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Research paper

Eulimdana clava (Nematoda: Filarioidea) infection in domestic pigeons (*Columba livia domestica*): Molecular characterization and pathological changes

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ABSTRACT

Filarial nematodes of the *Eulimdana* genus inhabit subcutaneous tissue of various avian species, mostly Charadriiforme birds. In domestic pigeons, *E. clava* is the only species recorded in the subcutaneous tissue in a number of isolated cases. In the present study, we discuss the morphology and histopathology of filarial nematodes recovered from subcutaneous tissue of domestic pigeons in Bosnia and Herzegovina. In total 110 pigeons were submitted to necropsy at the Department of Pathology of the Faculty of Veterinary Medicine in Sarajevo. At necropsy, in four pigeons (3.6%) numerous thread-like 0.9–2.1 cm long nematode parasites were observed in the subcutaneous tissue, peritracheal and periesophageal connective tissue. In one pigeon, the parasites were also found free in the body cavity around the heart and lungs. In addition, several 80–90 µm long microfilariae were noted in the tissue cross-sections. No significant lesions were observed associated with adult parasites or microfilariae. Based on morphology, host species and localization detected parasites were identified as *E. clava*. Molecular analyses of the *cox*1 and 12S rRNA nucleotide sequences herein generated revealed the close genetic relationship to other filarioid nematodes. The importance of the nematodes in pigeons and the lack of sequences in genetic databases for comparison of avian filarial parasites are emphasized.

1. Introduction

Avian filarial nematodes belong to the family *Onchocercidae* which comprises 16 genera. Among these, *Eulimdana* genus, initially referred as *Pelecitus*, is now recognized as a separate genus including 16 species that affect avian hosts from seven orders, including *Columbidae* family (Bartlett et al., 1985, 1989; Bartlett, 1992, 2009). Most of the *Eulimdana* species have a broad host range with a wide geographic distribution (Bartlett, 2009). Like all filarial nematodes, *Eulimdana* spp. have a life cycle that includes an invertebrate intermediate host or vector. The nematodes are transmitted by amblyceran (i.e. *Austromenopon limosae, A. haeopodis* and *Actornithophilusli mosae*) and ischnoceran (i.e. *Carduicepsc layae* and *Lunaceps numenii phaeopi*) chewing lice (Bartlett, 1993). Microfilariae present in the blood or the skin of the definitive hosts are ingested by the arthropod vector, where they continue their development to the infective third-stage larvae. The larvae migrate to the head and mouth of the arthropod and enter back into the definitive host's body, where they reach adult stage (Bartlett, 2009).

In most cases, adult worms are found only in juvenile, but not in adult birds, due to the phenomenon of ephemerality (Bartlett et al., 1985; Bartlett, 1992, 2009). In adult birds, microfilariae in the skin are the only evidence of infection. Adults of *Eulimdana* spp. reside in the subcutaneous tissue of the head and neck, in the peritracheal and periesophageal connective tissue or around the crop (Bartlett et al., 1985, 1989; Pizzaro et al., 1994; Bartlett, 2009). Usually, infections with *Eulimdana* spp. are subclinical, produce protective immunity, and have not been associated with any pathological changes in Charadriiforme birds (Bartlett et al., 1989; Bartlett, 2009).

The diagnosis and differentiation between species are mainly based on the host species, localization of the parasites and on morphological characteristics. However, identification of *Eulimdana* spp. by morphology is challenging due to a lack of literature data (Bartlett, 1992). Furthermore, molecular identification and comparison of the species are currently impossible because of the absence of nucleotide sequences in public databases, as recently seen in the study on filarial nematodes in Eclectus parrots (*Eclectus roratus*) in Taiwan (Huang et al., 2017).

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Eulimdana clava (Wedl, 1856) is considered as the only species parasitizing pigeons (*Columba livia,* Columbidae) (Eslami, 1987; Gharagozlou, 1988). Several isolated cases in domestic pigeons, reported as *Pelecitus clava* (Guidall and Settnes, 1968; Rutherford and Black, 1974; Pizzaro et al., 1994), were most likely misidentifications of *E. clava* (Bartlett, 2009). The reported clinical signs related to *E. clava* infection in pigeons were loss of feathers on the head, neck, back and wings, as a result of the microfilariae present in the skin (Eslami, 1987; Gharagozlou, 1988; Pizzaro et al., 1994), accompanied by marked edema, hemorrhages, thickening and folding of the skin, subcutaneous infiltrates of leukocytes, multifocal granulomas, burrowing of parasites in the crop and erosions of the crop (Guidall and Settnes, 1968; Eslami, 1987; Gharagozlou, 1988; Pizzaro et al., 1994).

The present paper reports the observation of *E. clava* infection in domestic pigeons from Bosnia and Herzegovina. The findings of parasite morphology and histopathological findings are discussed.

2. Material and methods

2.1. Bird collection

In the period from January 2010 to December 2016, 110 mostly young (less than one year old) domestic pigeons died due to different etiologies were presented for necropsy at the Department of Pathology, Faculty of Veterinary Medicine, University of Sarajevo. Birds originated form ten pigeon lofts in Sarajevo Canton (Central Bosnia).

