J Vet Res 70, 2026
DOI:10.2478/jvetres-2026-0060

Ancylostoma caninum and *Uncinaria stenocephala* – are these really the dominant hookworm species in Poland? An update of knowledge based on coproscopic and molecular investigations

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Received: April 16, 2026 *Accepted: November 12, 2026*

Abstract

Introduction: Dogs are vectors of many parasitic diseases. Invasions by members of the family *Ancylostomatidae* are among the most frequently diagnosed nematodoses in dogs in Poland. Standard diagnostics do not allow unambiguous distinguishing between *Uncinaria stenocephala* and *Ancylostoma caninum* helminthiases. The aim of the study was to evaluate the prevalence and geographical distribution of *A. caninum* and *U. stenocephala* in a population of dogs in Poland using molecular methods. **Material and Methods:** In the years 2021–2023, a coproscopic examination of 1,825 dogs originating from rural and urban areas as well as from animal shelters located in voivodeships in different regions of Poland was conducted. Faecal samples were investigated using the flotation method. Isolated eggs of hookworms were tested using PCR-restriction fragment length polymorphism analysis to determine the species of nematode. **Results:** Hookworm invasion was detected in 8.8% of the examined dogs. Molecular examination of 80 nematodes revealed *Uncinaria stenocephala* in 100% of cases. **Conclusion:** The obtained results show that the problem of nematode invasion by the *Ancylostomatidae* family in dogs in Poland is still current, and the species *Uncinaria stenocephala* definitely prevails.

Keywords: hookworms, *Uncinaria stenocephala, Ancylostoma caninum*, diagnostics, PCR.

Introduction

Hookworms are common blood-feeding nematodes found in both humans and animals in various parts of the world. Members of the family *Ancylostomatidae* causing invasions in carnivores include the following species: *Uncinaria stenocephala, Ancylostoma braziliense, Ancylostoma caninum, Ancylostoma ceylanicum*, and *Ancylostoma tubaeforme* (8, 12). In Europe, typical hookworms in dogs are *U. stenocephala* (Railliet, 1884) and *A. caninum* (Ercolani, 1859),

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which both have widespread distribution but each have different geographical ranges. The former predominates in the northern part of the continent, while the latter is more common in the southern regions, which is related to the climatic requirements of each species (6, 12).

In Poland, over the last 30 years, hookworm presence in dogs has been reported with prevalence ranging from 4% to 75% (3, 4, 7, 10, 13, 27, 32, 43, 44). The findings leave undetermined which of the species referred to predominates nationwide, as the authors of the available studies usually describe these invasions infections collectively, without species-level identification. Both in Poland and in other European countries, study results most often identify only "hookworms" or the family *Ancylostomatidae*, or use the designation *Ancylostoma/Uncinaria*, treating both nematode genera as causing a single parasitosis (4, 5, 6, 7, 13, 14, 23, 25, 31, 37, 40). This taxonomic irresolution results from standard diagnostic procedures, most commonly based on flotation techniques, which do not allow for unambiguous species identification of hookworms. For this reason, differential diagnosis of these invasions using molecular methods or coproculture of L1 larvae is recommended (16, 17, 21, 33, 43). In Poland, hookworm infections have so far been identified using PCR in foxes and dogs in the central-eastern part of the country (10, 17, 18).

However, the fundamental question remains whether species-level identification of hookworms in parasitological diagnostics is of importance. In the case of *U. stenocephala* and *A. caninum*, comprehensive diagnosis is relevant from both medical and veterinary perspectives. These species differ in pathogenicity, which results from their routes of invasion, the type of migration occurring within the host organism, and their zoonotic potential. Both larvae of *U. stenocephala* and *A. caninum* may enter the host either *via* the alimentary route or through the skin. However, the first of the species mentioned, for which the *per os* route predominates, is characterised by lower pathogenicity and causes subacute or chronic catarrhal enteritis when larvae reach the duodenal glands and mature sexually (2, 12). In contrast, *A. caninum* primarily invades the host *per cutis*, and may penetrate deeper layers of the intestinal mucosa and other tissues. Its larvae migrate *via* venous or lymphatic vessels, passing through the lungs to the pharynx and, once swallowed, reach the small intestine. As a result of both *per cutis* and *per os* invasion, a proportion of *A. caninum* and *U. stenocephala* larvae migrate somatically within the host, primarily to muscles and adipose tissue, where they complete development as hypobiotic larvae. In the case of *A. caninum*, this migration may also lead to transmission *via* the lactogenic route. Authors' opinions regarding the possibility of prenatal transmission from pregnant bitches are divided (2, 8, 20, 44). As a consequence of their different pathogenicity, *A. caninum* and *U. stenocephala* also cause nematodoses differing in clinical presentation. The former species' extensive skin penetration may result in epidermal inflammatory lesions, local allergic reactions and necrosis of the skin. Consequences of tracheal larval migration include microhaemorrhages, coughing, dyspnoea, and pulmonary inflammation (12, 35). Typically haematophagous, *A. caninum* nematodes ingest on average 50−200 µL of blood per day, and in heavy infection their presence may manifest as severe haemorrhagic enteritis and acute iron deficiency anaemia. In contrast, *U. stenocephala* nematodes feed on both blood and tissues, and in consequence blood loss caused by invasion with this species is considerably lower and amounts to approximately 0.3 µL of blood per day (2, 12).

Ancylostoma caninum also constitutes a higher zoonotic risk than *U. stenocephala.* Although these hookworms are regarded as primarily parasitising carnivores, cases of human invasion have been reported (16, 35). Both species may cause cutaneous *larva migrans* syndrome; however, in the case of *U. stenocephala*, such manifestations are recorded rarely. In contrast, in addition to cutaneous *larva migrans* syndrome, *A. caninum* is also associated with eosinophilic enteritis of the small intestine, eosinophilic pneumonia, and local inflammation of muscles and hair follicles (9, 35).

