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ORIGINAL PAPER

Epidemiological updates and economic losses due to *Taenia hydatigena* in sheep from Sardinia, Italy

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Abstract The aim of this study was to investigate the epidemiology and transmission of *Taenia hydatigena* in sheep and dogs from Sardinia and the economic estimation of losses due to this metacestodosis in lambs. A total of 7781 Sarda breed lambs were examined at abattoirs for the detection of *Cysticercus tenuicollis* or necrotic–haemorrhagic tracks of their migration. Morphological and molecular identification of parasites was carried out. Individual faecal samples from 300 dogs were examined for copromicroscopic investigations and coproELISA assay. An overall prevalence of 14.6 % for *T. hydatigena* cysticercosis was found in the examined lambs. In total, 10,807 parasitary tracks were found, with an abundance of 1.39 and an average intensity of 9.52. The molecular analysis of the isolates showed an overall pairwise nucleotide divergence for the *CO1* and *ND1* was of 0–3.1 and 0–3.3 %, respectively. Low intra- and interspecific variation was recorded for *C. tenuicollis* isolates used in this study which suggested the absence of differentiation. Microscopic examination of dog faeces showed a total prevalence of 31.3 % for endoparasites in the examined samples (94/300). Taeniid eggs were found in 8.3 % of the dogs. The results of the monoclonal antibody ATH4 ELISA test showed a prevalence of 11 % (33/300) for *T. hydatigena* coproantigens. The total economic costs related to cysticercosis amounted to almost € 330,000. The prevalence of *C. tenuicollis* in 14.6 % of 30–40-day-old lambs highlights the high parasitic pressure by *T. hydatigena* in the territory of Sardinia, Italy.

Keywords *Taenia hydatigena* · *Cysticercus tenuicollis* · Cysticercosis · Sheep · Dogs · Sardinia

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Introduction

Taenia hydatigena is a cosmopolitan and widespread parasite of canids that can infect a wide range of mammals with its larval stage which is historically referred to as *Cysticercus tenuicollis* (Murrell et al. 2005). The mature tapeworm lays eggs which pass out in the faeces of the host and are ingested by intermediate hosts during grazing. Ingested eggs hatch in the small intestine and later migrate to reach the liver and rarely in other organs such as the lungs and kidneys of the intermediate hosts (Scala and Marrosu 1997). In addition, mature cysticerci are usually found on the omentum, mesentery, peritoneum and, less frequently, on the pleura and pericardium. Migrating larvae can be found mostly in the liver parenchyma within 7–10 days and may cause traumatic hepatitis in young animals (Sweatman and Plummer 1957; Blažek et al. 1985). Most infections are chronic and asymptomatic and are not usually identified until slaughter (Christodoulopoulos et al. 2008). In heavy infections, the migrating larvae could produce intensive destruction of liver parenchyma with eosinophilic infiltration and severe inflammation that could be fatal (Trees et al. 1985; Edwards and Herbert 1980; Scala et al. 2014). In addition to constituting an animal health problem,

the parasitosis is a source of economic loss for the meat industry (Abidi et al. 1989; Scala et al. 2014).

Sardinia is an island territory of Italy, where 3,228,878 dairy sheep are raised using traditional methods (Ministero della Salute 2013). It could therefore constitute a useful model for the study of the epidemiology and transmission of *T. hydatigena* as well as that of other metacestodosis as previously done in Australia (Dew 1935; Thompson and Jenkins 2014).

Extensive grazing, the presence of shepherd dogs and numerous stray dogs and uncontrolled home slaughtering, along with the improper disposal of carcasses, are all factors that could favour the persistence of *T. hydatigena* as well as other important metacestodosis such as hydatidosis and coenurosis caused by *Echinococcus granulosus* and *Taenia multiceps*, respectively (Scala et al. 2006; Varcasia et al. 2009; Dore et al. 2014).

Reliable data on the epidemiology of *T. hydatigena* in Sardinia are scant and dated (Scala and Marrosu 1997), except for a recent report of acute fatal cysticercosis in lambs (Scala et al. 2014). The current study investigates the epidemiology and transmission of *T. hydatigena* in sheep and dogs in Sardinia by combining direct livestock examination at abattoirs, survey of dogs' coproantigen and also an economic estimation of losses due to this metacestodosis in lambs.

