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Research Note

Calicophoron daubneyi in sheep and cattle of Sardinia, Italy

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Summary

This study aimed to investigate the prevalence of paramphistomosis and confirm the species identity of rumen flukes from sheep and cattle of Sardinia (Italy), by molecular methods. From 2011 to 2014, 381 sheep and 59 cattle farms were selected and individual faecal samples were run on 15 sheep and 5 cattle for each farm, respectively. The prevalence at the slaughterhouse was calculated by examination of 356 sheep and 505 cattle. 13adult flukes collected from sheep and cattle and 5 belonging to the historical collection of Laboratory of Parasitology at the Department of Veterinary Medicine of Sassari, previously classified as *Paramphistomum* spp., were used for PCR amplification and sequencing of the ITS2+ rDNA. Previously classified *Paramphistomum leydeni* from South America were used as controls.
The EPG prevalence was 13.9 % and 55.9 % for sheep and cattle farms respectively. At slaughterhouses, paramphistomes were found in 2 % of the sheep and 10.9 % of the examined cows. Conversely to the latest reports, the sequences comparison showed that all the Sardinian rumen flukes belong to *Calicophoron daubneyi*.
Keywords: *Calicophoron daubneyi*; rumen fluke; paramphistomosis; sheep; cattle

Introduction

Paramphistomosis of ruminants is caused by different species of Paramphistomidae (Trematoda, Digenea), from several genus (*Paramphistomum*, *Calicophoron* and *Cotylophoron* etc.) (Eduardo, 1982). In most of cases, adult flukes inhabit the rumen and/ or reticulum without damage (Rolfe *et al.*, 1994), while immature flukes are found in the upper small intestine where can cause serious morbidity by protein loss, decrease of milk production and weight, oedema and eventually death (Horak, 1971; Rangel-Ruiz *et al.*, 2003, Rolfe *et al.*, 1994). Studies carried out in Europe (Mage *et al.*, 2002; Cringoli *et al.*, 2004 ; Rinaldi *et al.*, 2005; Díaz *et al.*, 2007; Rieu *et al.*, 2007; Foster *et al.*, 2008; Murphy *et al.*, 2008, González-Warleta *et al.*, 2013) have shown an increased prevalence in the last few years. This should probably been caused by the climate changes with warmer winters and wetter summers that positively influenced the life-cycle of the parasite (Gordon *et al.*, 2013). Furthermore animal movement should be also be implicated in the diffusion of the parasite as suggested by Taylor *et al.* (2012).
Moreover, several clinical cases have been described (Dorchies *et al.*, 2002; Deiana *et al.*, 1962; Millar *et al.*, 2012; Dorny *et al.*, 2011; Malrait *et al.*, 2015; Zintl *et al.*, 2014).
Sardinia island (39° 13’ 0’’ N, 9° 7’ 0’’ E) has the largest Italian sheep population (3,100,716 dairy sheep) and an important number of herds (264,925 cattle), which are mostly bred by traditional extensive methods (BDN Anagrafe Nazionale Zootecnica). To date, there is a lack of updated information about Paramphisto-

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mosis in domestic ruminants of this area; therefore, a prevalence study can be used as a model for paramphistomosis in the Mediterranean area.
Amphistome species in Sardinia were previously classified using standard morphological and histological methods, leading to the identification of *P. cervi* by Deiana *et al.* (1962) and later *P. daubneyi* and *P. microbothrium* by Sey and Arru (1977).
It is well known that the identification of amphistome's species can be quite difficult leading to a possible misclassification (Horak, 1971; Mage *et al.*, 2002), but nowadays, molecular biology techniques represents an additional tool for this purpose. PCR amplification, restriction fragment length polymorphism and sequencing of DNA segments with a high substitution rate, such as the internal transcribed spacers from the ribosomal DNA (rDNA), are the most frequent procedures for taxa recognition (Itagaki *et al.*, 2003; Otranto *et al.*, 2013; Rinaldi *et al.*, 2005; Sanabria *et al.* 2009).
Therefore, the aim of this study was to investigate the prevalence and distribution of Paramphistomidae in sheep and cattle farms in the Sardinia region using a coprological, necroscopic and molecular survey.

