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Research Note

Crenosoma striatum in lungs of European hedgehogs (*Erinaceus europeus*) from Portugal

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Summary

Crenosoma striatum is a host-specific metastrongiloid nematode causing respiratory tract disease in hedgehogs (*Erinaceus europaeus*). Since few studies have reported *C. striatum* in hedgehogs and little genetic data is available concerning this lungworm, this study aimed to determine the occurrence of *C. striatum* in a population sample of hedgehogs from Portugal, additionally providing morphological, histological and molecular data. From 2017 to 2018 a survey of infection was carried out in 11 necropsied hedgehogs. Worms were extracted from fresh lung tissues and microscopically evaluated. Molecular characterization of partial mitochondrial (12S rRNA) and nuclear (18S rRNA) genes was performed. The presence of lungworms in pulmonary tissues of five hedgehogs (45.5%) was detected. Morphological and histopathological analyses evidenced adult forms of nematodes consistent with *C. striatum*. Molecular characterization of 18S rRNA genes confirmed the classification as C. striatum. Also, novel genetic data characterizing the mitochondrial (12S rRNA) gene of *C. striatum* is presented.

This is the first report of *C. striatum* infection in hedgehogs of Portugal. The findings here reported provide new insights regarding the geographic distribution and the molecular identification of this lungworm species.

Keywords: *Crenosoma striatum;* hedgehog; lungworm; nematode

Introduction

European hedgehogs (*Erinaceus europaeus*) are hosts for a wide variety of parasites namely ticks, fleas, mites and helminths (Pfaffle *et al*., 2014). Several studies demonstrated various species of helminths parasitizing hedgehogs such as *Crenosoma striatum*, *Physaloptera clausa* and *Hymenolepis erinaceid* (Naem *et al*., 2015; Pavlovic & Savic 2017; Raue *et al*., 2017). *Crenosoma* (Nematoda: Metastrongyloidea) is a genus of lungworms of the family Crenosomatidae, and C*renosoma striatum* is a species known to invade trachea, bronchi, and alveolar ducts of hedgehogs (Beck 2007).

The European hedgehog is a synanthropic nocturnal species in Europe that feeds on gastropods like slugs and snails, and which act as definitive or paratenic host of several agents that pose a considerable risk for morbidity and mortality, particularly *C. striatum* (Gaglio *et al*., 2010; Hoseini *et al*., 2014; Riley & Chomel 2005). After a prepatent period of 21 days the worms become sexually mature and the first stage larvae (L1) can be found in the feces (Beck 2007). Hedgehog crenosomosis is generally characterized by weight loss, nasal discharge, increased respiratory effort, cough, and in severe cases death (Hoseini *et al*., 2014).

A growing body of literature have documented the presence of *C. striatum* in hedgehogs, namely in Turkey (Cirak *et al*., 2010),

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Poland (Mizgajska-Wiktor *et al*., 2010), Iran (Hoseini *et al*., 2014; Naem *et al*., 2015), Italy (Manzocchi *et al*., 2016), Serbia (Pavlovic & Savic 2017), Greece (Liatis *et al*., 2017) and Germany (Raue *et al*., 2017).

The aim of the present report was to evaluate the occurrence of *C. striatum* in a population sample of hedgehogs from Portugal., This study also describes the histological features associated with *C. striatum* pulmonary occurrence and provides the molecular characterization of partial 12S and 18S rRNA genes of *C. striatum*. To the best of our knowledge, no study identifying and characterizing *C. striatum* has ever been done in the Iberian Peninsula, and no characterization of the mitochondrial (12S rRNA) ribosomal gene has ever been performed.

Materials and Methods

From January 2017 to October 2018, all deceased hedgehogs (N=11) that were housed at a Rescue and Rehabilitation Center (RRC) in Porto, Portugal, were necropsied at the Veterinary Pathology Laboratory of ICBAS-UP. These animals had been collected from several Portuguese municipalities and sent for the RRC for rehabilitation. During the necropsy examination, representative samples of all macroscopic alterations detected, as well as others from apparently healthy tissues were collected, in order to identify the eventual cause of death and to evaluate the health status of the animals.

For parasitology, nematodes were extracted from fresh lung tissues for identification. Parasites were suspended in sterile saline (0.9 % NaCl) and microscopically examined under glass coverslips for morphological identification.

