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Morphological and molecular comparison of nematodes from the family Ancylostomatidae isolated from selected species of carnivorous mammals in central Poland

PAWEŁ GÓRSKI[1], EWA DŁUGOSZ[1], JUSTYNA BARTOSIK[1], PIOTR BĄSKA[1], JACEK ŁOJEK[2], WOJCIECH ZYGNER[1], JUSTYNA KARABOWICZ[1], MARCIN WIŚNIEWSKI[1]

[1]Department of Parasitology and Parasitic Diseases, Department of Preclinical Sciences, Institute of Veterinary Medicine, [2]Department of Animal Breeding and Production, Institute of Animal Sciences, Warsaw Agricultural University, Ciszewskiego 8, 02-786 Warszawa, Poland

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Górski P., Długosz E., Bartosik J., Bąska P., Łojek J., Zygner W., Karabowicz J., Wiśniewski M.

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Summary

The aim of the study was to morphologically and genetically compare parasitic nematodes from the genus Uncinaria found in different hosts. These common parasites belong to hookworm nematodes (family Ancylostomatidae). Worms were collected during dissection of mammals obtained from the Institute of Veterinary Hygiene in Warsaw, where the animals had been tested for rabies. The hosts of the nematodes were European badgers (*Meles meles*) from the family Mustelidae, as well as red foxes (*Vulpes vulpes*), dogs (*Canis familiaris*), and raccoon dogs (*Nyctereutes procyonoides*) from the family Canidae. The parasites were viewed under a microscope, and identified on the basis of their morphological features characteristic of the species *U. stenocephala*. Isolated, PCR-amplified, and sequenced rDNA segments containing internal transcribed spacer segments (ITS1 and ITS2) in the gene encoding 5.8S rDNA were also compared. No genetic or morphological differences were found between Uncinaria hookworms obtained from the four different host species. This means that wild raccoon dogs and badgers can be a source and reservoir for the hookworm *U. stenocephala* that infects dogs.

Keywords: *Uncinaria stenocephala*, Ancylostomatidae, carnivorous mammals, central Poland

The Ancylostomatidae family contains many species of nematodes known as hookworms. Infections with these bloodsucking parasites can cause serious health problems both in humans and animals, and are therefore studied by human and veterinary medicine. At least 700 million people are infected with hookworms worldwide (11). Domestic animals, such as ruminants, dogs and cats, also suffer from hookworm infections. The pathogenicity of infection results from the fact that parasites cause serious blood loss in their hosts, which can lead to anemia and even death. The most common species infecting dogs and wild canines, such as wolves (24, 25), red foxes (1, 2, 4, 21-23) and raccoon dogs (5), in Europe is *Uncinaria stenocephala*, which is also occasionally reported from cats (27), mustelids (19, 28), and even humans (14). The related hookworm species *U. criniformis* is a parasite of mustelids and has been reported from the European badger, the stone marten, and the European polecat (7, 17, 19, 26). In earlier studies, this nematode was also reported from members of the Canidae family (19, 29). Both these *Uncinaria* spp. have also been found in Poland. *U. stenocephala* is a common parasite of red foxes (*Vulpes vulpes*) and dogs living mainly in rural environments (1, 12, 15).

Nowadays, DNA sequencing methods are often used for species differentiation of Ancylostomatidae nematodes. Internal transcribed spacer ribosomal DNA (ITS) can be used as a reliable genetic marker for the identification of nematode species from the Strongylida suborder (3, 6, 8, 9, 13, 16, 20).

The aim of this study was to compare the morphology and ITS sequences of Uncinaria nematodes

obtained from four different host species belonging to the Canidae (dog, red fox, and raccoon dog) and Mustelidae (European badger) families.

Material and methods

Worms. Hookworms were collected from the intestines of experimental dogs between 1995 and 1998 during studies in the Department of Parasitology and Invasive Diseases of the Veterinary Faculty of the Warsaw University of Life Sciences (permission no. 1/95 of 20/06/1995 issued by the Rector of the Warsaw University of Life Sciences) and were stored frozen. The larvae used to infect the dogs were from a field strain (animal shelter). Other hookworms were isolated from the intestines of foxes, badgers, and raccoon dogs obtained from Institute of Veterinary Hygiene in Warsaw, where they had been tested for rabies. All animals came from the Masovia Voivodeship. Autopsies were carried out with safety precautions because of the risk of echinococcosis (10, 18). Worms of both sexes obtained during the examination were frozen in a small amount of distilled water and kept at a temperature of –70°C until further procedures. In total, 20 worms of both sexes (11 males and 9 females) were obtained from 3 dogs (*Canis familiaris*), 45 (26 males and 19 females) from 3 foxes (*Vulpes vulpes*), 21 (13 males and 8 females) from 3 raccoon dogs (*Nyctereutes procyonoides*), and 13 (8 males and 5 females) from 2 badgers (*Meles meles*). The morphology of all worms was analyzed in detail, and the body length of both sexes was measured under a microscope. The lengths of the esophagus and the male's spicula were also measured. Particular attention was paid to the shape of the dorsal rays of the male copulatory bursa, because they differ morphologically between the species of the genus Uncinaria. In order to compare the size of nematodes obtained from different host species, one-variable variance analysis was performed using the Statistical Package SPSS 24.0. The significance level $P \leq 0.01$ was considered as highly significant, and $P \leq 0.05$ as significant.