Materials and Methods

Copromicroscopic survey
The survey was carried out in Sardinia island (Italy), located in western Mediterranean sea, characterized by a temperate climate with long, dry and hot summer and short and mild winter; the rainfall is irregular, concentrated specially in winter and the average temperatures ranging from 15 °C to 18 °C. Local sheep (n=381) and cattle (n=59) farms were visited once from 2011 to 2014. Sheep were autochthonous Sarda breed, while cattle were of various breeds (Sarda, Brown-Swiss, Limousine, Charolaise and crossbreeds) and raised under extensive farming. The average (standard deviation) livestock was 351.48 (SD = 208.91) sheep and 51.38 (SD = 72.47) cattle per farm. Individual faecal samples were randomly collected from 15 ewes (> 3 years) in sheep farms and 5 cows (1 year) in cattle farms, directly from rectum. The faeces were pooled at the laboratory into 3 composite samples from each ovine farm and 1 composite sample from each cattle farm (Rinaldi *et al*., 2014). Composite samples were constituted by 5 equal parts of individual samples weight 2 grams. According to Nicholls and Obendorf (1994), this method simplifies the samples processing by saving time and still provides reliable results. The coprological study was carried out using the Flotac® double technique (Cringoli *et al*., 2010) with a sensitivity of 2 eggs per gram (EPG) of faeces, using a zinc sulphate solution (specific gravity = 1350) (Cringoli *et al.*, 2010).

Direct examination at the abattoirs
During the same period, 356 sheep and 505 cattle were examined in 4 abattoirs of the 4 historical provinces of Sardinia (Sassari, Nuoro, Oristano and Cagliari). Ewes (> 3 years) and cattle (both sexes) were divided into 3 groups: calves (males and females from 6 months to1 year), heifers (females from 1 to 3 years) and cows (females over 3 years old).
At the slaughterhouse, rumen and reticulum were removed from each animal. These organs were examined for the presence of Paramphistomidae and classified into 2 infection levels: ≤ 100 and >100 adult parasites recovered from each animal.
To perform the species identification, 10 % of the adult parasites were collected from each animal. Flukes were washed in physiological saline and stored in 70 % ethanol prior to molecular analysis.

Biomolecular survey
13 flukes isolated in the present survey and 5 belonging to the historical collection of Parasitology of Sassari (University of Sassari, Italy) (collected during the last 30 years and preserved in 70 % ethanol), and previously classified as *P. microbotrium* and *P. daubneyi*. (Sey and Arru, 1977) were processed. Additionally, 12 flukes from sheep and cattle previously classified as *P. leydeni* (Sanabria *et al.*, 2009, 2011) from Entre Ríos (32° 52'S; 59° 26'W) and Buenos Aires (34° 05'S; 59° 01'W) regions (Argentina) were processed for DNA extraction and included in the study as species holotype.
Genomic DNA was extracted from individual flukes, using the DNeasy extraction kit (Qiagen, Germany), according to the manufacturer's recommendations. DNA was eluted with 100 µl of nuclease-free water and stored at −20 °C prior to use. The Internal Transcribed Spacer 2 of the rDNA, plus the flanking 5.8S and 28S partial segments (ITS-2+) were amplified using the generic primers, ITS-2For 5'-TGTGTCGATGAAGAGCGCAG-3' and ITS-2Rev 5'-TGGTTAGTTTCTTTTCCTCCGC-3' as described by Itagaki *et al.* (2003). PCR was performed in a total reaction volume of 50 µl containing X µl of template and a PCR mix composed by 10x *Taq* Buffer, 2 mM of $MgCl_2$, 25 pmol of each primer, 200 µM of each dNTP and 2.5 U of Taq DNA Polymerase (5Prime). PCR was carried out in a GeneAmp PCR System 9700 (Applied Biosystems, USA) under the following conditions: initial denaturation of 94 °C for 5 min, followed by 40 cycles of 94 °C for 1 min, 53 °C for 1 min, 72 °C for 1 min and a final extension of 72°C for 10 min. PCR products were separated in 1.2 % agarose gels prepared in Tris–acetate–EDTA (TAE) buffer with GelRed (Cambridge Bioscience, UK) and visualized by an ultraviolet transilluminator. Ten amplicons were purified using the ChargeSwitch® PCR Clean-Up Kit (Invitrogen™) and submitted to MWG Eurofins for sequencing, and were analyzed, aligned and trimmed by the software MEGA 6. Sequences obtained were compared to the same segments of reference sequences deposited in GenBank, by means of nBLAST and MEGA 6. The sequences accounted for comparison were *C. microbothrium* (GU735639.1; GU735640.1; GU735644.1; GU735647.1; GU735649.1; GU735650.1; GU735651.1; GU735652.1; GU735653.1; GU735654.1; GU735655.1; GU735656.1), *Cotylophoron cotylophorum* (KC503917.1), *P. leydeni* (HM209065.1; HM209064.1; HM209066.1; HM209067.1), *P. cervi* (HM026462.1) and *P. epiclitum* (JF834888).