Nematode-infected animals were subjected to histological examination. Lung tissue samples were fixed in 10 % phosphate-buffered formalin (pH 7.0) for 24 h, routinely processed, embedded in paraffin wax, cut into 3 – 4-µm sections, and stained with hematoxylin and eosin (H&E). Slides were then analyzed using a Nikon Eclipse E600 microscope and tissues photomicrographs and measurements of the parasites for morphologic identification were taken using a digital image processing system (Nikon Digital DS-5M).

Genomic DNA from adult worms (one from each animal with lungworms) was extracted using a commercial kit (GRS Genomic DNA Kit – Tissue, Grisp, Portugal) in accordance with the manufacturer's instructions. Partial fragments of mitochondrial 12S rRNA (330 bp) and nuclear 18S rRNA (1700 bp) genes were amplified using two sets of primers (12SF: 5'-CGGGAGTAAAGTTTTGTTTAAACCG-3' and 12SR: 5'-CATTGACGGATGGTTTGTACCAC-3'; NC18SF1: 5'-AAAGATTAAGCCATGCA-3' and NC5BR: 5'-GCAGGTTCACCTACAGAT-3', respectively) (Latrofa *et al*., 2015). The PCR amplification was performed using KAPA Taq DNA polymerase (Kapabiosystems, Massachusetts, USA). Genomic fragments were amplified using the following conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 60 s; 55 °C for 60 s, 72 °C for 60 s; and a final extension at 72 °C for 7 min. The amplicons were purified (GRS PCR & Gel band purification kit, Grisp, Portugal) and bidirectionally sequenced, using the same primers as for PCR, employing the BigDye ® Terminator v3.1 Cycle Sequencing Kit [Applied Biosystems] in an automated sequencer (3130XL Genetic Analyzer, Applied Biosystems). Sequences were compared, using Basic Local Alignment Search Tool (BLAST – http://blast.ncbi.nlm.nih.gov/Blast.cgi), with those available in the GenBank database.

In order to investigate the phylogenetic relationship with other metastrongyloids, the sequences of mitochondrial and nuclear genes herein generated were aligned, using ClustalW, with those available in the GenBank database. Phylogenetic trees based on ribosomal 12S rRNA and 18S rRNA were constructed using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model and the Neighbor-Joining method based on the

Table 1. Characterization of necropsied hedgehogs lung lesions and cause of death.

Case nr	Gender	Season	Lung lesions	Concurrent lungworm	Cause of death
1	Male	Spring	Diffuse subacute bronchopneumonia	Yes	Parasitic pneumonia
2	Female	Autumn	Pulmonary edema	No	Heart chronic failure
3	Female	Winter	Hyperemia and pulmonary edema	No	Heart chronic failure
4	Male	Spring	Subacute multifocal to diffuse pneumonia	No	Pneumonia
5	Female	Spring	Multifocal chronic interstitial pneumonia	No	Chronic pneumonia
6	Male	Spring	Fibrinopurulent pleuropneumonia	No	Pleuropneumonia
7	Male	Spring	Diffuse subacute bronchopneumonia	Yes	Parasitic pneumonia
8	Male	Autumn	Diffuse subacute bronchopneumonia	Yes	Parasitic pneumonia
9	Male	Autumn	Diffuse subacute interstitial pneumonia	No	Pneumonia
10	Male	Autumn	Diffuse subacute bronchopneumonia	Yes	Parasitic pneumonia
11	Female	Autumn	Diffuse subacute bronchopneumonia	Yes	Parasitic pneumonia

