ICARDA

Science for resilient livelihoods in dry areas

Co-infection analysis

Analysis of co-infection with *Neospora caninum* and *Toxoplasma gondii* in Tunisian sheep

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Background

Toxoplasma gondii and *Neospora caninum* are two closely related protozoan parasites that are distributed worldwide (Panadero et al., 2010). They have similar life cycles with different definitive hosts, cats and dogs for *T. gondii* and *N. caninum,* respectively. Both parasites cause significant diseases and economic losses in the farming industry, with *T. gondii* primarily causing abortion and foetal abnormalities in sheep and goats, and *N. caninum* in cattle (Dubey, 2003). The majority of the studies carried out aimed to estimate the seroprevalence or the molecular prevalence of the two parasites in ruminants.

Co-infection with *N. caninum* and *T. gondii* has been detected in several species worldwide. It has been detected in rabbits (Hughes et al., 2008), domestic camelids (Pardini et al., 2008), cats (Hamidinejat et al., 2011 ; Thomasson et al., 2011), cattle (Huong et al., 1998 ; Ooi et al., 2000 ; Bae et al., 2000 ;Yildiz et al., 2009 ; Panadero et al., 2010; Gharekhani, 2014 ; Razmi et al., 2017), sheep (Romanelli et al., 2007 ; Panadero et al., 2010) and yaks (Liu et al., 2008).

The similarities and differences between these two parasites prompted us to study the co-infection of hosts. Our study was carried out to investigate the co-infection by *T. gondii* and *N. caninum* in meat and semen of Tunisian sheep.

Materials and Methods

Study area and samples collection

Meat samples

A total number of 198 meat samples (neck muscles) were collected from ewes slaughtered in the regional slaughterhouse of Béja. Béja district is a mountainous area in northern Tunisia with a mean altitude of 248 m, an average humidity of 22%, and a temperatures ranging between 15 and 42°C throughout the year (https://www.wolframalpha.com). The Sheep population in the district was estimated to 357,560 heads. The sheep belong to three breeds: Barbarine (59.6%), Noire de Thibar (20.7%) and Crossbred animals (19.7%).

Semen samples

Samples were collected during summer 2015 from four farms located in four Tunisian governorates (Jendouba, Kairouan, Zaghouan and Ben Arous) totaling 92 adult rams of different breeds and age using an artificial vagina and kept in identified sterile tubes for molecular study. All rams included in this study were used for natural mating. Data concerning altitude, annual rainfall, temperature and moisture in the four study regions are reported in Table 1.

Table 1: Charectristics of the studied regions

District	Region	Number of sampled rams	Bioclimatic zone	Mean altitude (m)	Annual temperature (°C)		Mean annual precipitations (mm)
					Mean	Min-Max	
Jendouba	Bousalem	19	Sub-humid	141	18	9.8-27.9	504
Kairouan	Oueslatia	9	Semi-arid	383	17.5	10.4-27.9	293
Zaghouan	Saouaf	42	Semi-arid	158	16.8	9.9-26.7	447
Ben Arous	El Mohammedia	22	Semi-arid	65	16.4	10.1- 26.5	455

DNA extraction

From meat samples

For each animal, a piece of meat sample (50 mg) was collected then washed with sterile distilled water and centrifuged at 16.000g for 6 min. DNA was extracted using Wizard® Genomic DNA purification kit (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions then stored at -20°C until analysed.

From semen samples

For each animal, 500 µl of semen was centrifuged at 13,000 g for 20 min. After discarding the supernatant, DNA was extracted using Wizard® Genomic DNA purification kit (Promega, Madison, WI, USA) according to the manufacturer's instructions then stored at −20°C until analysed.

Polymerase chain reaction (PCR)

All the samples were subjected to universal PCR to evaluate the DNA extraction success and evaluate the quality of the DNA. A set of primers (1A and 564R) targeting the hypervariable regions V1–V3 coding for 18S rRNA was used (Wang et al., 2014) (*Cf*. Table 2). PCR reactions were prepared with a mix consisting of 1x PCR buffer, 2 mM $MgCl_2$, 10 µM of each primer (1A and 564R), 0.2 mM of each dNTP, 2 U Taq Polymerase (Vivantis, Chino, California, USA), 1.5 µL of DNA template and distilled water for a total volume of 25 µL.