Materials and methods

Epidemiological survey and estimation of condemnation losses

A total of 7781 sarda breed lambs aged between 30–40 days were examined at *post-mortem* in 12 abattoirs of the four historical provinces of Sardinia (Sassari, Nuoro, Oristano and Cagliari), Italy, during the period from February to August 2014. The lambs, born and raised on the island, were part of 137 lots consisting of a variable number of lambs ranging from 7 to 331 individuals (M 57.21, SD 57.22). All lambs were naturally milked and then kept with their mothers at grazing for the total duration of their life.

At post-mortem the lungs, liver, omentum, mesentery and peritoneum were examined for the presence of *C. tenuicollis* or necrotic–haemorrhagic tracks of the migrating parasites. The right and left parts of diaphragmatic side, right and left parts of the visceral side and the Spigelian lobe of the liver were carefully inspected. The number and position of the parasites and their tracks were electronically recorded, and cysticerci in various degrees of development were removed from the liver and other sites for microscopical identification and further biomolecular investigations. Morphological identification of the parasites was performed according to the keys reported by Rostami et al. (2013). Molecular identification was performed in order to confirm morphological diagnosis particularly in situations when the identification of parasitic tracks proved difficult.

Molecular analysis

A total of 23 putative *C. tenuicollis* isolates collected from sheep livers during slaughter at Cagliari, Sassari and Nuoro Province abattoirs were included in this study. Genomic DNA was extracted from individual cysticerci using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany). Extracted DNA was used either immediately for PCR amplification or stored at −20 °C until used. Using 10–20 ng of template DNA, fragments within the *CO1* and *ND1* mitochondrial genes were amplified using primers JB3 (5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and JB4.5 (5′-AAAGAAAGAACATAATGAAAATG-3′) and primers JB11 (5′-AGATTCGTAAGGGGCCTAATA-3′) and JB12 (5′-ACCACTAACTAATTCACTTTC-3′), respectively, as described by Bowles and McManus (1993a, b) and Bowles et al. (1992).

Polymerase chain reaction (25 μl) was performed in 10 mM Tris–HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 250 μM of each dNTP, 25 pmol of each primer and 2 U *Taq* polymerase (Roche) under the following cycling conditions: 94 °C, 5 min (initial denaturation), followed by 30 cycles of 94 °C, 30 s (denaturation), 55 °C, 30 s (annealing), 72 °C, 30 s (extension), followed 72 °C for 5 min (final extension). PCR products were purified with a commercial kit (High Pure PCR product purification kit, Roche). PCR amplicons were purified using an Exo SAP-IT kit (Amersham Biosciences) and sequenced through an external service (MWG Eurofins), using the PCR primers. Nucleotide sequences were compared with those available in GenBank® (ncbi.nlm.nih.gov) using basic local alignment search tool (BLAST) analysis (National Centre for Biotechnology Information, ncbi.nlm.nih.gov). Phylogenetic analysis was carried out in MEGA version 6 (Tamura et al. 2013). Genetic distances for the *CO1* and *ND1* protein sequences were calculated using Kimura 2-parameter index with 1000 bootstrap replications to construct a neighbour-joining phylogenetic tree. Sequences of *T. multiceps* (*CO1* DQ309767 and *ND1* AY669089) from Sardinia were used as out-groups.

Economic losses

The estimation of the economic losses caused by *C. tenuicollis* was evaluated by collating the number of condemned lambs' livers, the official number of slaughtered animals provided by the Regional Government of Sardinia and the average price of lamb meat (€ 7.00/kg). Interestingly, lamb liver in Sardinia retails for the same price as the whole lamb carcass, and they are usually sold together for traditional and culinary purposes. The formula used to estimate total gross economic losses due

to condemnation per year in the entire Sardinian territory was as follows:

$$ACLC = (NSL \times PLC)/100 \times LC$$

where

ACLC	Annual cost of liver condemned
NSL	Number of slaughtered lambs per year in Sardinia (1,102,957)
PLC	Percentage of liver condemnation (found in the present survey)
LC	Mean price of lamb liver in Sardinian markets [(€ 7.00/g 1000)×280 g=€ 1.96], where 280 g is the average weight of a (Sarda breed) lamb liver.