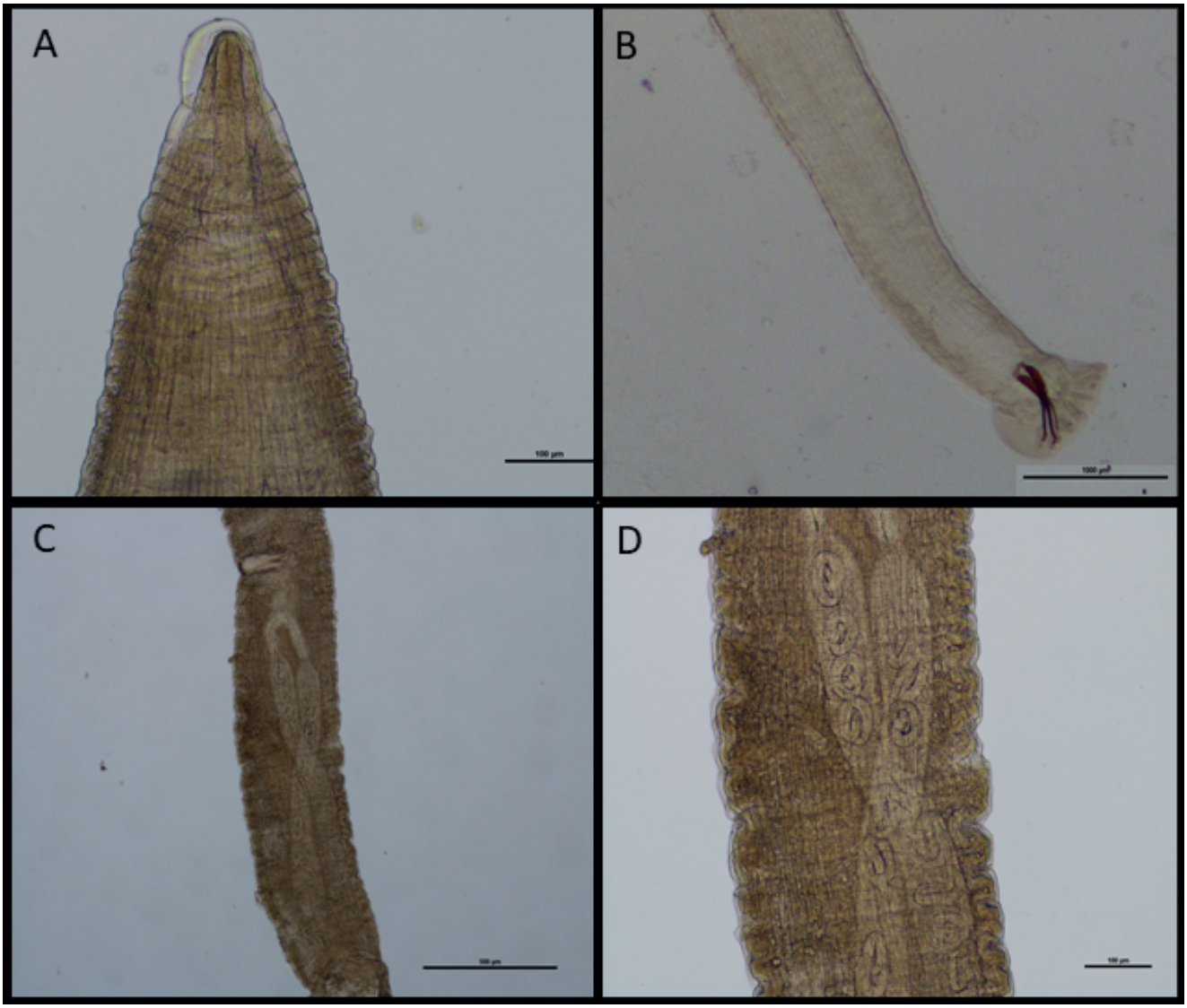

Fig. 1. Morphological characteristics for the identification of *Crenosoma striatum*.
A - rudimentary buccal capsule with a cross striation body cutile, B – copulatory bursa with two spicules, C – median vulva, D – eggs with ovoid shape that contain L1.

Kimura 2-parameter model, respectively, and bootstrap values are based on 5000 replicates, using MEGA X software (Kumar *et al*., 2018). For each analysis, the bootstrapped confidence interval was based on 5000 replicates.

Results and Discussion

During the aforementioned period, all the 11 necropsied hedgehogs displayed macroscopically pronounced and then microscopically confirmed, pulmonary changes (Table 1). Out of the 11 corpses, 5 (45.5 %) presented macroscopic evidence of nematodes with long, thin, simple and whitish colour bodies of approximately 6 mm in length. Microscopically, these presented bilaterally symmetrical bodies surrounded by a non-cellular and cross-striated cuticle, rudimentary buccal capsule, copulatory bursa with two spicules and a median vulva. Females depone a large number of ovoid eggs, that contained the first larval stage when being excreted (Fig. 1). Histologically, the presence of several worms within the bronchi and bronchiole surrounded by mucous coexisted with moderate to severe inflammatory reaction, with mixed inflammatory cell infiltrate, including neutrophils, macrophages, lymphocytes, plasma cells and variable number of eosinophils (Fig. 2). Hyperplasia of the bronchial epithelium and pulmonary oedema was also noticed. All together, these features were suggestive of crenosomosis.
PCR amplification of each target region from individual DNA samples resulted in amplicons of the expected size for both studied regions. Only a 12S and a 18S rRNA sequence from one nematode were retrieved and further compared with those available in

