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Short Communication

First mitochondrial and nuclear DNA sequences of *Lamanema chavezi* (Nematoda: Molineidae): Novel findings to improve its identification in feces from South American camelids

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ABSTRACT

Lamanema chavezi (Family Molineidae) is a parasitic nematode of South American camelids (SACs). A few studies have reported this parasite in SACs, mainly in domestic camelid species (llama and alpaca). Parasite identification by means of copro-parasitological methods is non-invasive and allows performing epidemiological studies. However, egg misidentification and difficulty to culture third-stage larvae do not allow identifying nematodes to species level. In contrast, molecular tools allow identifying eggs of gastrointestinal nematodes more accurately. However, the little genomic information available in databases for some species prevents an accurate diagnosis. In the present work, *L. chavezi* females present in feces of llamas from northwestern Argentina were molecularly characterized to obtain genomic information and improve parasitological diagnosis of *L. chavezi*-like eggs present in guanaco feces from southeastern Argentina. An 833-bp fragment of nuclear ribosomal DNA (rDNA) and a 434-bp fragment of the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene from both *L. chavezi* females and eggs were amplified and sequenced. Comparison between sequences from females and eggs showed 99–99.6% identity to rDNA and 99.5–96.1% to the *cox1* gene fragments, confirming egg morphological assignment. A higher divergence between sequences was observed in the *cox1* fragment, with a maximum variation of 3.9%. The examination of eggs found in guanaco feces from southeastern Argentina and their specific molecular identification represent the first record for this host in Argentine Patagonia and contribute to improving the diagnosis of gastrointestinal nematodes in SACs, mainly in wild camelids.

South American camelids (SACs) include both domestic (llamas, *Lama glama* Linnaeus 1758, and alpacas, *Lama pacos* Linnaeus 1758) and wild (guanacos, *Lama guanicoe* Müller 1776, and vicuñas, *Vicugna vicugna* Molina 1782) species. The guanaco is the most widely distributed, and its population is highest in Patagonia. However, in the last century, its geographic distribution has been reduced mainly due to the advances of the livestock and agricultural frontiers [1]. Guanacos and other SACs can be parasitized by host-specific nematodes present in their native habitats (*Graphinema aucheniae*, *Mazamastrongylus* (=*Spiculopteragia*) *peruvianus*, *Camelostrongylus mentulatus*, *Nematodirus lamae*, *Lamanema chavezi*, and *Trichuris tenuis*) [2]. However, since livestock was introduced, cattle have competed with guanacos for food resources and have also introduced new parasites in their distribution area.

Lamanema chavezi Becklund 1963 (Strongylida: Molineidae) is the only member of the genus *Lamanema*. The life cycle of *L. chavezi* is direct, involving ingestion of infective larvae (L3) by susceptible hosts during foraging. Larval stages L3 and L4 penetrate into the intestinal wall and migrate to the liver. Maturation to adult stage and sexual reproduction occur in the small intestine, and then eggs are released in feces [3].

A few studies have reported the identification of *L. chavezi* in current SACs, mainly in domestic camelid species [e.g. 4,5]. In addition, in wild camelids, *L. chavezi* infection has been reported in a single presumed case in guanacos from Chilean Patagonia [6], in guanacos and vicuñas from northwestern Argentina [7], and in guanacos from Peru [8]. The low prevalence of *L. chavezi* in SACs may be due to several ecological factors as well as to the misidentification of eggs found in feces. Morphological identification of *L. chavezi* eggs is difficult because of their similarity to eggs of *Nematodirus* species. In this sense, molecular tools are useful to identify eggs more accurately by comparisons with available genomic data of related nematodes. In the last years,

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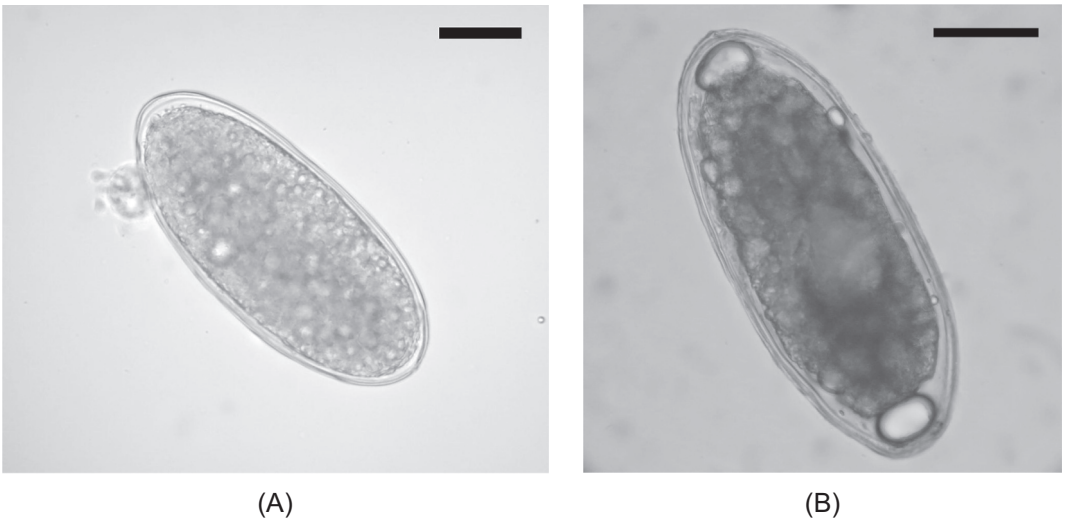

