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Some larval morphological characteristics of *Camelostrongylus mentulatus* and *Nematodirus spathiger*

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Abstract

Monitoring of gastrointestinal nematode parasites in ruminants (domestic and wild) is often based on fecal examination techniques, looking for excreted eggs and larval forms using morphological keys. These, are more available in domestic ruminants, in which helminths are widely studied, than in wild ruminants. This study tried to provide certain morphological elements that will help to recognize the L3 larvae of *Camelostrongylus mentulatus* and *Nematodirus spathiger* that could parasite either domestic or wild ruminants. For that, we resorted first to the culture of L3 larvae from fecal samples taken from African antelopes, and second by the microscopic characterization of each isolated larval morphological pattern previously identified by sequencing of its internal transcribed spacer (ITS-2) regions of the ribosomal DNA. The results of different microscopic captured images showed that *Camelostrongylus mentulatus* larva is 16 intestinal cells that measuring approximately 820 µm length, ≈ 25 µm wide, and ≈ 47 µm for its sheath tail extension and by this be closer to *Teladorsagia circumcincta* characteristics. For *Nematodirus spathiger*, it possesses 8 gut cells and measuring about 1020 µm long, ≈ 25 µm wide, and ≈ 143 µm for its sheath tail extension with specific tail appendages. Have done this, we were able to get some clarifications on the morphology of the studied larvae, and we believe thus that this study will contribute to the establishment of morphological identification keys especially for parasitic nematodes of wild ruminants.

Key words: Morphological characteristics, L3 larvae, *Camelostrongylus mentulatus, Nematodirus spathiger*, antelopes.

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1. Introduction

Gastrointestinal nematodes are common parasites of a wide range of mammals, especially herbivores. They have a direct cycle with two phases: parasitic phase and free-living phase. The parasitic phase during which the adult nematode is generally located in the digestive tract lumen of the parasitized host, where the female nematode lays eggs that will be excreted in the environment through the feces. The free-living phase takes place outside the animal, which starts with the hatching of eggs producing first stage larva that will molt to the second then the third-stage larva (L.3). This latter is the infective stage that will infect the host by oral route in the pasture when grazing and will molt inside the animal to the fourth then the fifth stage larva before becoming adult nematode (Anderson, 2000).

Ruminants are subject to such parasitic infections, especially when they are sequestered in limited space pasture dimensions that are continuously contaminated by L.3 infective larvae (Molento et al., 2016). Across the world, nematode parasites are also widely prevalent in wild ruminants (Hoberg et al., 2001). The health impact of the infection varies from subclinical to clinical manifestations, which could be exacerbated by sequestration stress-related factors, particularly when animals are held in captivity (zoos) or in semi-captivity (parks and reserves) conditions (Arneberg et al., 1998; Flach, 2008). The problem presents itself even more pointedly when dealing with threatened species, in which we cannot tolerate such health disorders, and we are therefore forced to adopt an ecologically adequate sustainable health management strategy combining diseases control and biodiversity preservation (Aguirre et al., 2002; Delahay et al., 2009).

The control of those nematodes, in wild ruminants, would necessarily entail possession of valid and non-invasive laboratory tools allowing us to identify them through their egg and larval forms. However, the currently available laboratory guides are developed for use in domestic ruminants and are generally based on morphological characteristics of eggs and infective larvae (Thienpont et al., 2003; Van Wyk & Mayhew, 2013). In addition to that, the molecular characterization is employed as an incontrovertible complement tool, to obtain reliable results for parasites identification and phylogenetic analysis, but not always affordable especially in developing countries (Gasser et al., 1993; Morgan et al., 2005).

The current study attempts to contribute to closing this gap by describing the larval morphological patterns of *Camelostrongylus mentulatus* and *Nematodirus spathiger* isolated from fecal cultures of the Sub-Saharan antelopes hosted at Souss-Massa National Park (SMNP) in Agadir (Morocco).

2. Materials and methods

Larvae recovery and morphological characterization

Animals are hosted at the Souss-Massa National Park, and ten fecal samples were picked up from the ground for each antelope species: *Addax nasomaculatus*, *Gazella dorcas,* and *Oryx dammah.* In the laboratory, two pools of five crumbled fecal samples were prepared in Petri dishes for each antelope species and submitted to the larval culture at 27 °C for 7 days for *Camelostrongylus mentulatus* L.3 larvae, and for 14 days for *Nematodirus spathiger* L3 larvae, and finally the larvae were recovered by Baermann funnel method, as preconized in Taylor et al., (2016). Larvae were observed under a digital optic microscope. Digital images were captured with Motic® Plus 2.0 software and processed using GIMP v.2.10.14 software. For each characterized pattern in its antelope host, ten individual candidate larvae were selected for further DNA extraction and molecular analysis.