In addition, the cost of the disposal of condemned offal as recommended by the government, which in Sardinia amounts to € 0.40 per kg, was included in the estimation of economic losses caused by *C. tenuicollis*. Obtained data were processed with software MiniTab®16.2, while abundance and average intensity values were obtained according to methods described by Bush et al. (1997).

Coproantigen survey of farm dogs

Individual faecal samples from 300 dogs from Sardinia were retrieved rectally when possible or collected from the defecation zone of the dogs. Each sample was frozen at −80 °C for 96 h to ensure any viable eggs were killed. Faecal samples were then divided into aliquots for copromicroscopic investigations and coproELISA assay. Each sample was initially checked macroscopically for the presence of adult worms and/or proglottids. Copromicroscopic examination was then carried out for each individual sample using sedimentation and flotation techniques with sodium thiosulfate solution (S.W. 1200) in order to detect helminth eggs as described by Varcasia et al. (2004).

Coproantigens (cAG) were extracted according to the protocol described by Allan et al. (1992) and subsequently stored at −20 °C until used. Faecal soluble antigens from *T. hydatigena* were detected using monoclonal antibody ATH4 produced by immunization with adult *T. hydatigena* E/S products (Carmona, data not published, 2014). Sandwich ELISA for coproantigen detection using ATH4 was performed following the protocol described by Malgor et al. (1997). Flat-bottomed microtitre plates were coated with protein A purified rabbit anti-*T. hydatigena* E/S products antibody (30 μg ml^{-1}) in carbonate–bicarbonate buffer (pH 9.6), overnight at 4 °C. The plates were blocked with 1 % BSA in PBS for 2 h at room temperature (RT) and then incubated with faecal supernatant (50 μl/well) for 2 h at RT. They were then loaded with 50 μl of hybridoma culture medium, and after 1 h, 50 μl/well of HRP-conjugated anti-mouse IgM (1:4000) was added for 1 h. Finally, 100 μl of *o*-phenylenediamine (4 mg/ml) and H_2O_2 (0.02 %) in citrate phosphate buffer (pH 5.0) was added for 10 min at 37 °C. The reaction was stopped through the addition of 50 μl of 4 N H_2SO_4. The ELISA microplates were read using a spectrophotometer at 405 and 492 nm, and the figures for each sample were transferred to a PC for statistical processing. The information was introduced into a database and processed with software SPSS v. 14.

Results

Epidemiological survey and estimation of economic losses

Prevalence

An overall prevalence of 14.6 % for *T. hydatigena* cysticercosis was found in the examined lambs (1135/7781). The lots of lambs positive for cysticercosis was 51.1 % (70/137), with an average prevalence of 25.2 % (SD=20.3). The prevalence rates stratified into classes between lamb lots are reported in Fig. 1. In total, 10,807 parasitary tracks were found, with an abundance of 1.39 and an average intensity of 9.52. In each parasitized liver, parasitary tracks ranged from 1 up to 97.

The number of tracks found in each liver was stratified into classes of infestation (Table 1), while the localization within the hepatic parenchyma of the parasitary tracks is shown in Table 2. The distribution of the lesions on the liver surface was not uniform (χ^2 for linear trend=459 707; p=0.0000); there was a significant difference between the involvement of the visceral (10.9 %) and the diaphragmatic side (13.7 %) (χ^2= 28.56; p<0.0001) (relative risk 1:26; 1:16<RR<1:37), as well as between the right (10.0 %) and the left portion (11.8 %) of the diaphragmatic side (χ^2=12.96; p=0.0003) (relative risk 1:18; 1:08<RR<1.29).

Molecular analyses

We amplified 388-bp *CO1* and 453-bp *ND1* fragments using DNA of putative *C. tenuicollis* isolates retrieved from Sardinian sheep livers. A blast search of all the 23 *CO1* (Cagliari=11; Sassari=9; Nuoro=3) and 21 *ND1* (Cagliari n=11, Sassari=9, Nuoro=1) nucleotide sequences gave a 99 % identity with *T. hydatigena* for both genes (accession numbers AB792722, AB792724, JN827307 and KF268023 for *CO1* and KC876043, HQ204207 and JN831270 for *ND1*).

