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Detection of *Toxoplasma gondii* and *Sarcocystis tenella* in indigenous Cornigliese sheep in Italy using serological and molecular methods☆

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ABSTRACT

The aim of the present study was to determine seroprevalence for *Toxoplasma gondii* by meat juice ELISA and evaluate the presence of *T. gondii* and *Sarcocystis* spp. within host tissues by histology, PCR and in vitro isolation, in the indigenous Cornigliese sheep breed in northern Italy. Seventeen out of 24 (70.8%) sheeps were positive for *T. gondii* by meat juice ELISA. Twenty sheep (83.3%) were positive by PCR for *T. gondii*, while 24/24 sheep (100%) were positive by PCR for *Sarcocystis* spp. Tissues cysts compatible with *Sarcocystis* spp. were visible in all animals on histology. PCR confirmed the presence of *T. gondii* after three weeks of in vitro culture on Vero cells in only one sample. Genotyping of *T. gondii* by RLFP with 5 markers showed a predominance of genotypes II/III. Sequence analysis of *Sarcocystis* spp. showed only the presence of *Sarcocystis tenella*.

T. gondii and *S. tenella* are present in a high percentage of Cornigliese sheep in northern Italy. Future studies should concentrate upon the reproductive and economic effects of these parasitic infections, in light of the necessary conservation of this local, indigenous sheep breed.

1. Introduction

The farming of indigenous sheep breeds is an important economic and cultural activity in many parts of the Mediterranean and genetic diversity offered by these breeds plays an important role in production and resistance to disease (Worwa et al., 2010; Billinis et al., 2012; Salamon et al., 2014). The Cornigliese sheep breed is indigenous to northwestern Italy. It is transhumant and is known for the excellent dye uptake of its wool, for the quality of its milk for cheese production and for its meat (Ceccobelli et al., 2015). It is currently considered at risk of extinction and counts approximately 1600 head (Ceccobelli et al., 2015).

Toxoplasma gondii and *Sarcocystis* spp. are coccidian parasites that can cause reproductive failure in sheep (Dubey and Lindsay, 2006). Acute infection by *Sarcocystis tenella* (syn. *Sarcocystis ovicanis*), one of the most pathogenic species, has also been implicated in severe forms of encephalomyelitis and respiratory disease in young sheep (Schock et al., 2012), while chronic infections can lead to reduced quality of wool, meat and milk (Heckeroth and Tenter, 1999). Furthermore, *T. gondii* is considered one of the most important and worldwide distributed food-borne parasitic zoonoses and ingestion of undercooked lamb or raw dairy products has been identified as a risk factor for human disease (McAllister, 2005).

The aim of the present study was to determine seroprevalence for *T. gondii* with meat juice Enzyme Linked Immunosorbent Assay (ELISA) and evaluate the presence and genotype of *T. gondii* and *Sarcocystis* spp. within host tissues by histology, PCR and in vitro isolation, in Cornigliese sheep.

2. Materials and methods

2.1. Animal sampling

Hearts were obtained from 24 adult Cornigliese sheep from three herds located in the province of Parma. Samples were obtained at slaughter between March and April, 2014 and hearts were stored at 4 °C and processed within 24 h of slaughter.

2.2. Histology

Tissue samples from collected hearts were fixed in a buffered 7% formalin solution for 48 h. The samples were then cut into

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Table 1
Results for *Sarcocystis* spp. and *T. gondii* prevalence in the 24 samples of Cornigliese sheep analyzed. The table summarizes results obtained with the ELISA test, culture, PCR and histology.

Sample	Elisa test	In vitro (Vero cells)	PCR		Histological exam for *Sarcocystis* spp.
			529 bp	18S rRNA	
1	–		+	+	+
2	+		+	+	+
3	+		+	+	+
4	+		+	+	+
5	+		+	+	+
6	–		+	+	+
7	+		+	+	+
8	Doubt		+	+	+
9	–		–	+	+
10	–		+	+	+
11	–		+	+	+
12	Doubt		+	+	+
13	+		+	+	+
14	+		–	+	+
15	+		+	+	+
16	+		+	+	+
17	+		–	+	+
18	+		+	+	+
19	+		–	+	+
20	+	–	+	+	+
21	+	–	+	+	+
22	+	+	+	+	+
23	+	–	+	+	+
24	+	–	+	+	+
No. of positive	17/24	1/24	20/24	24/24	24/24

0.5 cm-thick sections, dehydrated with serial dilutions of ethanol and xylene and processed into paraffin. After 24 h of incubation at 56 °C, the slides were rehydrated and stained with hematoxylin and eosin for morphological examination.

