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Molecular identification of *Nematodirus spathiger* (Nematoda: Molineidae) in *Lama guanicoe* from Patagonia, Argentina

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Summary

The guanaco (*Lama guanicoe*) is the major inhabitant and the largest wild artiodactyl in Patagonia. The introduction of invasive species into its ecological niche poses ecological risks, since invasive species may introduce harmful parasites to this native species. In this work, filariform larvae of the *Nematodirus* genus were found in feces of guanacos from the Perito Moreno National Park in Argentina. All species were characterized according to morphological features and molecular analyses using ribosomal DNA (rDNA). For the molecular analysis, rDNA fragments were amplified by PCR and then sequenced. The results of the BLASTN comparison threw a 99 % of identity with *Nematodirus spathiger* and 97 % with *N. helvetianus*, suggesting that *N. spathiger* is the infecting parasite. *Nematodirus spathiger* together with *N. filicollis* and *N. battus* causes diarrhea and deaths in sheep and, in some cases, in South American camelids. The availability of more accurate diagnostic methods such as PCR could improve the control measures for gastrointestinal helminthiasis.

Keywords: filariform larvae; coproparasitology; PCR; ribosomal DNA (rDNA)

Introduction

The guanaco (*Lama guanicoe*) is the major artiodactyl that inhabits in Patagonia. Until the Pleistocene-Holocene Transition, other large artiodactyls inhabited Patagonia along with the guanaco. At least five archaeological sites from Southern Patagonia show an association between *L. guanicoe* and *L. gracilis* (extinct species) (Cajal *et al*., 2010). The guanaco population extends from Peru at 8 ºS to the Isla Navarino (archipelago of Tierra del Fuego, Chile) at 55 ºS (Wheeler, 1995). Nowadays, guanacos occupy a 40 % of the original area that they inhabited at pre-Hispanic times (Franklin *et al*., 1997), with about 840,000 individuals in all (González *et al*., 2006), from which 400,000 inhabit in Patagonia (Amaya *et al*., 2001).
Parasites act as density-dependent factors regulating host population sizes (Anderson & May, 1979), particularly the wild species population (Tompkins & Begon, 1999). Wild animals are susceptible to pressures from human activity (direct or indirect). For instance, the invasive species, which are product of human activity, that are placed into the guanaco ecological habitat may introduce harmful parasites to this, and others, native species (Prenter *et al*., 2004). These invasive species, therefore, may cause a high impact in wildlife (Scott, 1988).
A wide range of parasites have been reported for *L. guanicoe* from Patagonia by coproparasitological studies, necropsy examinations or through gastrointestinal lavages. Nematodes of the Trichostrongyloidea Superfamily are the gastrointestinal helminthes most frequently reported in guanacos: *Cooperia* (Larrieu *et al*., 1982; Navone & Merino, 1989), *Trichostrongylus* (Larrieu *et al*., 1982; Navone & Merino, 1989; Karesh *et al*. 1998; among others), *Marshallagia* (Navone & Merino, 1989; Karesh *et al*., 1998; Beldomenico *et al*., 2003), *Haemonchus* (Navone & Merino, 1989), *Nematodirus* (Larrieu *et al*., 1982; Navone & Merino, 1989; Beldomenico *et al*., 2003; Correa *et al*., 2012; among others) and *Ostertagia* (Navone & Merino, 1989). *Nematodirus battus*, *N. filicollis, N. spathiger* and *N. lanceolatus*, within the *Nematodirus* sp., have been reported through gastrointestinal lavage inspection (Larrieu *et al*., 1982).
The life cycle of the Trichostrongyloids parasites involves a rhabditiform larva (L1), which moults and matures in the infective filariform larva (L3). These stages are found in the pasture and the infective form, L3, enters in the susceptible host during its feeding, closing the cycle when it matures in the host intestine (Anderson, 2000). Hence, these parasites represent a risk to wild artiodactyls because of their eating habits (Leguía & Casas, 1999).

The specific diagnostic is important to study the transmission patterns of specific parasites in order to evaluate the introduction and movement of gastrointestinal helminthes of wild and domestic invasive species. The aim of this work was to identify the genus and species of larvae isolated in coproparasitological samples of wild guanacos from Patagonia. The analysis of genus and species was performed through molecular tools of DNA purification and PCR amplification of ribosomal DNA fragments (rDNA).