The overall pairwise divergence for all the *CO1* sequences ranged from 0 to 3.1 % whereas that for the *ND1* sequences was 0 to 3.3 %. *CO1* sequence variation within *C. tenuicollis* populations from Cagliari and Sassari were 0 and 0.2 %, respectively. The mean nucleotide distances within *ND1* sequences for Cagliari and

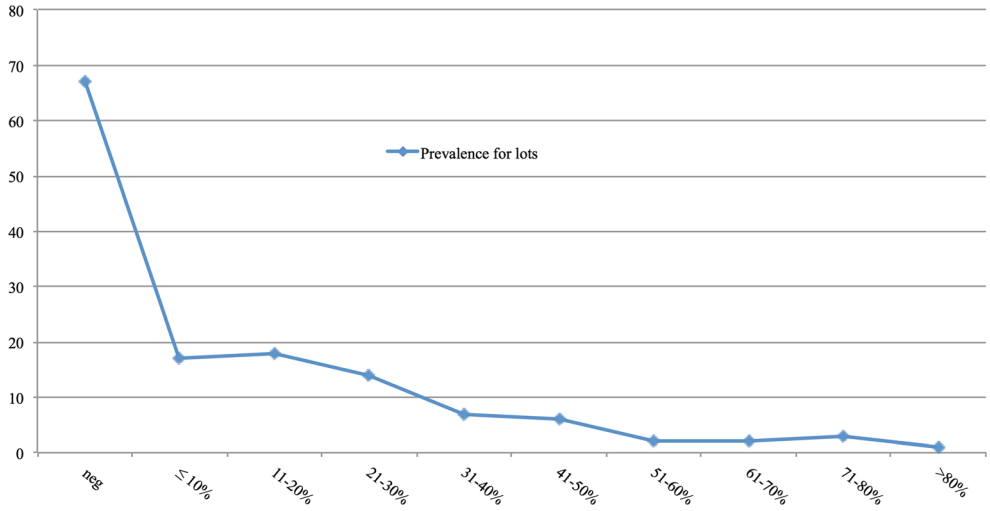

Fig. 1 Prevalence rates of hepatic track lesions due to *Cysticercus tenuicollis* stratified into classes between Sardinian lamb lots

Sassari metacestode populations were 0.8 and 1.3 %, respectively. Interspecific variation as estimated using mean distances between groups of *C. tenuicollis* from the Sardinian Provinces of Cagliari and Sassari were 0.1 and 1.1 % for the *CO1* and *ND1*, respectively.

The low variation within and between isolates from Sardinian Provinces was also evident in the phylogenetic trees which had similar topologies (Figs. 2 and 3). The *CO1*-derived tree showed isolates from different regions within Sardinia sharing the same clade. The most variable isolate was Th51 (from Nuoro) followed by Th6 (from Sassari). When Th51 isolate was excluded from the analysis, the pairwise distance for the *CO1* sequences was 0–0.8 %. The pairwise distance between all isolates and Th51 (excluding Th6) was 2.3 % and that between Th51 and Th6 was 3.1 %. Similarly, the short branches of the *ND1* phylogenetic tree support the presence of small changes between the isolates although unlike the *CO1* layout, the isolates appear to arrange into distinct clades. However, clades were not specific to any province with isolates seen intermingled across the entire tree. Due to their small sample size, isolates from Nuoro Province were included only in the generation of phylogenetic trees.

Economic losses due to liver condemnation as a result of cysticercosis amounted to € 315,622.2 per year. The cost for the proper disposal of condemned offal was € 18,035.5 per year. This brings the total economic costs related to cysticercosis due to *T. hydatigena* infection to € 333,657.7.

Coproantigen survey of farm dogs

Microscopic examination of dog faeces showed a total prevalence of 31.3 % for endoparasites in the examined samples (94/300). Taeniid eggs were found in 8.3 % of the dogs, while regarding other endoparasites, Ancylostomids (13.3 %), whipworms (6 %), ascarids (5.3 %) and *Isospora* sp. (3 %) were found. Nevertheless, this method did not allow us to make a specific diagnosis for *Taenia* spp., because of the great similarity between the eggs of the parasites that belong to this taxon. The results of the monoclonal antibody ATH4 ELISA test showed a prevalence of 11 % (33/300) for *T. hydatigena* coproantigens.