DNA extraction, PCR and sequencing

The DNA was extracted from each one unique larva using QIAGEN QIAamp DNA Mini Kit (QIAGEN Lake Constance GmbH, Germany) after maceration by zirconium beads. PCR reactions were carried out using universal primers (NC1: 5'ACGTCTGGTTCAGGGTTGTT-3' and NC2: 5'-TTAGTTTCTTTTCCTCC GCT-3') described by Gasser et al., (1993). The reactions were performed using the AgPath-ID™ PCR kit (Ambion®-Applied Biosystems) in a total volume of 50 µl per reaction, which contained 10 µl of DNA extract, 2 µl of each 10 µM primer, 2 µl of 25X RT-PCR Enzyme Mix, 25 µl of 2X RT-PCR Buffer and 9 µl of water. The thermal profile began with an initial denaturation at 94 °C for 10 min followed by 40 cycles, 45 secs each, of denaturation at 94 °C, annealing at 55°C and extension at 72 °C, with a final extension at 72 °C for 5 min. PCR amplicons were visualized by electrophoresis in a 2 % agarose gel; positive samples were identified by the presence of a band of ≈ 300 bp. The positive products were sequenced, by a sequencing provider service (Sanger method), using the same primers. Generated sequences were blasted for searching similarities in the NCBI GenBank database and phylogenetically analyzed using MEGA v7.0 software (Kumar et al., 2016). Three sequences (MH047837-MH047845-MH047853) for *Camelostrongylus mentulatus*, three sequences (MH047829, MH047836, MH047851) for *Nematodirus spathiger* and one sequence (MH047834) for *Teladorsagia circumcincta* from local sheep, were deposited in GenBank.

3. Results and discussion

Molecular analysis

Phylogenetic analyses allowed us to identify two different isolates larval patterns, which are *Nematodirus spathiger*: MH047829 from *Gazella dorcas*, MH047836 from *Oryx dammah* and MH047851 from *Addax nasomaculatus*; and *Camelostrongylus mentulatus*: MH047837 from *Gazella dorcas*, MH047845 from *Oryx dammah* and MH047853 from *Addax nasomaculatus*; in addition to MH047834 identified as *Teladorsagia circumcincta* from local domestic sheep. The three sequences of *N. spathiger* cluster well with the reference sequences (MG651880 and Y14012) with which they show when aligned together, an overall p-distance score of 0.006, corresponding to 99.4 % of identity. The same for *C. mentulatus* sequences, that cluster in the same clade with reference sequences (Y14819, KY930444, and MG651891), with which they show, when aligned, an overall p-distance score of 0.007, corresponding to 99.3 % of identity. Figure 1 shows the relationship tree for each parasite species.

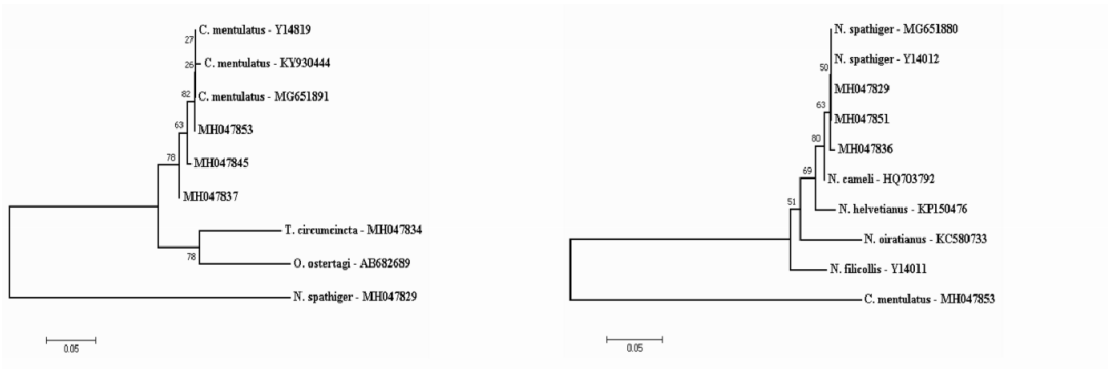

Fig. 1. The phylogenetic relationships of *Camelostrongylus mentulatus* (left) and *Nematodirus spathiger* (right) based on ITS-2 sequences of the rDNA, using the Neighbor-Joining method with the Tamura 3-parameter method. The percentages of replicate trees in the bootstrap test (1000 replicates) are shown next to the branches. The analysis involved 9 nucleotide sequences for *C. mentulatus* (3 from antelopes, one from local domestic sheep and 5 from Genbank) and 10 nucleotide sequences for *N. spathiger* (3 from antelopes and 7 from Genbank). Evolutionary analyses were conducted in MEGA7.

