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Research Note

Cox-1 gene sequence of *Spirometra* in Pampas foxes from Argentina

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Summary

The parasites of the genus *Spirometra* belong to one of the twelve genera of the family Diphylobothriidae, with several species of zoonotic importance whose definitive hosts are carnivorous mammals. In Argentina, few reports have described these parasites in wild carnivores. Morphological studies of the adult stage obtained from necropsy allow the distinction between *Diphylobothrium* and *Spirometra*. A less invasive method of identification is the analysis of the parasite eggs; however, the morphometric similarities between close genera and species and alterations in egg preservation affect the identification. In Argentina, molecular tools have been used as a non-invasive and accurate method to increase the information about *Spirometra* and to improve its identification. In the present study, DNA was extracted from *Spirometra* eggs from Pampas foxes and a 450-bp fragment of the mitochondrial cytochrome C oxidase subunit 1 (*cox1*) gene was sequenced. The sequence obtained, which is the first *Spirometra* DNA sequence from Argentina, was deposited at GenBank. Comparison by BLASTN analysis between the sequence obtained and the sequences from GenBank showed 93 % identity with *S. proliferum* and 89% with *S. erinaceieuropaei*.

Keywords: non-invasive methods; wild mammals; Diphylobothriidae eggs; PCR; cytochrome C oxidase subunit 1 gene

Introduction

Cestodes of the family Diphylobothriidae include twelve genera with several species of zoonotic importance, such as *Diphylobothrium* (Cobbold, 1858), *Spirometra* (Faust, Campbell & Kellogg, 1929) and *Diplogonoporus* (Lönnerberg, 1892) (Kuchta *et al.*, 2008). According to the last revision of the genus *Spirometra* (Kamo, 1999), there are four recognized species: *S. erinaceieuropaei* (Rudolphi, 1819), *S. mansonoides* (Mueller, 1935), *S. pretoriensis* (Baer, 1924) and *S. theileri* (Baer, 1924). The plerocercoid larvae (spargana) of *Spirometra* can cause sparganosis, a serious parasitic zoonosis. This human disease can arise from intake of invertebrates containing proceroid larvae or of reptiles or amphibians containing plerocercoid larvae. The larvae can remain in the subcutaneous tissue and sometimes invade other tissues, including the central nervous system (e.g., Magnino *et al.*, 2009; Shirakawa *et al.*, 2010). Ijima (1905) reported the proliferative human sparga-

nosis caused by the larval cestode *Sparganum proliferum*, but did not find the adult stage of the parasite. According to morphological (Ijima 1905) and molecular analyses, *S. proliferum* is considered a species different from but closely related to *S. erinaceieuropaei* (Miyadera *et al.*, 2001; Okamoto *et al.*, 2007; Bennet *et al.*, 2014). The life cycle of *Spirometra* sp. has been widely studied because of the importance of plerocercoids as agents of human sparganosis (Denegri & Reisin, 1993). Besides, interestingly, plerocercoids of *S. erinaceieuropaei* secrete a growth hormone-like factor stimulating overgrowth in mammals (Hirai, 2003). The definitive hosts of these cosmopolitan species are carnivorous mammals, specifically felines and canines. In addition, they have two intermediate hosts that are necessary to complete the parasitic cycle: a copepod (*Cyclops* spp.) and any vertebrate except fishes i.e., humans and other mammals, amphibians, reptiles and birds (Denegri & Reisin, 1993).