Materials and methods

The feces of guanacos were collected in the Río Robles valley in the Perito Moreno National Park, Santa Cruz province, Argentina. The samples were taken by members of the National Park Board of Management during the month of May (2011). Groups of 3 to 8 dry pellets were rehydrated with 0.5 % aqueous trisodium phosphate (Callen and Cameron, 1960). Then, the pellets were crushed and disintegrated with 0.2 % v/v tween 20 by vortex for 1 min. The processed pellets were sieved using 290 µm nylon mesh and they were allowed to settle for 3 h when sediment was formed. This recovered pellet was kept in 70 % v/v Ethanol at 4 ºC.

Microscopic examinations for parasite identification were performed under a light microscope (100x magnification). A total of 30 µl of sediment were mixed with a glycerin drop to avoid desiccation of the preparation and this mix was then placed on slides. No coverslips were used. Larvae were manually isolated with a capillary tube that acts as a Pasteur pipette and then they were washed by subsequent transfers of a drop of sterile phosphate buffered saline (PBS) 1X on the slide. The larvae were continuously kept in 2 µl of sterile PBS 1X.

The DNA extraction was performed by lysis-PCR method (Gasser *et al*., 1993) with modifications. Briefly, a final volume of 25 µl containing fragments of larvae was agitated by vortex during 1 min in 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 $MgCl_2$) with a subsequent ice incubation for 10 min. Then, the samples were digested with 400 µg/ml of proteinase K (Biobasic) for 2 h at 56 ºC and over night at 37 ºC. Finally, the processed samples were boiled for 15 min to inactivate the proteinase K and 50 µl of sterile ultrapure water were added. After centrifugation at 2,000 X g for 5 min, the supernatant was kept at -20 ºC for subsequent use in PCR. A negative control of DNA extraction was included.

For the molecular identification of larvae, the rDNA fragment including the 18S 3´end, ITS-1, ITS-2, the 5.8S subunit or the 28S 5' end was amplified by PCR using the primers NC5 (forward) and NC2 (reverse) (Gasser *et al*., 1996). PCR amplifications were performed by triplicates with 2 µl of DNA in a final volume of 25 µl. The amplifications were made using 0.65 units of *Taq* DNA Polymerase (Fermentas) in 10X *Taq* DNA polymerase buffer (Fermentas), 2 mM of $MgCl_2$ (Fermentas), 200 µM of each dNTP (Finnzymes) and 0.4 mM of each primer. PCR conditions were as follows: an initial denaturing step at 94 ºC for 3 min, followed by 40 cycles at 94 ºC for 1 min, 55 ºC for 1 min, and 72 ºC for 1 min, and a final extension step at 72 ºC for 10 min. Negative PCR controls were added. Triplicates of the specific fragment were sequenced and the obtained sequences and chromatograms were analyzed using the BioEdit v7.2.0 program. The obtained consensus sequences 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).

Results and discussion

The optical microscopy observation allowed us to identify nematode infective filariform larvae with a shell, which probably belongs to the *Nematodirus* genus (Molineidae Family) (Fig. 1) because of the tail length and the shape of its anterior region (Niec, 1968; van Wyk & Mayhew, 2013).

Three 849 bp identical sequences corresponding to rDNA fragments amplified by PCR were obtained from the three analyzed larvae. The BLASTN analysis of each consensus sequence with nonredundant nucleotide sequences from the NCBI databases showed a 99 % of identity with other *Nematodirus spathiger* sequences (AF194144.1, AF194143.1 and AF194128.1) and 97 % of identity with *N. helvetianus* sequences (AF194127.1, AF194142.1, AF194141.1 and JQ828846.1). Both species are very similar genetically, showing differences of 3.9 % between the ITS-2 nucleotide sequences (Newton *et al*., 1998). Our results are in line with previous findings in the literature (Newton *et al*., 1998). Therefore, the larvae found from guanaco feces in the present research were identified as *N. spathiger* and the sequences were submitted in the GenBank (KF305647.1).

Generally, the *Nematodirus* sp. parasites have been identified by methods based on morphological characters of adult males such as the spicule tips, copulatory bursae and the distribution of bosses on the inside surface of the copulatory bursa (Becklund & Walker, 1967). Lichtenfels and Pilitt (1983) created a key to distinguish between females and males of the different species of the *Nematodirus* genus according to cuticular ridge patterns. However, the identification of *Nematodirus* species based on morphological characters is difficult when immature parasite remains from feces are studied. Therefore, the culture of larvae from fresh feces is useful to obtain filariform larvae for diagnosis, but unfortunately the culture frequently fails.