Morphological characteristics

The sheathed L.3 larvae, molecularly identified as *Camelostrongylus mentulatus*, are square-headed larvae, possessing 16 gut cells, and being on average ≈ 820 µm long for ≈ 25 µm approximately wide, and ≈ 46 µm for the length of the sheath tail extension. The tail itself is uniform and does not show any relief (Fig. 2).

For the sheathed L.3 larvae of *Nematodirus spathiger*, they are head rounded having eight intestinal cells measuring approximately ≈ 1020 µm long and ≈ 25 µm wide. Its sheath tail extension measures ≈ 143 µm with a tail shape that shows a specific rising appendage amid terminal reliefs (Fig. 3 & 4).

Fig. 2. Images of *Camelostrongylus mentulatus* third-stage larva, showing the morphology of the sheathed larva with 16 intestinal cells (a), the exsheathed square head (b) and the sheath tail extension (c)

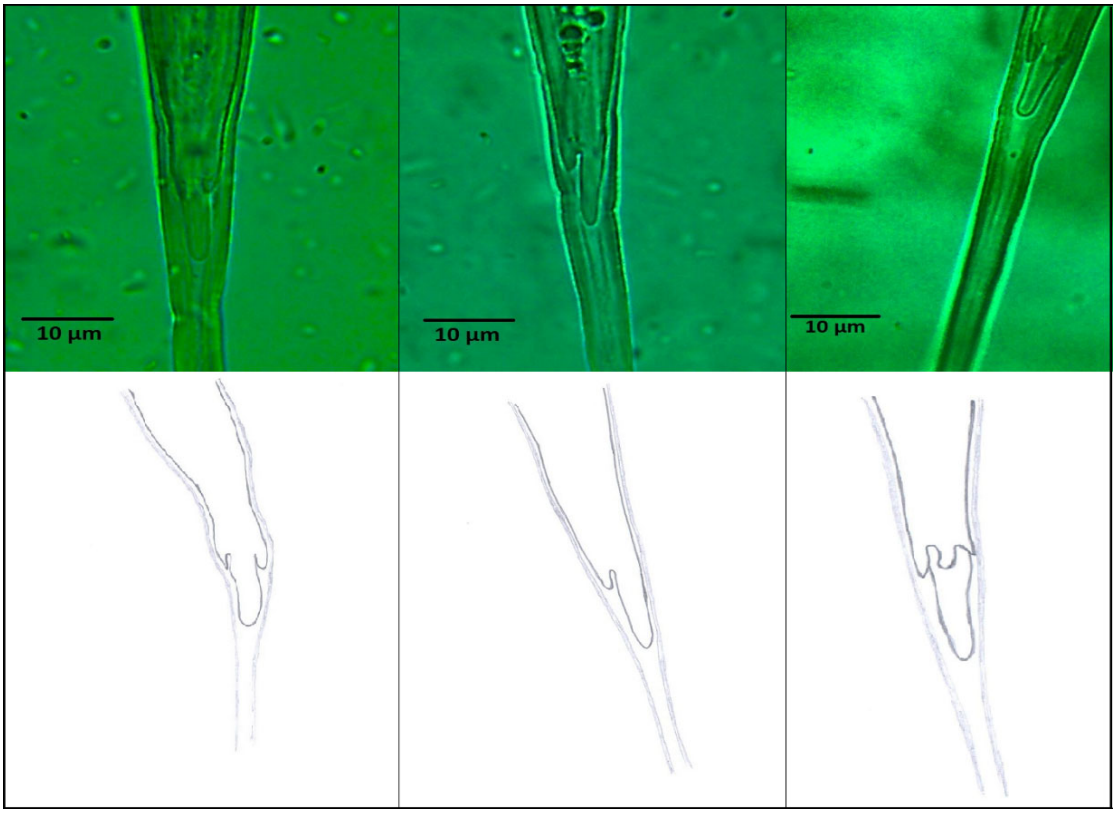

Fig. 3. Photos of different views (dorsal, lateral, ventral) of *Nematodirus spathiger* third-stage larva tail and their correspondent diagrammatic representations

We used also this opportunity to take pictures of *Nematodirus* egg evolution (Fig. 4).