In Argentina, there are few reports on *Spirometra*, mostly in domes-

tic definitive hosts. Santa Cruz and Lombardero (1987) found *S. mansonoides* in cats, whereas Venturini (1980, 1989) and Denegri and Reisin (1993) reported *S. erinaceiropaei* in cats and dogs. In wildlife, Martinez *et al.* (2010) identified *S. mansonoides* eggs in the felids *Felis pardalis*, *F. yagouaroundi*, *Panthera onca* and *Puma concolor*. Other researchers have described *Spirometra* sp. within the community of endoparasites of the Pampas fox (*Lycalopex gymnocercus*) from La Pampa Province (Reigada *et al.*, 2012) and recently Scioscia *et al.* (2014) identified adult specimens as *S. erinaceiropaei* in Pampas foxes from Buenos Aires Province. Sparganosis diagnosis in the definitive hosts relies on morphological studies of the adult stage obtained by necropsy. In fact, the genera *Diphyllbothrium* and *Spirometra* can be distinguished based on differences in the uterus; in *Spirometra* the uterus is coiled spirally, whereas in *Diphyllbothrium* it is rosetted. The microscopic observation of eggs isolated from feces also allows the distinction between *Spirometra* (pointed ends) and *Diphyllbothrium* (rounded ends) (Mueller, 1936). Only *S. erinaceiropaei* and *S. mansonoides* have been compared in detail and Muller (1936) identified these specimens as separate species based on characters of the uterus and vagina of the adult stage. The species identification of eggs by means of light microscope observation can be difficult because of the morphometric similarity between close genera. Possible morphological alterations because of preservation procedures also make the identification difficult (Milano & Oscherov, 2005; González, 2012). In contrast, the molecular tools applied in isolated eggs are fast and less invasive for the identification of these parasites in wild animals. Furthermore, molecular tools provide additional information to complement the identification of the species of the genus *Spirometra*.

Most genetic studies have been performed in Asia with diagnostic purposes (e.g., Koonmee *et al.*, 2011; Boonyasiri *et al.*, 2013; 2014) and for phylogenetic analysis (e.g., Miyadera *et al.*, 2001; Okamoto *et al.*, 2007; Zhang *et al.*, 2014). Until now, there are no reports on molecular studies based on the genus *Spirometra* in Argentina. Thus, with the aim to increase the information about *Spirometra* from South American wild mammals, in the present work we improved a non-invasive and accurate method based on molecular tools.

Materials and Methods

Adults of *S. erinaceiropaei* were isolated from small intestines of 12 Pampas foxes and identified by morphological studies (see Scioscia *et al.*, 2014). *Spirometra* sp. eggs were found in the feces of all foxes through Ritchie's sedimentation (Young *et al.*, 1979) and Sheather's flotation (Benbrook & Sloss, 1965). Only one of the *Spirometra* sp. positive samples with high concentration of eggs was selected for the analysis. Eggs were isolated and disrupted manually under a light microscope using a capillary tube. Broken eggs were preserved in 2 µl of sterile PBS 1X at -20 °C until their use.

Three different pools of 3 or 4 eggs were processed for molecular analysis. DNA was extracted according to Gasser *et al.* (1993) with modifications. Briefly, eggs were immersed in 50 µl of compatible lysis-PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.8, 0.4 % v/v

Nonidet P-40, 0.8 % v/v Tween 20, 2.5 mM MgCl₂). The samples were digested with 260 µg/ml of Proteinase K (Biobasic) for 2 h at 56 °C and then at 37 °C overnight and then boiled for 15 min for Proteinase K inactivation and subsequently centrifuged at 2,000 Xg for 5 min. The supernatants were kept at -20 °C until use. A negative control of DNA extraction was included.

A 450-bp region of the mitochondrial cytochrome C oxidase subunit 1 (*cox1*) gene was amplified using already available primers: JB3 5' TTTTGGGCATCCTGAGGTTTAT 3' and JB4, 5' TAAAGAAAGAACATAATGAAAATG 3' (Bowles *et al.*, 1992). PCR was performed in 25 µl containing 2 µl of DNA sample, 200 µM of each dNTP (Finnzymes), 0.4 µM of each primer and 0.65 units of Dream Taq DNA Polymerase (ThermoScientific) in 1X Dream Taq Buffer containing 2 mM of MgCl₂. The PCR conditions were as follows: an initial denaturation step (94 °C for 3 min), 40 cycles at 94 °C for 45 s (denaturation), 49 °C for 45 s (annealing), and 72 °C for 45s (extension), and 72 °C for 10 min (final extension). The negative control was included in all PCR experiments.