Only 15 % of the cAG-positive samples were at the same time positive by coprological examination (4 to *Taenia* spp. and 1 to *Dypilidium caninum*), while 27.2 % of cAG-positive samples were positive for other endoparasites (ascarids, Ancylostomids, *Isospora* sp.). The remaining 60.6 % cAG-positive samples were negative following copromicroscopic examination.

Discussion

The prevalence of *C. tenuicollis* in 14.6 % of 30–40-day-old lambs highlights the high parasitic pressure on the territory of Sardinia by *T. hydatigena*. Indeed, the recovery of necrotic–

Table 1 Number of tracks due to *Cysticercus tenuicollis* in Sardinian sheep livers stratified into classes of infestation

Classes per number of tracks	From 1 to 5	From 6 to 10	From 11 to 15	From 16 to 20	From 21 to 25	From 26 to 30	From 31 to 35	From 36 to 40	>40
Prevalence of infected lambs (%)	49.7	21.9	11.1	5.9	4.0	2.5	1.0	1.2	2.7
Number of lambs	564	249	126	67	45	28	11	14	31

Table 2 Localization of necrotic–haemorrhagic tracks due to *Cysticercus tenuicollis* within hepatic parenchyma of Sardinian sheep livers

Hepatic localization	Number (*N*°) of positive lambs	Prevalence (*P*) of positive lambs (%)	Odds ratio
Caudate lobe (Spiegel lobe)	323	4.2	1.00
Right visceral surface	617	7.9	1.99
Left visceral surface	667	8.6	2.16
Right diaphragmatic surface	779	10.0	2.57
Left diaphragmatic surface	919	11.8	3.09
Total of the visceral surface	848	10.9	2.82
Total of the diaphragmatic surface	1067	13.7	3.67

haemorrhagic tracks in the liver, presumably caused by larvae acquired at least 10 days before in subjects still lactating or weanling, would be due to the licking of the breast when sucking milk or lapping structures or grass polluted by eggs of *T. hydatigena*.

The diffusion of cysticercosis by *C. tenuicollis* in Sardinia seems to be uniform according to our data; in fact a high number of lots of lambs from all territories of the island were positive to the infection. The recovery of farms with prevalences up to 80 %, and with the number of parasitary tracks in the liver greater than 40, constitutes a serious sanitary problem, where probably farm dogs have regular free access to animals' infected offals.

In the latter cases, it is not inconceivable that the lesions present in the liver may also cause in lambs alterations of haematological and biochemical parameters (significant decrease in red blood cell count, haemoglobin, pace cell volume and total protein), as detected by Radfar et al. (2014) in young goats naturally infested with *C. tenuicollis*, with possible negative repercussions on weight gain. The recently reported finding of an acute outbreak of cysticercosis by *T. hydatigena*, causing about 24 % of deaths out of 21–60-day-old female lambs (Scala et al. 2014), confirms the high parasitic pressure in Sardinia. Compared with a similar survey carried out in Sardinia in the period 1993/1994 (Scala and Marrosu 1997), a highly significant increase in prevalence rates from 12.1 to 14.6 % (χ^2= 15:05; p=0.0003) was detected.

The high number of sheep bred with extensive methods; the presence of a strong relationship between dogs, sheep and humans on the farms; and the low commercial value of adult sheep meat which indirectly induces home slaughtering support together with other social and economic factors the persistence of cysticercosis in Sardinia as well of other metacestodosis of zoonotic interest, like cystic echinococcosis (Scala and Mazzette 2009; Varcasia et al. 2011).

The post-slaughter examination of livers allowed the detection of lesions especially on the diaphragmatic side of the organ, and in particular on its left portion. It is highly recommended that veterinary inspectors check the left diaphragmatic lobe carefully for parasitary tracks caused by *C. tenuicollis* in the livers of slaughtered lambs. Moreover, this inspection is