PCR was performed in an Applied Biosystem 2700 thermocycler with the following conditions: initial denaturation at 94°C for 5 minutes followed by 25 cycles (94; 59 and 72°C for 50 seconds each) and a final extension at 72°C for 10 min. (Wang et al, 2014). The amplicons were visualized by electrophoresis in 1% (w/v) agarose gel mixed with 0.05% ethidium bromide in TAE buffer.

A nested PCR was performed with four oligonucleotides to amplify a 279 bp *N. caninum* DNA fragment belonging to ITS1 gene and coding for the 18S-5.8S rRNA according to the protocol of Buxton et al. (1998).

A primary PCR was performed with 0.15 µM of each primer (NN1: 5'- TCAACCTTTGAATCCCAA -3' and NN2: 5'- CGAGCCAAGACATCCATT -3') in a total reaction volume of 25 µl consisting of 3 µL of DNA sample, 1x PCR buffer, 2.5 mM $MgCl_2$, 200 µM dNTP each, 1 U of Taq Polymerase. The amplification was carried out in a thermocycler under the following cycling conditions: 95°C for 5 min, followed by 26 cycles (denaturation at 94°C, annealing at 48°C and extension at 72°C for 1 min each) and a final extension at 72°C for 5 min. We added 2 µl of the amplicons as template for the nested PCR using the same mixture as primary PCR and 0.2 µM of each inner primer (NP1: 5'- TACTACTCCCTGTGAGTTG -3' and NP2: 5'- TCTCTTCCCTCAAACGCT -3') and amplified for 26 cycles of 60 sec at 94°C, 30 sec at 48°C and 30 sec at 72°C, with final extension cycle increased to 5 min. A positive and negative controls consisting of *N. caninum* DNA and nuclease-free water were added for each reaction, respectively. The amplicons were visualized by electrophoresis in 1.8% (w/v) agarose gel mixed with 0.05% ethidium bromide in TAE buffer.

The 114 base-pairs of *T. gondii* B1 gene was amplified via PCR using two primers: a forward one: B22 (5'- AACGGGCGAGTAGCACCTGAGG AGA-3') and reverse one: B23 (5'- TGGGTCTACGTCGATGGCATGAC AAC-3').

The PCR reaction was carried out in a final volume of 25 µl consisting of 10×PCR buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 0.6 mg BSA, 10 pmol of each primer, 1U Taq polymerase and 6 µl of

sample DNA. The PCR amplification profile involved two initial preliminary steps of 2 and 6 min at 50°C and 95°C, respectively followed by 40 cycles of 30 s at 94°C, 30 s at 57°C and 1 min at 72°C adding 1 s/ cycle. A final elongation step of 7 min at 72°C completed the PCR run. PCR products were resolved in 3% agarose gel and visualized under UV light (Boughattas et al., 2014).

Statistical Analyses

Differences in infection prevalences were tested by the chi-square Mantel-Haenszel test with Epi Info 6 software ($p<0.05$) threshold (Schwartz, 1993).

Results

Co-infection in meat samples

Among the 198 analysed meat samples, only 8/198 animals were positive for both, *T. gondii* and *N. caninum*, corresponding to an overall prevalence of 4.04%. Statistically significant differences were observed according to age (p = 0.002), breed (p = 0.009) and locality (p = 0.003). The highest prevalence (10 ± 7%) was observed in sheep over 1 year of age.
The co-infection rate was significantly higher in sheep from Téboursouk and Thibar (12 ± 0.88; p = 0.003). Compared to Barbarine animals (2.5 ± 2.8) the prevalence of co-infection was higher in Noir de Thibar animals (12.2 ± 10; p = 0.009) (Table 2).