DNA isolation and amplification. Whole nematodes (all specimens obtained from their hosts) were homogenized, and DNA was isolated using Genomic DNA Prep Plus from A & A Biotechnology according to the manufacturer's instructions. DNA samples were frozen for further analysis. rDNA fragments containing the internal transcribed spacer segments (ITS1 and ITS2) in the gene encoding 5.8S rDNA were amplified. NC5 (5'-GTAGGTGAACCTGCGGAAGGATCATT-3') and NC2 (5'-TTAGTTTCTTTTCCTCCGCT-3') primers were used (8). PCR reactions were run in 50 µl of reaction mix containing reaction buffer, 0.2 mM $MgCl_2$, 0.2 mM of deoxynucleotide mix, 0.2 µM of each primer, 1 U of Taq Polymerase, and 5 µl of DNA template under the following conditions: 3 minutes of denaturation at 94°C, 35 cycles of denaturation (30 sec, 94°C), annealing (30 sec, 55°C), extension (45 sec, 72°C), and final extension (5 min, 72°C). PCR products were separated by agarose gel electrophoresis. DNA bands of about 800 bp in length were excised from the gel, and DNA was isolated from gel slices using A&A Biotechnology Gel-Out Kit. PCR products were then sequenced in a commercial laboratory (Genomed, Warsaw, Poland). Nucleotide sequences were analyzed using the BLAST program on the website of the National Center for Biotechnology (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results and discussion

The dimensions of male and female nematodes are presented in Table 1. Highly significant differences were found between the body lengths of worms isolated from different hosts ($P \leq 0.004$). Parasites of both sexes obtained from badgers were significantly smaller then those obtained from other hosts. The differences in the lengths of nematodes obtained from dogs, foxes, and raccoon dogs are not statistically significant. Features, such as esophageal and spicule lengths, proved to be typical of the *U. stenocephala* species. The dorsal rays

Tab. 1. The significance of differences in the mean body lengths of *Uncinaria* sp. from dogs, foxes, raccoon dogs, and badgers

Host species of *Uncinaria* sp.	Average body length (both sexes)	The significance of length differences in relation to *Uncinaria* from other hosts	
		Host species	P
Dog	8.2685	Fox	0.994
		Raccoon dog	0.218
		Badger	0.002**
Fox	8.3241	Dog	0.994
		Raccoon dog	0.147
		Badger	0.001**
Raccoon dog	8.0157	Dog	0.218
		Fox	0.147
		Badger	0.042*
Badger	7.3323	Dog	0.002**
		Fox	0.001**
		Raccoon dog	0.042*

Explanation: The level of significance: $P \leq 0.01$ highly significant**, $P \leq 0.05$ significant*

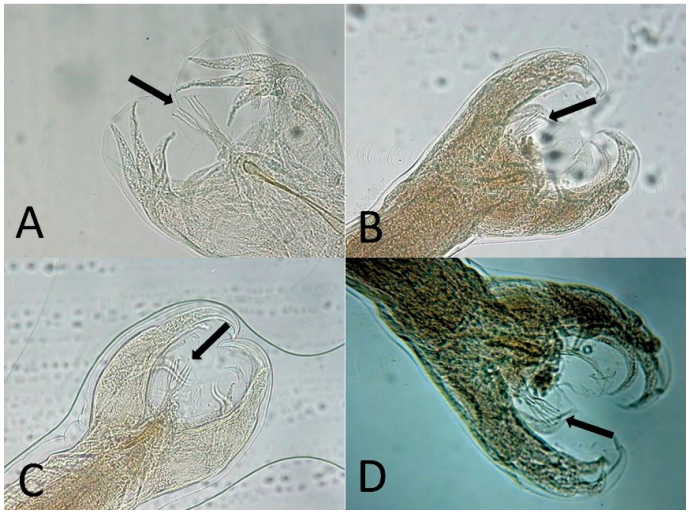

Fig. 1. Dorsal rays of male copulatory bursa of *Uncinaria* sp. from different hosts
Explanation: *Uncinaria* sp. from: A – dog, B – fox, C – raccoon dog, D – badger

of the male copulatory bursa were found to be tricuspid in all males. Such a structure was observed in male worms obtained from all host species (Fig. 1).