Triplicates of the specific fragment were sequenced and chromatograms were analyzed using BioEdit v7.2.0 (copyright © 1997 – 2013, Tom Hall, Ibis Biosciences). The consensus sequences obtained were compared with the GenBank sequences by using the BLASTN algorithm (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) of the National Center for Biotechnology Information (NCBI, USA). Molecular phylogenetic analysis was conducted in MEGA6.0.6 (Tamura *et al.*, 2013). Sequence alignment was performed with *cox1* sequences from GenBank of *Spirometra* sp. (AB015753.1, AB480297.1, GQ999951.1, JQ267473.1) and *Diphyllbothrium* sp. (AB544064.1, AB605762.1, AY972071.1) and the sequence of *Spirometra* obtained in this work (*Spirometra* Pampas fox_KC812049.1). The evolutionary history was inferred by using the Neighbor-Joining (NJ) method. The evolutionary distances were computed using the Maximum Composite Likelihood method. The bootstrap consensus tree was inferred from 1,000 replicates. The *cox1* sequence of *Echinococcus multilocularis* (JF906152.1) was used as an outgroup.

Results and Discussion

Light brown eggs with evident cap and pointed ends attributable to the genus *Spirometra* (Fig. 1) were isolated from fecal samples from Pampas foxes. However, neither the morphological features nor the measures (length and width) of eggs allowed us to determine the species. Many published studies have failed to differentiate the genus of operculate eggs morphologically and have thus only reported the presence of eggs of the family Diphyllbothriidae (Milano & Oscherov, 2005; González *et al.*, 2012).

Molecular analysis allowed detecting the 450-bp specific fragment of the *cox1* gene in the three pools of eggs and the sequence of this fragment was identical in all cases. This sequence was submitted to the NCBI (KF572950.1) as the first nucleotide *Spirometra* sequence from Argentina.

Comparison by BLASTN analysis between the sequence obtained in this study (*Spirometra* sp. from Pampas foxes) and the sequences from GenBank showed 93 % identity with *S. proliferum* (AB015753.1) and 89 % with *S. erinaceiropaei* (GQ999951.1).

Fig. 1. Egg of *Spirometra* sp. Scale Barr: 35 µm

The molecular phylogenetic analysis of the *cox1* sequence from *Spirometra* sp. from Pampas foxes and available sequences of *Spirometra* sp. and *Diphyllbothrium* sp. deposited at GenBank supported the BLASTN results (Fig. 2). *Spirometra* sp. from Pampas foxes grouped in the clade of the genus *Spirometra* but formed a sub-clade with *S. proliferum*. The *S. erinaceieuropaei* sequences made up other sub-clade and the *Diphyllbothrium* species formed a separate well-supported clade. The sequence found in the present study could not be compared with *cox1* sequences from other *Spirometra* species from Argentina or South America because they are not available in genomic databases. In addition, the only available *S. mansonioides* *cox1* sequence (AF096239.1) was not used in the phylogenetic analysis because of its low identity (~42 %) with the *Spirometra* sequences analyzed (data not shown). Nevertheless, in Argentina, *S. mansonioides* has been reported only in felids (Santa Cruz & Lombardero, 1987; Martínez *et al.*, 2010).

The *Spirometra* adults obtained from the same Pampas foxes studied in this work had been previously identified as *S. erinaceieuropaei* by morphological analysis (Sciencia *et al.*, 2014). In the present work, the analysis of the 450-bp fragment of the *cox1* gene did not allow us to confirm the species of *Spirometra*. Previous studies have widely used the *cox1* gene as genetic marker to identify the genus and species of *Spirometra* parasites (Miyadera *et al.*, 2001; Nakao *et al.*, 2007). A molecular analysis based on *cox1* has shown that *S. sparganum* isolates (from Venezuela and Japan) are genetically different from those of *S. erinaceieuropaei*. Furthermore, the analysis of the same *cox1* gene fragment studied in the present study showed more divergence between *S. erinaceieuropaei* and *S. proliferum* (89 % identity) than between different isolates of *S. erinaceieuropaei* (98 %) and within *S. sparganum* (99 %) (Miyadera *et al.*, 2001). Based on this background, the identity percentages of the BLASTN analysis by comparing the *cox1* sequence of *Spirometra* sp. of this work with those of *S.*

