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Research paper

Pathological lesions in the lungs of red deer *Cervus elaphus* (L.) induced by a newly-described *Dictyocaulus cervi* (Nematoda: Trichostrongyloidea)

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ABSTRACT

The large lungworms of the genus *Dictyocaulus* are causative agents of parasitic bronchitis in various ungulate hosts, including red deer. Recently, the red deer-derived lungworm *D. cervi* was described and separated from *D. eckerti*. Little is known of the transmission patterns, epidemiology, geographical distribution and pathogenicity of *D. cervi*. Histological examinations were performed on 22 formalin-fixed lung tissue samples of hunted red deer. Exclusively, *D. cervi* adults were derived from 15 red deer and confirmed molecularly (GenBank accession: MH183394). *Dictyocaulus cervi* infection was associated with various degrees of lung pathology, including interstitial pneumonia, bronchitis and bronchiolitis with an influx of eosinophils, lymphocytes, plasma cells and macrophages; massive hyperplasia of lymphoid follicles within bronchiolar tissue, and hyperplasia of the bronchial and bronchiolar epithelium. Furthermore, emphysema, atelectasis and lung tissue congestion were noted. Interestingly, interstitial and subpleural fibrosis was seen in adult *Dictyocaulus*-negative samples, suggesting either a prepatent phase of *Dictyocaulus* infection or infection/coinfection with protostrongylid nematodes.

1. Introduction

Large lungworms from the genus *Dictyocaulus* Railliet and Henry, 1907 (Nematoda: Trichostrongyloidea) are causative agents of parasitic bronchitis (dictyocaulosis, husk) in various ungulate hosts, including domestic and wild ruminants (Eysker, 1994; Divina et al., 2002; Mahmood et al., 2014; Pyziel, 2014). The life cycle of *Dictyocaulus* lungworms is direct, with adults being present in the small and large airways of a host, where they produce embryonated eggs that hatch in the airways or in the large intestine. First stage larvae (L1) are passed in the faeces of the host into the environment to develop into infective L3 (Panuska, 2006). A new host can be infected orally, and the ingested larvae migrate through the body to reach the lungs as L4 at the seventh day after infection (Panuska, 2006). According to Corrigall et al. (1982) the main clinical signs of parasitic bronchitis, which include coughing and dyspnea, occur during the prepatent phase, between days seven and 25 of infection (Panuska, 2006). They result from the immunological reaction to the appearance of larvae within the alveoli and to their migration towards the bronchiole and bronchi. During the patent phase, between days 25 and 55 of infection, clinical signs escalate; intense coughing, loss of condition, harsh respiratory sounds with rhonchi, and emphysematous crackling can be diagnosed (Panuska, 2006). Dictyocaulosis is a potential threat to the biodiversity and also to the development of the game industry (Gortázar et al., 2006; Hoffman and Wiklund, 2006).

According to the first systematic revision of the genus (Skrjabin et al., 1954), *D. eckerti* was described as a collective species infecting various cervid hosts including red deer. However, this classification had to be revised following the morphological and molecular description of *D. capreolus* Gibbons and Höglund (2002) from roe deer and moose (Höglund et al., 1999) and *D. cervi* Pyziel et al. (2017) from red deer (Pyziel et al., 2015). During this revision, the *D. cervi* lungworm of red deer with unique ribosomal SSU, ITS2 and mitochondrial *cox*1 and *cox*3 sequences (Pyziel et al., 2018), was separated from *D. eckerti*.

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The aim of the study was to report the pathological findings of lung tissue associated with infection of a newly-described *D. cervi*, a lungworm of red deer in northern-east Poland.

2. Material and methods

2.1. Specimen collection

Examinations were performed on 22 individuals of red deer (*Cervus elaphus*) culled as part of a deer management strategy during the autumn and winter 2017–2018 hunting season in Puszcza Piska Forest (53° 40′ 1.61″ N, 21° 26′ 44.31″ E): an area where *D. cervi* has previously been described from red deer (Pyziel et al., 2017).

The respiratory tracts of freshly-hunted animals were dissected and investigated for the presence of adult *Dictyocaulus* lungworms. The trachea, bronchi, and bronchioles were cut open and specimens were isolated one by one with the use of dissecting needles. Additionally, dissected respiratory tracts were immersed into beakers filled with tap water, the sediment allowed to settle to the bottom, and the fluid decanted. Afterward, the sediment was investigated under a stereomicroscope to recover individuals overlooked during dissection. In any lungworm-positive case, the worms were collected and preserved in 70% ethanol for further study.

2.2. Histopathological investigation

Samples of the pulmonary tissue were taken both from adult lungworm-positive lungs, around the location of the parasite, and from adult lungworm-negative lungs, and these were fixed in 10% buffered formalin. The formalin-fixed lung samples were submitted to the Department of Pathology and Veterinary Diagnostics, Faculty of Veterinary Medicine, Warsaw University of Life Sciences-SGGW for histopathological investigation. The lung samples were dehydrated through graded ethanol and xylene baths, and embedded in paraffin wax. Sections of 4 µm were stained with hematoxylin and eosin (HE). Parasite-positive specimens were also stained with Periodic acid according to the Schiff method (PAS). Additionally, several samples were stained with Masson's trichrome and Ziehl-Nielsen (ZN). Microscopic evaluation was performed using an Olympus BX43 light microscope (Olympus, Japan).