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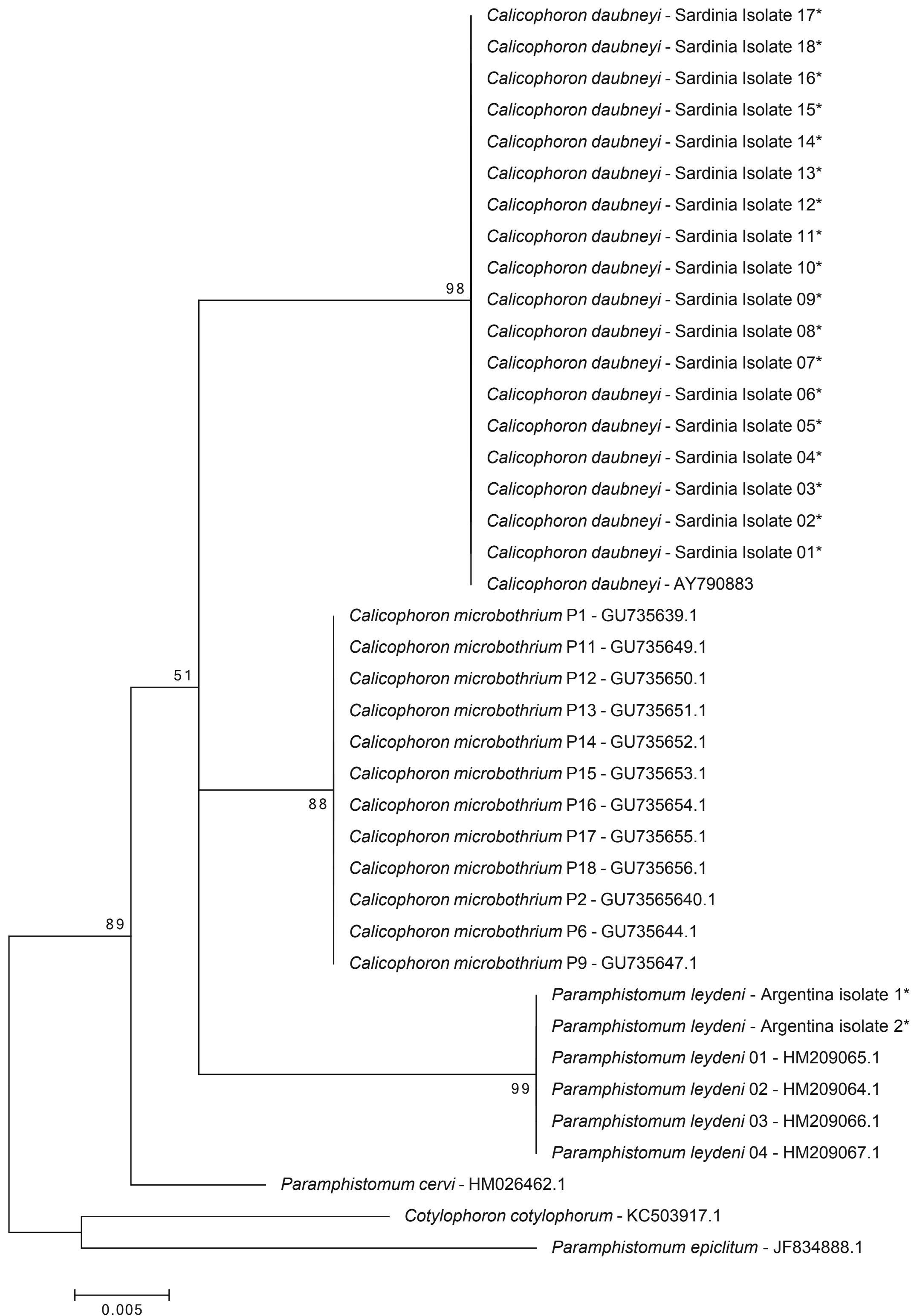

Fig. 1. The phylogenetic tree, show as Sardinian samples clustered all together with *C. daubneyi* and were separated to other species from the genus *Calicophoron, Cotylophoron* and *Paramphistomum*. Sequence originated by this study are marked with (*), while others are referred with Genbank reference numbers. The Sardinian *C. daubneyi* DNA sequence was deposited in Genbank with reference number: KR072562

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Data Analysis
Chi Square Test was performed to compare the prevalence between sheep and cattle. As egg elimination is not normally distributed, Mann-Whitney *U* test was employed (p < 0, 01) to compare EPG values between sheep and cattle. The relationship between age and the prevalence of paramphistomosis in slaughtered cattle was explored by Chi Square Test and odds ratio (OR) values (Sanchís *et al.*, 2013). Statistical analyses were performed using Minitab 16.2 (Minitab Inc. USA) and Epi Info 7 (CDC, USA).