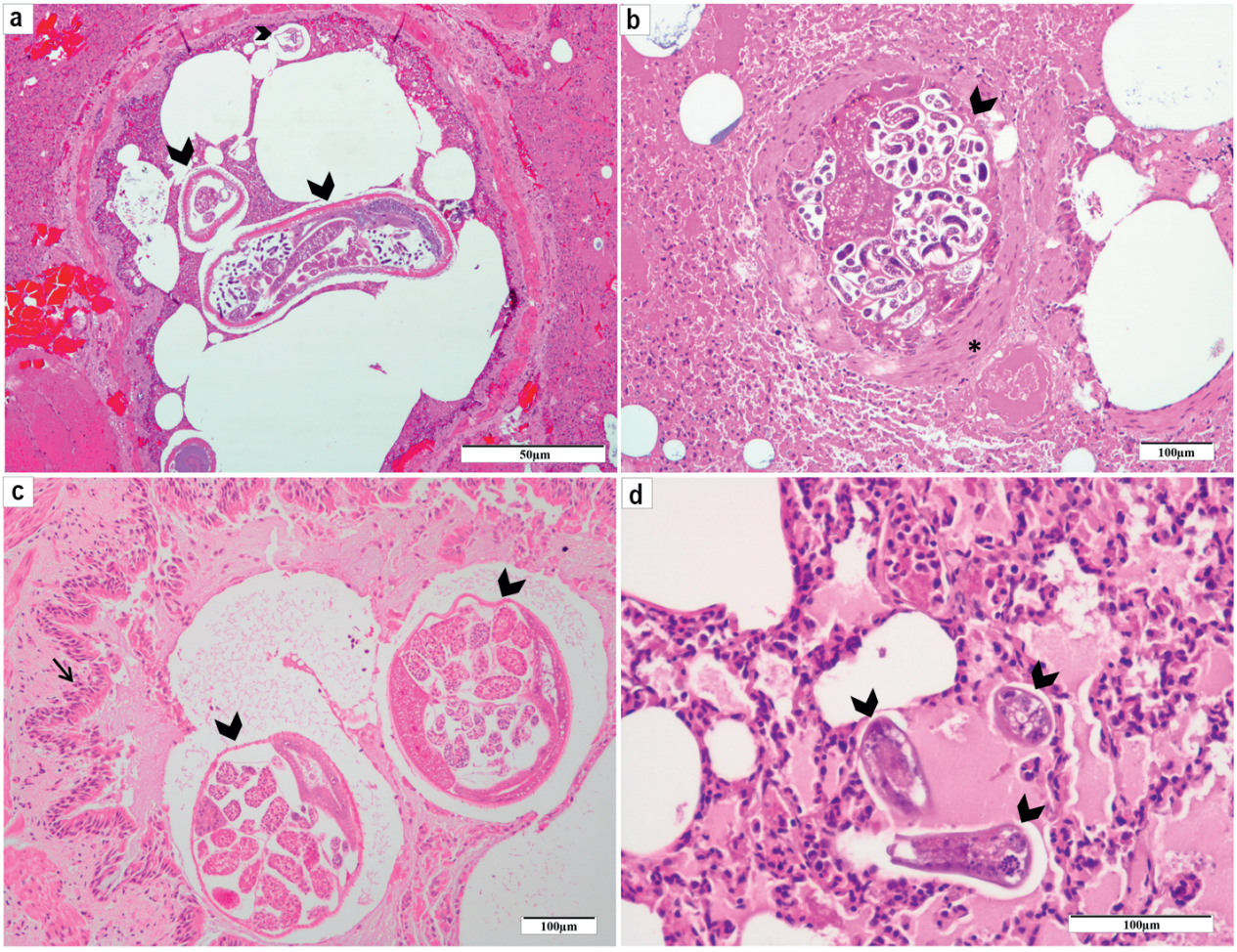

Fig. 2. a) and b) Presence of worms (arrowhead) within the bronchi and bronchioles. The alveoli are oedematous and contain abundant mixed inflammatory infiltrate and smooth-muscle hyperplasia (*) around the bronchiole is observed (case 7). c) Hyperplasia of the bronchial epithelium (arrow). Presence of intraluminal worms mixed with mucus and increased number of macrophages (case 1). d) Alveoli are filled with worms, proteinaceous material (oedema) and inflammatory infiltrate composed of neutrophils, eosinophils, macrophages, lymphocytes and plasma cells (case 8).