Fig. 1. (A) Egg obtained from the uterus of a *Lamanema chavezi* female. (B) Egg isolated from feces of guanaco (*Lama guanicoe*) from Chubut Province, Argentina, tentatively assigned to *L. chavezi*. Bar = 40 μm.

data on nuclear and mitochondrial DNA sequences of several gastrointestinal nematodes of ruminants have increased. For instance, the mitochondrial genomes of the molineids *Nematodirus spathiger* and *Nematodirus oiratianus* [9] have been published. However, a few DNA sequences of SAC-specific nematodes, corresponding to ribosomal DNA sequences of *Camelostrongylus mentulatus*, are available in gene databases (https://www.ncbi.nlm.nih.gov/nuccore/).

Based on the above, the aim of the present work was to obtain the first nuclear and mitochondrial DNA sequences of *L. chavezi* to improve the identification of eggs isolated from camelid feces by noninvasive methods. This approach aims to extend the information about gastrointestinal parasites that could infect wild camelids.

A dead llama (*Lama glama*) with gastrointestinal symptoms belonging to a livestock farm (25° 05′35,00_S, 65° 34′03,72_W) of Chicoana department, Salta Province, northwestern Argentina was necropsied. Three adult females identified as *L. chavezi* according to their morphological characters (see below) were isolated from the small intestine. Adult length ranged from 12 to 15 mm and adults showed a defined cephalic vesicle and a small oral cavity with a dorsal conical tooth. *L. chavezi* males have very chitinized spicules, gubernaculum and characteristic bursa, while females contain a caudal spine and a didelphys uterus containing eggs with shape and size typical of *L. chavezi* [10]. Eggs in the distal end of the uterus of one of the three females were extracted.

On the other hand, feces from guanaco were collected in the Provincial Reserve *Cabo Dos Bahías* (44°55_S, 65°31_W), Chubut Province, southeastern Argentina during the 2018 summer season. Parasite eggs present in the feces were concentrated by spontaneous sedimentation. Sediments from guanaco feces containing eggs were observed under light microscope (400× magnification). Eggs tentatively attributable to *L. chavezi* by morphometric characters were manually isolated using a capillary tube under light microscope (100×). Three different egg pools (with three or four morulated/larvated eggs) were kept at −20 °C for molecular identification. Measurements of 20 eggs (400×) isolated from one mature female and 20 eggs isolated from guanaco feces were statistically compared. A multivariate analysis of variance (MANOVA) was performed using the software Past 3.17.

DNA from the three *L. chavezi* females was isolated for molecular characterization, as described in Petrigh and Fugassa [11], with modifications applied to adult stages: 30 μl of final volume for lysis and proteinase K incubation, and then 80 μl of ultrapure water (Invitrogen) for proteinase K dilution. On the other hand, DNA from the three different egg pools was isolated from guanaco feces as described in Petrigh et al. [12] A negative control was included in each DNA purification experiment.

Specific nuclear and mitochondrial DNA fragments were amplified by PCR and sequenced. The ribosomal DNA (rDNA) fragments including the 18S 3′ end, the complete internal transcribed spacer region (ITS-1 and ITS-2) sequences, the 5.8S subunit, and the 28S 5′ end were amplified by using oligonucleotide primers NC5 (forward) and NC2 (reverse) specific of a wide range of strongylid nematodes [13]. In addition, when the rDNA sequences could not be obtained, primers NC1 (forward) and NC2 (reverse) were used to amplify the ITS-2 fragments [14]. The mitochondrial fragments of the cytochrome *c* oxidase subunit 1 (*cox1*) gene were amplified by using primers JB3 (forward) and JB5 (reverse) [15], designed to amplify a specific *cox1* fragment of free-living and parasitic nematodes. A PCR reaction was performed in 25 μl containing 2 μl of DNA sample, 200 μM of each dNTP (Thermo Scientific), 0.4 μM of each primer, 0.65 units of Go*Taq* DNA Polymerase (Promega) in 1× Go*Taq* Buffer green, and 3 mM of $MgCl_2$. The PCR conditions were the same as those reported in Petrigh and Fugassa [11]. A negative control was included in all PCR experiments. Specific DNA fragments were sequenced and chromatograms were analyzed using BioEdit v7.2.0 (copyright © 1997–2013, Tomm Hall, Ibis Biosciences). The consensus sequences obtained were compared with the Non-redundant Nucleotide Database by using the BLASTN algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of the National Center for Biotechnology Information (NCBI, USA). To test identities between all sequences, a pairwise analysis was carried out by using the LALIGN program (https://embnet.vital-it.ch/software/LALIGN_form.html).