Results

Copromicroscopic survey
Sheep farms showed a prevalence of 13.9 % (53/381) and an average of 59.6 (SD = 106.01) EPG. Just two pools from one farm (0.5 %) were over 250 EPG (zootecnic risk EPG value according to Ambrosi et al., 1995).
On the other hand, cattle showed a prevalence of 55.9 % (33/59) and an average of 80.2 (SD = 121.84) EPG; whereas another two pools from two different farms (3.4 %) were over 250 EPG. The difference between sheep and cattle farms prevalence was statistically significative ($\chi^2 = 57.37$; $p < 0.0001$) but it wasn't the same for the EPG's averages comparison ($p = 0.269$). EPG averages over 250 showed no statistical difference between sheep and cattle ($\chi^2 = 2.02$; $p = 0.155$).

Direct examination at the abattoirs
Overall, 7 of the 356 sheep examined (2 %) had adult flukes in the rumen and reticulum, and number of parasites in all positive animals is ≤ 100. Regarding cattle, adult Paramphistomidae were found in 10.9 % (55/505) of slaughtered animals, with a number of flukes ≤ 100 in 65.5 % (36/55) and > 100 flukes in 34.5 % of the examined cows (19/55). Regarding the age of slaughtered cattle, the prevalence was 6.6 % (OR = 1.00), 13.1 % (OR = 1.86) and 18.6 % (OR = 2.64) for calves (10/152), heifers (29/251) and cows (16/102) respectively, showing a significant relation between the age and the amphistome's burdens (χ^2 for trend = 5.409; p = 0.020).

Biomolecular survey
The sequence analyses of the ITS2+ segment from each fluke showed a constant 428 bp amplicon, including the complete ITS-2 fragment (282 bp) plus the two partial flanking segments corresponding to the genes coding for the 5.8S rRNA (99 bp) and 28S rRNA (47 bp). All the Sardinian isolates showed no intra-specific variation (homology = 100 %).
As well, they were 100 % homologous when compared to *C. daubneyi* (AY790883.1) reported from Southern Italy by Rinaldi *et al.*, (2005). The next best match (97.2 % homology) was *P. cervi*, (HM026462.1).
All *P. leydeni* samples from Argentina were coincident among them and had a 100 % homology compared to previous reported sequences of this species (HM209064) (Sanabria *et al.*, 2011). Compared to this South American amphistomes, the Sardinian *C. daubneyi* isolates showed the following differences: six transition in positions 227, 286, 301, 302, 303 and 339, three transversions in positions 114, 275 and 367 and a deletion behind the position 304 of the Sardinian sequences.
Compared to its genus counterparts, Sardinian isolates had a 98 % homology with *C. calicophorum* (GU133057.1) and 97 % homology with *C. microbothrioides* (AB056570.1).
The sequences of Sardinian Paramphistomidae showed a pairwise distance of 0.022 to *C. microbothrium*, 0.045 to *C. cotylophorum* and 0.052 to *P. epiclitum* (Tamura *et al.*, 1993). The phylogenetic tree (Fig. 1) show as Sardinian samples clustered all together with *C. daubneyi* and were separated to other species from the genus *Calicophoron*, *Cotylophoron* and *Paramphistomum* (Fig. 1).