2.3. *Meat juice serology for* T. gondii

Meat juice was obtained by freezing approximately 50 g of myocardial tissue, sampled from the apex, at −20 °C for 18–24 h, followed by defrosting at room temperature. The ELISA test was carried out according to the manufacturer's instructions for meat juice (Id Screen® toxoplasmosis indirect multi-species, Idvet, Switzerland). Samples were considered positive with a Sample/Positive control percentage (S/P%) calculated from the optical densities measured at 450 nm (S/P% = OD sample/OD positive control × 100) greater than 50%. Samples with S/P% ≤ 40% were considered negative, between 40% and 50% were considered doubtful and ≥50% were classified as positive.

2.4. *DNA extraction and identification of* T. gondii *and* Sarcocystis *spp. by polymerase chain reaction*

DNA was extracted from all the samples. Briefly, hearts were surface-sterilised by submersion in 70% ethanol. Using sterile scalpel and forceps, 50 g of tissue were sampled and blended with the addition of 125 ml of PBS. 200 μl of blended tissue were used for the DNA extraction carried out using a commercial kit (PureLink™ Genomic DNA Mini Kit-Invitrogen).

T. gondii infection was confirmed by a nested-PCR targeting a 529 bp region, using the primers TOX4 and TOX5, as described by Homan et al. (2000). The sequence is a 200- to 300- fold repetitive fragment in the genome of *T. gondii*; and has been shown to be more sensitive than the 35-copy B1 gene and is able to discriminate *T. gondii* from that of other parasites. Amplification was performed by 7 min incubation at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C and a final 10 min incubation at 72 °C. The products were fractionated on a 2% agarose gel, stained with ethidium bromide and visualized by UV transillumination.

For *Sarcocystis*, Restriction Fragment Length Polymorphism (RFLP) was used for the identification of the parasite, through amplification of the 18S rRNA gene target, followed by a digestion with the restriction enzyme BseD (Ho et al., 1996).

2.5. *In vitro isolation and PCR of* T. gondii *on Vero cells*

In vitro isolation of *T. gondii* was carried out on ELISA-positive samples, according to Zintl et al. (2009), with slight modifications. Briefly, 50 g of heart muscle were collected and blended with 125 ml of PBS, kept warm at 37 °C, then 250 ml of a pepsin solution (pH 1.1–1.2) was added, as suggested by Dubey (1998). This solution was maintained a 37 °C in a shaking water bath for one hour. Two-hundred-fifty milliliter of the homogenate were filtered with two gauzes and centrifuged at 1200 × *g* for 10 min. The supernatant was poured off and the pellet resuspended with 20 ml of sterile PBS (pH 7.4); the pH was neutralized with around 10 ml of sodium bicarbonate (1.2% w/v pH 8.3–8.4). The homogenate was centrifuged again as done before and the pellet resuspended with 10 ml of a saline solution containing penicillin, streptomycin, and Fungizone® (2%) (Gibco® 100x antibiotic–antimycotic). One milliliter of this solution was put inside a 75 cm^2 T-flask seeded with Vero cells with a confluence around 80% cultivated in RPMI 1640 (Euroclone spa, Italy) supplemented with 10% FBS (Euroclone spa, Italy). Visual inspection of seeded material was carried out on all samples. For five samples, after three hours incubation at 37 °C, 5% CO_2, the homogenate was poured off and new fresh medium with a lower percentage of FBS (2%) added to the infected monolayer. The medium was changed after 24 h and then after 3–5 days for the following three weeks as suggested by Zintl et al. (2009). At three weeks post-seeding, DNA was extracted as described above and PCR carried out for *T. gondii.*

2.6. T. gondii *genotyping and sequence analysis for* Sarcocystis *spp.*

Seven samples positive for the 529 bp region of *T. gondii* were genotyped by a multiplex multilocus nested PCR–RFLP to identify