Morphometric analysis showed that the eggs isolated from the uterus of the *L. chavezi* female (Fig. 1A) and those found in guanaco feces were similar (Fig. 1B). The measures were 167.5–197.5 (183.9 ± 7.71; $n = 25$) × 77.5–110-0 (84.55 ± 7.91; n = 25) μm and 167.5–196.25(180.25 ± 7.15; $n = 20$) × 67.5–82.5(73.69 ± 4.52; n = 20) μm, respectively. Statistical comparison between eggs isolated from females and those isolated from guanaco feces showed no significant differences ($p = 0.1878$, $\alpha = 0.05$). Eggs obtained from *L. chavezi* females were colorless, whereas eggs isolated from guanaco feces were brown-yellowish probably due to the fecal pigments, as in the case *Nematodirus battus* eggs [16].

In the present work, we were able to obtain the first genomic information of *L. chavezi* by sequencing an 833-bp fragment of rDNA, a 320-bp fragment of ITS-2 and a 434-bp fragment of *cox1* from female isolates (GenBank Accession numbers: MG598426, MG598418-

MG598420, and MG598415-MG59847, respectively). Ribosomal DNA sequences analyzed by the BLASTN algorithm showed 79–78% identities with other genera of the family Molineidae: *Nematodirus* spp. and *Oswaldocruzia filiformis*. Nevertheless, similar identities, between 81 and 79%, with genera of other families from the order Strongylida (*Travassostrongylus* sp., *Spiculopteragia houdemeri*, *Teladorsagia circumcincta*, *Ostertagia* sp., among others) were also observed.

So far, the taxonomic classification of *L. chavezi* based on morphological characters of adult females has been controversial. Durette-Desset [17] considered *L. chavezi* as a transitional link between the subfamilies Nematodirinae and Molineinae, whereas Rickard and Hoberg [18] transferred this species to the subfamily Molineinae, and did not consider it as an intermediate form between Nematodirinae and Molineinae. The last re-description supported the taxonomic location into the subfamily Molineinae [10]. However, in the present work, the genomic information did not support the inclusion of *L. chavezi* in the family Molineidae. Thus, more sequences of molineids are necessary to complement the taxonomic classification.

DNA sequences obtained from *L. chavezi* females were used to identify the *L. chavezi*-like eggs isolated from guanaco feces. Ribosomal DNA sequences from eggs (MG598427 and MG598428) were highly similar to those from eggs obtained from *L. chavezi* females. Sequences from eggs from females and those from guanaco eggs showed identities between 99.9 and 99.6-%. Therefore, egg pools from guanaco feces were assigned to *L. chavezi*, representing the first accurate identification of eggs morphologically similar to this molineid.

A preliminary study was then performed to analyze the genetic variability between *L. chavezi* isolates from northwestern llama and southeastern guanaco. Since ITS regions are the most variable nuclear loci in nematodes [19], ITS (ITS-1 and ITS-2) sequences have been widely used to identify nematode species [e.g., 14]. Thus, a fragment of ITS-2 was amplified and sequenced from eggs from guanaco feces and nucleotide sequences were submitted to GenBank (MG598424 and MG598425). Pairwise analysis between the ITS-2 sequences from *L. chavezi* isolates from northwestern llama and southeastern guanaco showed up to 1% divergence. The *cox1* gene sequence is more accurate than ITS sequences to identify closely related species due to the fast evolution rate by nucleotide substitution accumulation. Thus, it is a useful tool to identify cryptic species [19]. A 434-bp *cox1* gene fragment from all isolates of *L. chavezi* from guanaco eggs was amplified and sequenced (MG598421-MG598423). Comparison between sequences from llama and guanaco showed divergences from 0.5% to 3.9%. Differences between 0.2 and 0.7% were obtained between isolates from guanaco, whereas differences of 0.7%, 3.7% and 3.9% were obtained among the three isolates from llama, showing that these differences within isolates from the same area do not respond to geographical variation.

Parasitological studies previously performed in guanacos from the Provincial Reserve *Cabo dos Bahías* [20,21] reported the presence of Molineids (identified as *Nematodirus* sp.) and trichostrongylids but not that of *L. chavezi*. This work is reporting the nucleotide sequences of *L. chavezi*, providing the first partial characterization of mitochondrial (*cox1*) and nuclear (rDNA, ITS-2) genes available in public databases. These results also allowed obtaining the first molecular copro-diagnostic in SACs. Specifically, the examination of the eggs found in guanaco feces of Chubut Province, Argentina, and the molecular identification of *L. chavezi* represent the first record for this host in Patagonia.

Currently, the diagnosis of gastrointestinal nematodes in SACs is based on fecal egg counts and nematode larval culture. These coproparasitological studies offer a non-invasive tool for the parasitological monitoring of guanaco populations. However, the one-step PCR and sequencing reported in the present work represent a simple and more accurate method to complement the morphometric identification of eggs from feces, contributing to improving copro-parasitological studies of guanacos in Patagonia.

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