Discussion

Paramphistomosis was described in Sardinia for the first time by Deiana *et al.* (1962), but the present report constitutes the first epidemiological survey of sheep in Sardinia Island. The coprological prevalence found in sheep farms in Sardinia (13.9 %) was similar to that reported in Southern Italy (10.2 %) by Cringoli *et al.* (2012) and in India (7.4 %) by Maitra *et al.* (2014), but relatively low, compared to other reports, like Gupta et al. (1985), who reached up to 52 % in sheep and goats from India. In cattle farms, the prevalence of 55.9 % seems to be increasing if compared to the survey carried out in the same region by Scala *et al.* (1997a) 18 years ago, where a prevalence of 19.6 % was found; The values found here are in according to the report of Cringoli *et al*. (2012), (55.7 %), while are lower compared to Spanish (61 %) and Uruguayan (69 %) reports as described by Sanchís *et al.*, (2013). The slaughterhouse's survey revealed a very low prevalence of paramphistomosis in sheep (2 %), similar to findings from Iran (0.041 %) (Tehrani *et al.*, 2015) and Turkey (4.43 %) (Ozdal *et al.*, 2010) but was much lower compared to records from Ethiopia (25 %) (Sissay *et al.*, 2007) and India (36.2 %) (Godara *et al.*, 2014). The lower values obtained from sheep coprological analysis and in slaughterhouse's survey could be probably due to the fact that in this species the infection seems to be less frequent than in cattle (Rojo-Vázquez *et al.*, 2012). Regarding cattle, the 10.9 % prevalence found was slightly lower compared to that found about 20 years ago by Scala *et al.* (1997b), (16.9 %) and also compared to French (20 %) (Szmidt-Adjidé *et al.*, 2000) and Spanish (18.8 %) (González-Warleta *et al.* 2013) reports, while were higher compared to the findings reported in Castilla y León, Spain (6.2 %) by Ferreras *et al.*, (2014): indeed these Spanish regions have a very cold and dry climate that may influence the parasite life-cycle. The lower prevalence reported in our study could be related to the climate of the region that could affect the biology of the intermediate hosts, compared to other areas with a more humid environmental conditions. Furthermore the management may also influence the diffusion of the rumen flukes:

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for example longer periods at pasture could induce an higher exposure to the parasite. The relation between the cattle age and the amphistome's burdens seems to evidence a time-dependent cumulative effect, as observed by Ferreras *et al.*, (2014) suggesting that repeated exposures do not confer protections against reinfections. By the way, Horak (1971), proposes that cattle are better hosts than small ruminants, since cattle amphistomes matures early, reach a larger size and number in rumen compared to sheep and goat, as well as has a shorter prepatent period. This might allow to mature flukes to live much time, even years in the same animal without damage.

The coprological results of sheep and cattle, together with the abattoirs prevalence seem to globally indicate an increasing trend considering the previous reports. This can lead to consider Paramphistomosis is an emerging parasitosis onto the island.

The present survey has allowed to identify *C. daubneyi* in Sardinia, reinforcing other reports from Mediterranean areas such as Southern Italy, France, Spain (Ferreras *et al.*, 2014; Paraud *et al.*, 2009; Rinaldi *et al.*, 2005) and other European countries like Belgium (Malrait *et al.*, 2015), UK (Gordon *et al.*, 2013) and Ireland (Zintl *et al.*, 2014). Since it can be considered as synonymous of *P. daubneyi*, the other species reported by Sey and Arru (1977), *P. microbotrium*, was not found here.

In this case, molecular techniques improved the morphological methods of classification, suggesting that the genus *Paramphistomum* is not currently present in Sardinia. This fact points out once again the need of both methods for an accurate identification. Similarly, Gordon *et al.*, (2013) found that *C. daubneyi*, and not *P. cervi* as previously thought, is the most common species in the UK.

As well, sheep and cattle seems to be infected by the same species as also found in Southern Italy by Rinaldi *et al.* (2005), at least for this molecular marker.

This species identification also clears some epidemiological features in one of the biggest Mediterranean islands. While *Paramphistomum* spp. mainly have as intermediate hosts *Planorbis* spp. and *Bulinus* spp. (Pavlović *et al.*, 2012; Spence *et al.*, 1996) which lives in aquatic permanent habitats, *Calicophoron* spp. have as main intermediate host *Galba truncatula*, which could live in temporary habitats (Abrous *et al.*, 2000; Augot *et al.*, 1996).

In addition *Galba truncatula* is also the intermediate host of *Fasciola hepatica*, and this snail species can also harbor double infections by both genus (Abrous *et al.*, 1999; Abrous *et al.*, 2000; Augot *et al.*, 1996).

Considering a rising trend in the amphistomes prevalence, some specific issues might have contributed to this. The large number of anthelmintic treatments implemented against *F. hepatica*, might allowed to *C. daubneyi* to successfully compete (and win) for *G. truncatula*, however this must be verified by snails prevalence studies. Additionally, this fluke is often underestimated in the routine diagnosis and have no specific registered treatments available in Italy (Scala *et al.*, 1999).

The presence of *F. hepatica* is currently investigated by the official veterinaries during the slaughterhouse's inspections, while unfortunately, the prestomachs inspection is not quite frequent.

In conclusion, this is the first study on Paramphistomidae in sheep from Sardinia Island, where the only species identified in this species and in cattle was *C. daubneyi*, which would be at least the most prevalent. This results may help to further studies about infection seasonality, habitat of the intermediate host, host-trematodes interactions and control strategies for paramphistomosis.

Competing interests

The authors declare that they have no competing interests.

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