2.2. Pathological examination

At necropsy, the skin, subcutaneous tissue, and all the visceral organs were examined *in situ*. Samples of brain, heart, lungs, liver, spleen, kidney, esophagus, trachea, proventriculus, gizzard, intestine, bursa of Fabricius and bone marrow were collected for histopathology. The samples were fixed in 10% neutral buffered formalin for 24–48 h and routinely processed for histopathology. Sections (4–6 µm) were stained with hematoxylin and eosin (H&E) and examined under the light microscope.

2.3. Identification of parasites

Parasites observed in the subcutaneous tissue were collected and stored in 70% ethanol for morphological and molecular analyses. Prior to microscopic examination specimens were cleared in lactophenol. Morphometric characteristics of the parasites were observed and measured microscopically with a calibrated ocular micrometer. The length of the parasites was expressed in millimeters (mm), and microscopic measures in micrometers (µm) as mean and range in parentheses. Major measures were adopted from Bartlett et al. (1989) (Table 1), with exception of maximum width and the distance between the anus and posterior extremity (because the anus was not readily visible in our specimens).

2.4. DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted separately from eight adult worms i.e., two worms from each infected pigeon by using a commercially available extraction kit (High Pure PCR Template Preparation Kit, Roche Diagnostics, Germany) and following the manufacturer's instructions. The PCR assays were performed according to previously published protocols (Casiraghi et al., 2001; Latrofa et al., 2015). A 689 bp long fragment of the mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) gene was amplified using the primers NTF (5′-TGATTGGTGGTTTTGG TAA-3′) and NTR (5′-ATAAGTACGAGTATCAATATC-3′) (Casiraghi et al., 2001). The samples were additionally tested by PCR using Fila_12SF (5′-CGGGAGTAAAGTTTTGTTTAAACCG-3′) and Fila_12SR (5′-CATTGACGGATGGTTTGTACCAC-3′) primers which amplify an approximately 300 bp fragment of the 12S ribosomal RNA (12S rRNA) gene (Latrofa et al., 2015). All PCR reactions were carried out in a final volume of 25 µl using 5 × Green Reaction Buffer and GoTaq G2® Polymerase (Promega, Germany). Purified PCR products were sequenced in both directions by the company Microsynth, Austria.

3. Results

3.1. Pathological findings

At necropsy, 46 white, thread-like and 9–21 mm long (mean 15.6 mm) adult nematodes were observed in the subcutaneous tissue of the neck, peritracheal and periesophageal tissue of four pigeons (3.6%) (Fig. 1a). In one of them, the same parasites were also recovered in the body cavity, from the connective tissue and spaces surrounding the lungs and heart. Histopathology revealed multiple 300–400 µm cross-sections of nematode parasites in the connective tissue enclosing the esophagus and trachea. A moderately thick cuticle, coelomyarian musculature, small intestine and microfilariae filled uterine tubes were noticed (Fig. 1b). A small number of 80–90 µm long and 3–4 µm thick microfilariae with rounded posterior and pointed anterior end were observed free in peritracheal and periesophageal connective tissue (Fig. 1c), as well as in the blood vessels of that region. In one pigeon, numerous microfilariae were recorded in the blood vessels of interlobular or interalveolar septa of the lungs (Fig. 1c) as well as in the blood vessels of the liver. Moderate peritracheal and periesophageal fibrosis and edema without inflammatory reaction were noticed in the vicinity of adult worms. However, there were no lesions associated with microfilariae.

3.2. Parasitological findings

Microscopic examination of the parasites cleared in lactophenol revealed that all were females based on the presence of microfilariae in the uterus. Parasites had moderately thick striated cuticle throughout the body. Central oval opening surrounded with four papillae (Fig. 2c) was observed at pointed, bluntly "V" shaped and slightly bulbously dilated anterior extremity. Simple, well developed and muscular esophagus ends at a clear esophageal-intestinal junction. Nerve ring was clearly discernible (Fig. 2a). The loops of convoluted genital tubes filled with microfilariae were visible extending from the posterior extremity to the esophageal-intestinal junction, or sometimes to the nerve ring anteriorly. Vulva and vagina were located post-esophageal with slightly visible lips (Fig. 2d). The length of the vagina was obscured by uterine loops. The small intestine was also visible in the anterior fourth of the body. Body width was uniform over the middle third of the body and slightly increasing toward the extremities. Posterior extremity was round "U" shaped and divided with a shallow cleft (Fig. 2b). Anus, when visible, was sub-terminal (Fig. 2b). Based on the localization, morphological and morphometric characteristics (Table 1) these parasites were identified as *E. clava*.

The parasites are deposited in the parasite collection of the Natural History Museum Vienna, Austria (inventory number: NHMW EV 20502), and additional specimens are kept at the Department of Pathology, Faculty of Veterinary Medicine in Sarajevo, Bosnia and Herzegovina.

3.3. Molecular characterization

In order to molecularly confirm the species identity, two different PCR assays and subsequent sequencing were performed. All sequences generated in the present study (*cox*1 and 12S rRNA) were identical to each other. However, BLAST search of the *cox*1 sequences revealed a low level of nucleotide similarity to those available in GenBank® database and displayed only 87.4% (559/639 bp) to 87.7% (522/595 bp) identity to the closest nucleotide sequences of *Loa loa* derived from