The identification of parasites is facilitated by molecular techniques since they provide useful tools for taxonomic classification of a wide range of parasites. In this regard, the rDNA has been used as a genetic marker for the differentiation between genera, including species of different taxa (Holterman *et al*., 2006; Carreno *et al*., 2012). For instance, *N. spathiger*, *N. helvetianus*, *N. filicollis* and *N. battus* have been identified through the PCR amplification and sequencing of the internal transcribed spacer (ITS)-2 (Newton *et al*., 1998). In addition, PCR amplification and

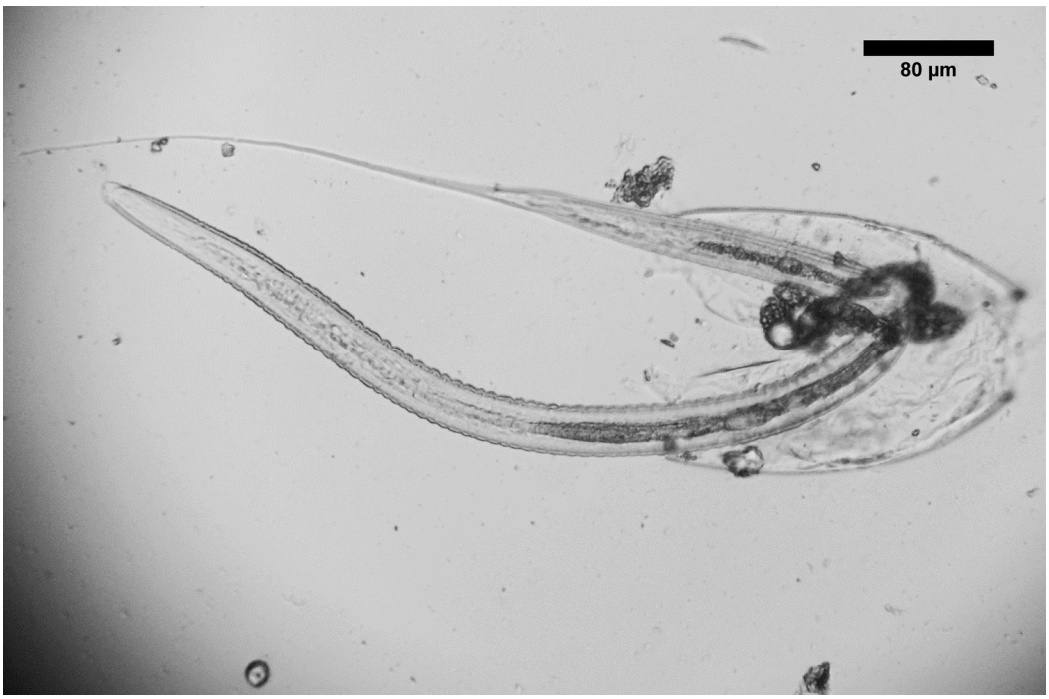

Fig. 1. *Nematodirus* filariform Larvae L3 found by light microscopy. A – 400x. Scale Bar = 80 µm.

sequencing of rDNA regions containing the 3´ end of the 18S gene, ITS-1, ITS-2, the 5.8S subunit and the 5´end of the 28S gene have been widely used in *Nematodirus* sp. identification (Nadler *et al*., 2000). This rDNA fragments have been used in specific diagnosis of Trichostrongyloidea parasites found in wild and domestic ruminants (Gasser *et al*., 1999; Nadler *et al*., 2000).

In the present research, we demonstrated that the rDNA amplification is useful for the specific diagnosis of filariform larvae previously identified as *Nematodirus* sp. by optic microscopy.

The effects of *Nematodirus* spp. on the host vary according to the intensity of the infection. For instance, *N. spathiger* with *N. filicollis* and *N. battus* cause diarrhea and deaths in sheep and, in some cases, in South-American camelids (Leguía & Casas, 1999; Marquardt *et al*., 1959). *Nematodirus* spp. larva hatching is associated with the possibility of feces to disintegrate so that the eggs that are captured within them can be released (Gibson & Everett, 1981). The rain is important factor for disintegration of feces but it does not increase egg hatching or filariform larvae migration. Studies done in USA in similar latitudes of Perito Moreno National Park showed survivability through the time indicating that direct sunlight and high temperature of soil are the most limiting factors (Marquardt *et al*., 1959). Therefore, these parasites are highly resistant to adverse conditions in the environment.

The infection of *N. spathiger* parasites of guanacos from Perito Moreno National Park could be explained by the invasion by sheep of the ecosystem wherein the guanaco inhabits and the effect of resistant infective forms in the environment. These findings warn about the difficulty in the control of parasitism derived from invasive species as sheep within a protected area and particularly in species with high mobility as the guanaco. The availability of more specific identification methods such as PCR together with the morphological analysis could help in improving the diagnosis to these parasitic diseases. Besides, the knowledge of these parasitic species would allow the design of strategies for the prevention and control of disease outbreaks, which will contribute to the conservation of wildlife.

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