Belonging to the subfamily of Ostertaginae, *C. mentulatus* is a parasite nematode of the abomasum in many ruminant hosts (Anderson, 2000). Responsible for causing ostertagiosis like syndrome (Jones et al., 2018), it is worldwide prevalent, in small ruminants (Carrau et al., 2017), in camelids (Rashid et al., 2019), in girafids (Kyriánová et al., 2017), and in antelopes (Pauling et al., 2016). When analyzing the morphological characteristics of the third-stage larva of *C. mentulatus*, we noticed that the shape and the dimensions are so closer to those of *Teladorsagia circumcincta* third-stage larva, which is also an Ostertaginae parasite nematode mainly encountered in sheep. In fact, in Van Wyk and Mayhew, (2013), the reported total length of *Teladorsagia circumcincta* is about 830 µm and 35 µm for its sheath tail extension. Such larval morphological similarity, especially for the tail extension, is also noticed between *Teladorsagia* sp. and *Trichostrongylus axei* in previous studies in domestic ruminants (Hansen & Perry, 1994; Van Wyk & Mayhew, 2013), which makes recognizing these larvae difficult and recourse to the molecular identification is, therefore, essential (Roeber & Kahn, 2014).

Very prevalent globally, *N. spathiger* is a parasite nematode of the small intestine, widely shared between domestic and wild ruminants (Hoberg et al., 2001). It has been reported in *Lama guanicoe* (Petrigh & Fugassa, 2014), in domestic bovids (Oliver et al., 2014; Zhao et al., 2014), in many African wild bovids (Junker et al., 2015), and in Sub-Saharan antelopes (Said et al., 2017). The infections due to *N. spath-*

iger may go generally unnoticed except in juveniles where the larval stages migration cause tissue lesions within the intestinal mucosa, which is expressed by diarrhea and dehydration (Kaufmann, 2013). The morphology of *N. spathiger* L.3 infective larva was previously described in many studies in domestic ruminants, and can easily be distinguished from other *Nematodirus* (*N. fillicolis*, *N. battus*, *N helvetianus*) by its specific appendages on the larva tail (Audebert et al., 2004; Taylor et al., 2016); nevertheless, the current study may be considered as additional scientific support, especially in antelopes.

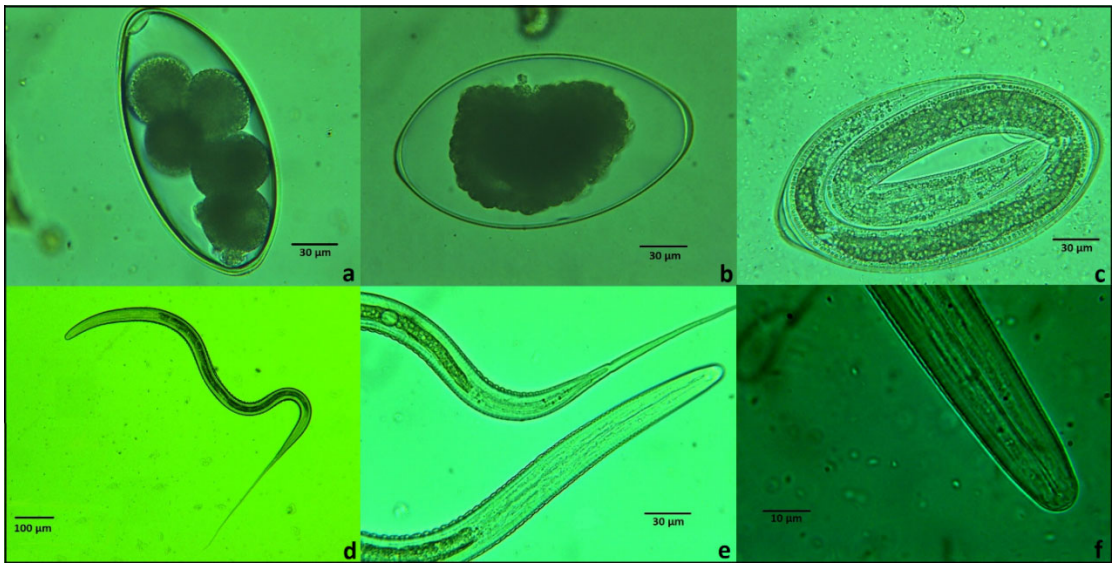

Fig. 4: Images showing *Nematodirus spathiger* egg evolution (8 blastomeres egg (a), morulated egg (b), larvated egg (c)), the third-stage larva pattern (d), the head and tail (e), and head morphology (f)

4. Conclusions

The control of such nematode parasites, which wild animals could be hosts and vehicles, must pass through their identification and monitoring. Thus, even though the molecular tool is more efficient for species identification and genome comparisons, the use of morphological characteristics as a tool for parasites identification is still low cost and more accessible for researchers, particularly in developing countries. Hence, we estimate that the current work, as a piece of the puzzle, will contribute to enriching the morphological and molecular data on ruminant parasitic nematodes.

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