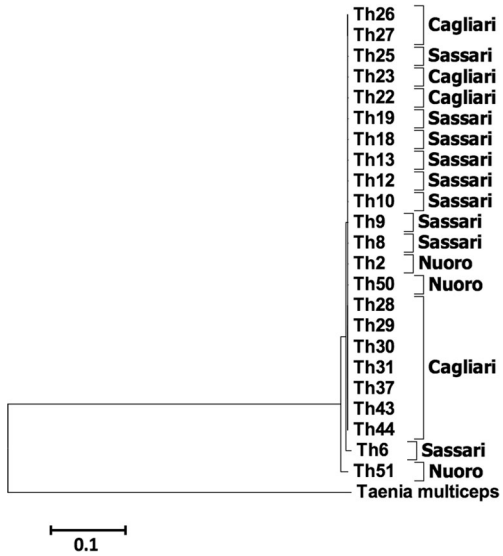

Fig. 2 Neighbour-joining phylogenetic tree constructed in MEGA 6 using *CO1* sequences of *Cysticercus tenuicollis* from Sardinian sheep. *Taenia multiceps* from Sardinia (accession no. DQ309767) was included as an out-group

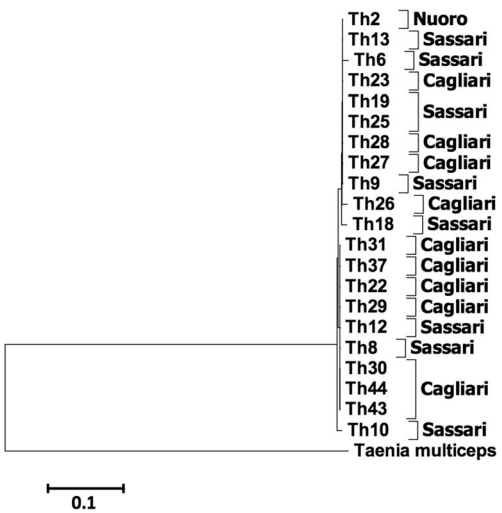

Fig. 3 Neighbour-joining phylogenetic tree constructed in MEGA 6 using *ND1* sequences of *Cysticercus tenuicollis* from Sardinian sheep. *Taenia multiceps* from Sardinia (accession no. AY669089) was included as an out-group

not always routinely performed for reasons related to the fast slaughter timing as the organ is usually examined as part of the carcass and not isolated as in other categories of animals slaughtered.

Pairwise variation within the *CO1* Sardinian *C. tenuicollis* isolates seen in this study (0–3.1 %) was lower than that described for Iranian sheep (0.3–3.4 %) (Rostami et al. 2013). Similarly, higher pairwise divergence values for *ND1* sequences of *T. hydatigena* from Poland, Ukraine and Wales (0.4–5.5 %) were previously reported (Kedra et al. 2001). The low intra- and interspecific variation observed in this study suggests the absence of genetic differentiation for sheep isolates of *C. tenuicollis* from Sardinia.

The coproantigen ELISA using monoclonal antibody generated against excretory secretory products from adult *T. hydatigena* showed a poor correlation with *Taenia*-positive faeces with only 4 out of 33 concordance while 60 % of cAG-positive faecal supernatants were helminth-free by coprology. Prevalences reported in the present survey for *T. hydatigena* coproantigens in dogs of Sardinia (11 %) seem to highlight a parasitic pressure slightly lower than those reported for the same parasite in Albania (16 %) (Xhaxhiu et al. 2011) and China (19.7 %) (Wang et al. 2006).

Although further studies are needed, particularly using experimentally infected dogs and necropsied stray dogs, data obtained in this study suggests that this sandwich ELISA lacks adequate sensitivity and specificity required to carry out accurate epidemiological investigations.

The condemnation of parasitized livers due to cysticercosis caused important economic losses, reaching almost € 330,000, if we considered the loss of income from sales and the costs of offal disposal. This amount seems to be underestimated due to the fact that the officially recorded number of slaughtered lambs is probably lower than the actual figure. The number of female sheep in Sardinia are at least 2,000,000 according to official data (Ministero della Salute 2013), and these give birth each year almost to the same number of lambs. Of these animals, only 20 % (approx 400,000 individuals) will constitute the replacement quota and so will be bred by farmers to substitute older sheep. The remaining lambs (approx 1600.00 individuals) are all slaughtered at 30–40 days of age. This number was not consistent with the official data provided by abattoirs and Regional Government of Sardinia, which reported that 1,102,957 lambs are slaughtered per year, with approximately 500,000 lambs slaughtered elsewhere (home, clandestine, unofficial slaughtering).

The control at slaughtering of these parasites in lambs could play an important epidemiological role that could be used also for control and prevention not only of cysticercosis but also of other metacestodosis like cystic echinococcosis and coenurosis that have the same life cycle.

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