GenBank dataset by BLAST analysis. From the 12S rRNA target PCR, a 177 nucleotide stretch sequence was obtained and showed the highest BLAST nucleotide identity with that of *C. vulpis* (i.e. 83.73 %, KU641458), since no 12S rRNA of *C. striatum* has ever been deposited in GenBank.

The phylogenetic analysis of the 12S rRNA sequence herein obtained and those of other metastrongyloids showed a cluster with *C. vulpis* (Fig. 3).

From the 18S rRNA target PCR analysis, a 321 nucleotide sequence was retrieved showing the highest BLAST nucleotide identity with that of *C. striatum* (i.e. 99.69 %, KP941434), found in hedgehogs from Germany (Lange *et al*., 2018). Phylogenetic tree based on 18S rRNA also showed that the obtained sequence clustered with *C. striatum* (Fig. 3). Sequences were deposited in GenBank with accession numbers MN149537 (12S rRNA) and MN097947 (18S rRNA).

C. striatum is a host-specific nematode with a high infection prevalence amongst hedgehogs (Pfäffle 2010). Lung disease associated with this nematode is one of the most observed reasons for health support in these mammals and it is associated with respiratory distress up to cardiac failure as a direct result of heavy worm burdens (Hoseini *et al*., 2014). The present report identifies *C. striatum* in *E. europeus* hedgehogs rescued from various Portuguese municipalities and strengthens the phylogenetic interpretation of this species. The genetic data here described corroborates the morphological classification of *C. striatum* and provides a first nucleotide sequence for a partial 12S rRNA gene of this nematode. To broaden the knowledge on the biology of this parasite, particularly its life cycle, further molecular studies are also needed on intermediate hosts.

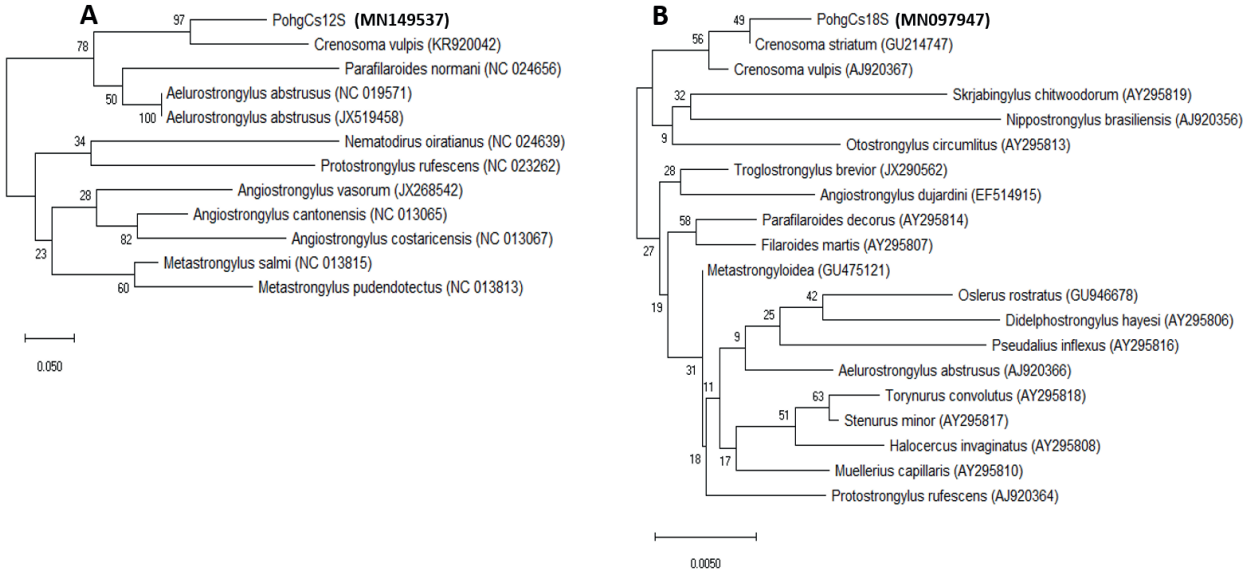

Fig. 3. Phylogenetic trees based on ribosomal 12S rRNA and 18S rRNA were constructed using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model (A) and the Neighbor-Joining method based on the Kimura 2-parameter model (B), and bootstrap values are based on 5000 replicates.

Conflict of Interest

Authors state no conflict of interest.

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