a significantly higher proportion of *T. gondii* seropositive animals in females and older sheep. Some authors established an association between *T. gondii* infection and age (Guimarães *et al.* 2013). Other ones found that the most significant factors associated with *T. gondii* infection in sheep were age and gender (Cosendey-KezenLeite *et al.* 2014).

Even if climate plays an important role in the conservation of oocysts of *T. gondii*, because occurrence locations of *T. gondii* infection are related to higher temperature, lower precipitation and lower altitude compared to the absence locations of *T. gondii* (Kantzoura *et al.* 2013), this study did not reveal a difference in infection prevalence between the two regions. Although some authors found differences in prevalence values according to age and region, results of this survey did not bring out these differences. This may be due to the sampling. In fact, the small number of animals did not detect statistically significant differences related to some risk factors.

Infections caused by *T. gondii* were confirmed in ewes using a nested PCR followed by sequencing of the *ITS1* region of the parasites' rDNAs. All sequences shared 97–99% identity with sequences from other strains present in GenBank. In fact, the two *T. gondii* sequences were identical to many ones, namely America, Norway, China, Brazil and Germany, and had a high genetic homology with sequence from Brazil. *Toxoplasma gondii* from sheep showed high genetic diversity. In Tunisia, sheep meat is highly infected by *T. gondii* representing though a major risk for the consumer especially for pregnant women who should be informed about this risk and the specific prevention majors that they should practice (avoid manipulation of lamb meat

and consumption of undercooked meat). Further studies are needed to improve our knowledge on different genotypes of *T. gondii* that infect Tunisian sheep population.

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Conflicts of interest

The authors declare no conflicts of interest in relation to this work.

Contributions

M Rouatbi, M Gharbi and M Rekik conceived and designed the experiments. M Rouatbi, S Amairia and MR Rjeibi performed the experiments. M Rouatbi, Y Amdouni and S Sammoudi involved in the collection of samples. M Rekik, M Gharbi and M Rouatbi wrote the manuscript.

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