Table 2: Association between the prevalence of co-infection *with Neospora caninum* and *Toxoplasma gondii* and risk factors in meat samples

Risk factors	***Parameters***	***Positives/examined (%± SE)***	***P value***
Age (an)	<1	1/126 (0.8 ± 1,5)	0.002*
	≥1	7/72 (10 ± 7)	
Breed	Barbarine	3/118 (2.5 ± 2.8)	0.009*
	Noire de Thibar	5/41 (12.2 ± 10)	
	Cross breed	0/39 (0)	
Locality	Amdoun	1/56 (1.8 ± 0.35)	0.003*
	Téboursouk and Thibar	7/59 (12 ± 0.88)	
	North Béja	0/32 (0)	
	South Béja	0/51 (0)	

*SE=standard error, * = p<0.05*

Co-infection in semen samples

Among the 92 analyzed semen samples, only 6/92 animals were positive for both, *T. gondii* and *N. caninum*, corresponding to an overall prevalence of 6.52%. The present study highlighted the association between the locality and co-infection prevalence with higher infection rates observed in rams from Bou Salem (15.8 ± 16.4; p = 0.04) (Table 3).

Table 3: Association between the prevalence of co-infection with *Neospora caninum* and *Toxoplasma gondii* and risk factors in semen

Risk factors	***Parameters***	***Positives/examined (%± SE)***	***P value***
Number of mating seasons	≤ 2	1/49 (2 ± 0.4)	0.06
	> 2	5/43 (11.6 ± 9.6)	
Breed	Barbarine	6/82 (7.3 ± 5.6)	0.37
	Other	0/10 (0)	
Locality	Bou Salem	3/19 (15.8 ± 16.4)	0.04*
	Oueslatia	0/9 (0)	
	Saouaf	0/42 (0)	
	El Mohammedia	3/22 (13.6 ± 14.3)	

*SE=standard error, *=p<0.05, ***=p<0.001*

Discussion

In this study we investigated the co-infection by *N. caninum* and *T. gondii* in meat and semen samples.

Co-infection by *N. caninum* and *T. gondii* has been detected in several species worldwide. It has been detected in rabbits (Hughes et al., 2008), domestic camelids (Pardini et al., 2008), cats (Hamidinejat et al., 2011 ; Thomasson et al., 2011), cattle (Huong et al., 1998 ; Ooi et al., 2000 ; Bae et al., 2000 ;Yildiz et al., 2009 ; Panadero et al., 2010; Gharekhani, 2014 ; Razmi et al., 2017), sheep (Romanelli et al., 2007 ; Panadero et al., 2010) and yaks (Liu et al., 2008).

Several studies have been carried out to study the co-infection in cattle with a co-infection rate varying between 0.5 and 3.68% (Huong et al., 1998; Ooi et al., 2000; Bae et al., 2000; Panadero et al., 2010; Gharekhani, 2014; Razmi and Barati, 2017).

In small ruminants, few studies have been carried out. In goats, as far as we know, no study has been carried out, whereas for sheep two studies were performed in Spain and Brazil that estimated the prevalence of co-infections in sheep to 3.68 and 5.2%, respectively (Romanelli et al., 2007; Panadero et al., 2010).

In the present study, co-infection by *N. caninum* and *T. gondii* was detected in 4.04 and 6.52% of the analyzed meat and semen samples, respectively. The prevalence of co-infection estimated in our study is in the same range as the published studies (Romanelli et al., 2007; Panadero et al., 2010). These low prevalence could be explained by the sensitivity of small ruminants to infection by *N. caninum* and *T. gondii* than other species (González-Warleta et al., 2008).

In semen samples, the rate of co-infection was significantly higher in Bou Salem and Mouhamadia (15.8 and 13.6% respectively) (p = 0.04) in comparison with other districts. This could be attributed to the climatic conditions in these two localities characterized by high rainfall and relatively low temperature which enhances oocysts' viability in the environment (Yan et al., 2016). There was no statistically significant difference in prevalence; according to breed and number of **mating seasons**, the small sample size could explain the absence of any difference.

In Meat samples, the co-infection rate was significantly associated with age (p=0.002), breed (p=0.009) and locality (p=0.003). The higher prevalence was observed in Noire de Thibar (12.2 ± 10) compared to Barbarine breed (2.5 ± 2.8).

The present study highlighted the association between age and infection prevalence with lower infection rates observed in younger sheep compared to older animals. This association with age

indicates the presence of horizontal transmission by ingestion of sporulated oocysts (Figliuolo et al., 2004). This route of transmission predisposes to a high risk of abortion associated with infection with *N. caninum* and *T. gondii* in ruminants.

Further studies are needed on bigger number of animals to explain the presence of differences between the age categories and the breed.

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