Fig. 2. Molecular Phylogenetic Analysis. Neighbour-Joining analysis of 450-bp *cox1* gene sequences of *S. erinaceieuropaei* from Pampas fox (this work), *Spirometra* sp., and *Diphyllbothrium* sp. from GenBank. Bootstrap consensus tree was inferred from 1,000 replicates. Outgroup: *Echinococcus multilocularis*

erinaceieuropaei and *S. proliferum* from GenBank was not enough to define the species. Some intra-specific variation, although independent of the geographic distribution or host, has been observed in *S. erinaceieuropaei* (Okamoto *et al.*, 2007; Liu *et al.*, 2012). An analysis of the nucleotide variation performed with the same *cox1* fragment of isolates from Asian dogs and *Diphylobothrium* species has revealed less than 2.6 % of variation within *S. erinaceieuropaei* and 0.1 to 1 % within *Diphylobothrium* species (Miyadera *et al.*, 2001; Okamoto *et al.*, 2007). Besides, in platyhelminth parasites, the *cox1* gene accumulates substitutions more quickly than the ribosomal internal transcribed regions. The fast introgression and bi-parental inheritance of mitochondrial DNA can lead to divergent lineages within species (Vilas *et al.*, 2005). In this sense, to confirm the morphological result with molecular information, other genetic markers as *nad4* and *cytb* should be included in future studies (Liu *et al.*, 2012).

Most molecular studies of *Spirometra* sp. have been carried out in Asia, and 71 sequences of the *cox1* gene have been reported. In contrast, only three sequences of this gene have been reported in Australia. No molecular analysis of *Spirometra* parasites had been performed in South America. Thus, in this study, we present the first molecular characterization of *Spirometra* sp. eggs from Pampas foxes from Buenos Aires Province, Argentina.

Wild animals play a central role in maintaining the life cycle of *Spirometra* sp. In this regard, the identification of *Spirometra* species in the potential definitive host is necessary. Some *sparganum* larvae may invade other tissues, causing sparganosis, with the most severe disease occurring when larvae invade the brain (cerebral sparganosis). In Argentina, three cases of sparganosis have been diagnosed. The three cases were diagnosed in individuals from bordering countries. Two of the cases occurred in adults, one with brain localization (Boero *et al.*, 1991) and the other with cutaneous localization (De Roodt *et al.*, 1993), whereas the third case occurred in a child (Jones *et al.*, 2013). In Argentina, no local cases of sparganosis have been detected yet.

Of all the cases of sparganosis reported, cerebral sparganosis represents 2.3 % but its impact on our environment is relativized due to the absence of typical clinical manifestation and because the diagnosis is postsurgical and histopathological (Duggal *et al.*, 2011). On the other hand, it is necessary to assess the presence of these parasites in animals that are in close contact with humans. Furthermore, the knowledge about *Spirometra* sp. prevalence in wild animals from Argentina is necessary because of the ongoing changes in the environment that affect parasite ecology and transmission dynamics. Recently, a prevalence of 21.8 % has been reported in Pampas foxes from rural areas of Buenos Aires province (Scioscia *et al.*, 2014). Pampas foxes inhabit areas highly modified by extensive ranching and agricultural activities (Lucherini *et al.*, 2004). This situation influences these animals and its environment. In that sense, humans and Pampas foxes often share the same niche, and thus humans are exposed to the parasites present in these wild mammals. This could represent a risk for human health. The morphological description of the *Spirometra* adult allows the identification of the genus and species. However, in order to obtain *Spirometra* spp. adult specimens, the definitive host must be dead to extract and examine the small intestine. In this sense, egg

isolation from wild mammal feces results a faster and less invasive method. However, egg species determination through morphological characterization is difficult, but the molecular tools provide additional information to complement identification of the genus and species of *Spirometra*. In addition, the use of non-invasive methods through parasite egg identification from feces is helpful to know the status of this zoonosis and the distribution and frequency of the main parasites in the ecosystem.

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