2.3. DNA extraction, amplification, and sequencing

Genomic DNA was extracted from adult lungworms using the Nucleospin Tissue DNA Extraction Kit (Macherey-Nagel, Düren, Germany; catalogue no. 740952.50) according to the manufacturer's protocol. We took 5 worms per host if the intensiveness of infection was ≥5 individuals. In other cases the DNA was isolated from a single worm. A partial region of the 18S rRNA gene (ca. 1600 bp) of the ribosomal gene array was amplified with the use of a forward primer NF50 (5′-TGAAATGGGAACGGCTCAT-3′-) and reverse primer BNR1 (5′-ACCTACAGATACCTTGTTACGAC-3′) according to Pyziel et al. (2017).

The amplicons were purified with the use of the Nucleospin gel and PCR clean-up kit (Macherey-Nagel) and eluted with 30 µl of laboratory-pure PCR water. Purified PCR products were sequenced in both directions by Genomed S.A. (Warsaw, Poland). The DNA fragments were assembled into contigs using ContigExpress software (New York, USA).

3. Results

3.1. Macroscopic examination

The lungs were pale pink with scattered cherry red foci, airy and soft. In a few cases, foreign bodies (digest foreign matter) were detected in the bronchi.

Individual adult *Dictyocaulus* lungworms were found in the bronchi and bronchioles of 15 of the 22 examined red deer. The mean intensity of infection was 12.6 ± 10.5 (95% CI = 6.4–18.8), and the range of infection was 1–39 worms per host. The airways filled with worms were plugged with greenish mucus.

3.2. Nucleotide sequences

The study resulted in 67 completely homologous nucleotide sequences (5 sequences/worms x 13 hosts, and 1 sequence/worm x 2 hosts). All large lungworm-positive individuals were infected exclusively with *D. cervi* (GenBank accession no. MH183394).

3.3. Histopathological findings

3.3.1. Adult lungworms-positive cases (n = 15)

The occurrence of *Dictyocaulus* infection was associated with various degrees of histopathological lesions in the lungs, regardless of intensity of parasitic infection or the presence of various developmental stages of worms. In samples from 12 animals, either cross-sections, longitudinal sections or oblique sections of the parasites in different stages of development were encountered. Sections of adult and young adult *Dictyocaulus* were seen in the lumen of bronchi or bronchioles (Fig. 1A), whereas larvae of the lungworms rich in PAS-positive deposits (Fig. 1B) were detected multifocally in alveoli (Fig. 1C), surrounded by inflammatory cells. Additionally in sections from 12 animals, interstitial pneumonia (Fig. 1D) was noted with the dominant presence of mononuclear cells (i.e., lymphocytes, plasma cells and macrophages), and with a lesser contribution of eosinophils.

Moreover, all peribronchial and peribronchiolar sections were infiltrated with inflammatory cells, mainly with eosinophils, lymphocytes, plasma cells and less often with macrophages (Fig. 1E), and in some cases they were swollen. Additionally, a perivascular inflammatory infiltrate was also noted. In most of the sections, examples of massive hyperplasia of the lymphoid follicles within bronchiolar tissue were observed (Fig. 1F), together with hyperplasia of bronchial and bronchiolar epithelium, accompanied by sloughing of the hyperplastic epithelium of small airways into the bronchiolar lumen (Fig. 1G). Additionally, cyst-like dilated goblet bronchiolar cells were also observed (Fig. 1H). Moreover, smooth muscle and connective tissue hyperplasia was noticed in the perivascular, peribronchial and peribronchiolar regions. In one section, some pulmonary arteries were found to be obstructed by a foreign body with a honeycomb structure, suggesting the presence of a nematode cuticle (Fig. 1I). In addition, thickened alveolar septa were detected, as was congestion of the interstitial tissue, admixed with hemosiderin pigment (Fig. 1J).

In each case the lung tissue was emphysematically altered, and in 13 animals, emphysema was accompanied by atelectasis accompanied by inflammatory lesions (Fig. 1K).

3.3.2. Adult lungworms-negative cases (n = 7)

In each histological section, lungworm larvae surrounded by inflammatory cells were detected interalveolarly (Fig. 2A), very often within haemorrhagic pulmonary parenchyma with hemosiderin deposits (Fig. 2B). Most of the lesions were found to correspond to previously described types, such as inflammation of the peribronchiolar and perivascular tissues (Fig. 2C), hyperplastic changes of bronchiolar epithelium and smooth muscles, emphysema and atelectasis, thinning of the alveolar septa (Fig. 2D), and interstitial pneumonia. In one tissue section, a larval stage of a lungworm was found inside the peribronchiolar lymphoid follicle (Fig. 2D). In contrast, interstitial and subpleural fibrosis was exclusively noted in the tissue sections of the lungs where no adult *Dictyocaulus* sp. was found during dissection (Fig. 2E and F).