rDNA sequences obtained during the study were submitted to GenBank under the following accession numbers: OP811911 (UVV – fox isolate), OP811912 (UCF – dog isolate), OP811913 (UNP – raccoon dog isolate) and OP811914 (UMM – badger isolate). BLAST analysis showed the highest homology of all fragments to *U. stenocephala* sequences, thus confirming the result of the morphological examination. The raccoon dog isolate was the most homologic (98.71%), and the dog isolate showed the lowest homology (96.36%). rDNA isolates from all hosts showed lower homology to the corresponding fragments originating from *U. cf. hamiltoni*, *Ancylostoma ceylanicum*, *U. sanguinis*, *Ancylostoma duodenale*, and *U. lucasi*. Detailed results of the BLAST analysis are shown in Table 2.

The only morphological difference between worms that parasitized different hosts was the length of their bodies. The remaining features, especially the details of the male copulatory bursa structure, did not differ between nematodes isolated from different host species. The differences in the mean body length of the worms collected from dogs, foxes, and raccoon dogs were found to be insignificant, whereas parasites isolated from badgers were significantly smaller. This can be explained by the fact that members of the Canidae family are the preferred hosts for *U. stenocephala* (9), unlike badgers, which belong to the Mustelidae family. *U. stenocephala* probably does not find optimal living conditions in the badger's intestine and therefore grows to a smaller size. Statistically significant differences within the *U. stenocephala* species depending on the host were observed previously (16, 30). Tricuspid dorsal rays of the copulatory bursa are characteristic of *U. stenocephala*. *U. criniformis*, a related species, has dorsal rays divided into two parts (29). Although the internal transcribed spacer segments do not undergo fast evolutionary processes, and the ITS1 and ITS2 sequences are only homogenous within the species, molecular determination depends on the presence of these sequences in the database. For example, the *U. criniformis* sequences are not available in the database. Consequently, on the basis of the morphology, it can be concluded that all worms exhibited features typical of the *U. stenocephala* species. Nematodes from the genus Uncinaria found in the gut of the red foxes belonged to the species *Uncinaria stenocephala*, as did those from the dogs' intestines. Moreover, the same species of hookworm was found during the sectional study of racoon dogs and badgers. So far, mammals from the Mustelidae family have not been identified as hosts of *U. stenocephala* in Poland. A previous study showed that mustelids from the Białowieża Forest were infected with *Uncinaria criniformis* (17). However, those results were based on the identification of eggs found in animal feces. It is very likely that the eggs were misidentified and in fact belonged to *U. stenocephala*. In view of the above, extreme caution is needed in identifying eggs of this nematode genus as *U. criniformis* in faeces of mammals from the Mustelidae family. Parasitism of the nematodes *U. stenocephala* in wild canines (fox, raccoon dog) and mustelids (badger) also has veterinary importance because it means that wild mammals can carry hookworms that infect domestic dogs.

Tab. 2. Results of the BLAST analysis of nucleotide sequences

Host	Isolate	Species	Homology	Access number
Fox	UVV	*U. stenocephala*	97.67%	AF194145.1
		U. cf. hamiltoni	92.37%	HE962184.1
		A. ceylanicum	91.58%	LC036567.1
		U. sanguinis	91.19%	KF690669.1
		A. duodenale	91.06%	EU344797.1
		U. lucasi	90.93%	HQ262141.1
Dog	UCF	*U. stenocephala*	96.36%	HQ262055.1
		U. cf. hamiltoni	91.46%	HE962184.1
		A. ceylanicum	91.06%	LC036567.1
		A. duodenale	90.67%	EU344797.1
		U. sanguinis	90.41%	KF690669.1
		U. lucasi	90.16%	HQ262141.1
Raccoon dog	UNP	*U. stenocephala*	98.71%	AF194145.1
		U. cf. hamiltoni	93.16%	HE962184.1
		A. ceylanicum	92.64%	LC036567.1
		U. sanguinis	92.25%	LC036567.1
		A. duodenale	92.12%	EU344797.1
		U. lucasi	91.99%	HQ262084.1
Badger	UMM	*U. stenocephala*	98.05%	AF194145.1
		U. cf. hamiltoni	92.50%	HE962184.1
		A. ceylanicum	91.97%	LC036567.1
		U. sanguinis	91.58%	KF690669.1
		A. duodenale	91.45%	EU344797.1
		U. lucasi	91.32%	HQ262141.1

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Corresponding author: Paweł Górski DVM, PhD, Ciszewskiego 8, 02-786 Warszawa, Poland; e-mail: pawel_gorski@sggw.edu.pl