Research Note

Calicophoron daubneyi in sheep and cattle of Sardinia, Italy

G. Sanna¹, A. Varcasia*¹, S. Serra¹, F. Salis², R. Sanabria³, A. P. Pipia¹, F. Dore¹, A. Scala¹, 4

¹Laboratory of Parasitology, Veterinary Teaching Hospital, Veterinary Department, University of Sassari, Italy, 
*E-mail: varcasia@uniss.it; ²Veterinary Practitioner, Martini Zootecnica, Italy; ³Veterinary Faculty, National University of La Plata, Buenos Aires, Argentine. National Scientific and Technical Research Council (CONICET), Argentine; ⁴Inter-University Center for Research in Parasitology (CIRPAR), Via della Veterinaria 1 – 80137, Naples, Italy

Article info

Received April 22, 2015
Accepted July 13, 2015

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. 13 adult 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., 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-
miosis 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 Deliana 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 represent 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 faecal samples were processed by saving time and still provides reliable results. The final transcribed spacers from the ribosomal DNA (rDNA), are the ones that have DNA segments with a high substitution rate, such as the internal transcribed spacers of *P. microbothrium* 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 MgCl₂, 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 amplions 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; HM209066.1; HM209067.1), *P. cervi* (HM026462.1) and *P. epiclitum* (JF834888).

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. microbothrium* 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 MgCl₂, 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 amplions 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; HM209066.1; HM209067.1), *P. cervi* (HM026462.1) and *P. epiclitum* (JF834888).
Fig. 1. The phylogenetic tree shows 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.
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 significant ($\chi^2 = 57.37; p < 0.0001$) 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 ($\chi^2$ for trend = 5.409; $p = 0.020$).

Biomolecular survey
The sequence analyses of the ITS2+ fragment 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. epilictum (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 accordance 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:
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 live 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 pre-stomachs 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.

Acknowledgements

The research was partially funded by Regional Government of Sardinia, Italy – Prot. CRP-60126 (L.R. 7, 2007), year 2012. The authors thank Mr. Francesco Salis, Lab Technician of Veterinary Parasitology (University of Sassari, Italy) for the technical support in copromicroscopic examinations.

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of species occurring in ruminants.