Studies conducted to date on the species distribution of hookworms in different regions of Poland are fragmentary in nature and based on standard coproscopic methods. For an accurate assessment of the actual epidemiological situation, it is necessary to supplement current knowledge using molecular techniques. Therefore, the aim of the present study was to evaluate the prevalence and geographical distribution of *A. caninum* and *U. stenocephala* in a population of dogs originating from various regions of Poland.

Material and Methods

Sample collection. The study material consisted of faecal samples collected from 1,825 dogs between April 2021 and April 2023. The dogs, of various ages, originated from urban areas (596), rural areas (74), and animal shelters (1,155) located in northern, southern, eastern, and western voivodeships of Poland. The study area is shown in Fig. 1.

Coproscopic examination and egg isolation. Faecal samples were examined using the flotation method according to Fülleborn as modified by Willis (19). Based on the coproscopic results, the prevalence of invasion by both hookworm species was determined. Hookworm eggs were isolated using a flotation–decantation technique with the authors' own modification (10). Subsequently, the eggs were suspended in 0.9% saline solution and stored at −80°C for further analysis. Randomly selected eggs originating from 80 dogs from different regions of Poland were used for molecular analyses. Eggs were stored in 0.9% saline solution. Prior to DNA isolation, samples were centrifuged (2,000 x *g*, 10 min), resuspended in 300 µL of nuclease-free water, and pre-treated with three cycles of heating (70°C, 10 min), followed by immediate freezing (−80°C, 10 min).

Isolation of DNA. Genomic DNA was isolated from the samples using a precipitation method as instructed by the manufacturer of the Sherlock AX Kit (A&A Biotechnology, Gdańsk, Poland). The DNA precipitate was resuspended in 10 µL of PCR-grade water and stored at −80°C until further analysis.

Amplification of hookworm DNA by PCR. Reaction mixtures were prepared using 15 µL of 2× Phire PCR Master Mix (Thermo Fisher Scientific, Vantaa, Finland), 3 µL of each primer (10 µM), 90–180 ng of DNA template and PCR-grade water. The primers were forward CDCTSF (5'-GTGCTAGTCTTCACGACTTTG-3') and reverse CDCTSR (5'-CATGTTGCAATATATTCTGATCT-3'), designed by Merino-Tejedor *et al.* (30) to amplify a 570-bp fragment of the internal transcribed spacer (ITS) region of the rDNA. These primers are a modification of the RTGHFI/RTABCR primers originally designed by Traub *et al.* (41, 42). Twenty-six samples' isolated DNA was destined for amplification, which was carried out under the following conditions: initial denaturation at 98°C for 5 min; 35 cycles of 98°C for 5 s (denaturation), 58°C for 5 s (annealing), and 72°C for 15 s (extension); and a final extension at 72°C for 1 min. All reactions were performed using a Biometra TAdvanced thermal cycler (Analytik Jena, Jena, Germany).

Restriction fragment length polymorphism (RFLP). To differentiate hookworm species present in the tested samples, 10 µL of each PCR product was transferred to a new, clean tube immediately after amplification and subjected to digestion with the *Hinf*I restriction enzyme (Thermo Fisher Scientific, Vilnius, Lithuania) following the manufacturer's protocol. Samples were incubated at 37°C for 1 h, after which the reaction was terminated by heating at 60°C for 20 min.

Gel electrophoresis. Digested and undigested DNA samples were analysed by gel electrophoresis on a 2.0% agarose gel containing SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, Carlsbad, CA, USA). The O'GeneRuler Express DNA Ladder (Thermo Fisher Scientific, Vilnius, Lithuania) was used as a molecular size marker. Each run included known positive and negative controls, as well as a no-template control.

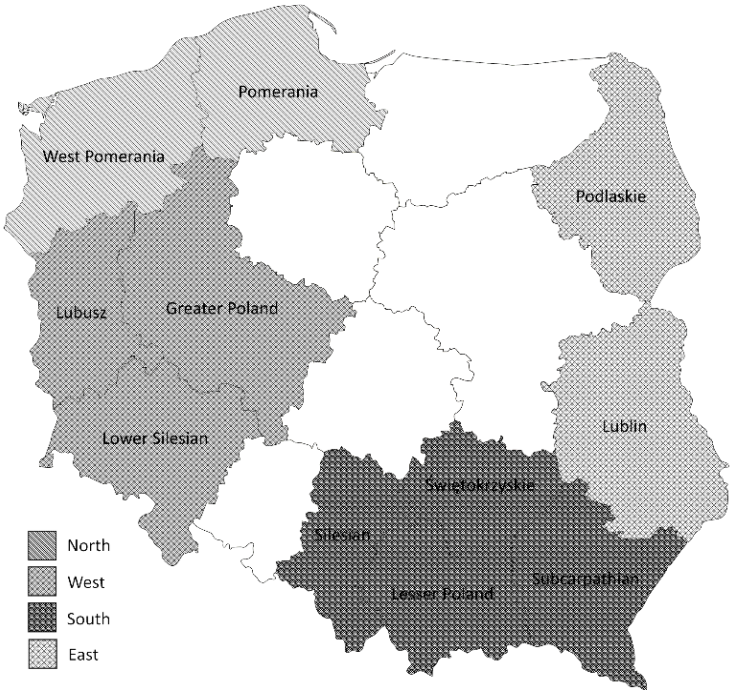

Fig. 1. Voivodeships in which samples were collected

Results

Of the 1,825 faecal samples examined, 160 (8.8%) were detected to have hookworm eggs. The PCR-RFLP analysis was performed on 80 randomly selected egg samples, and in every corresponding stool specimen, a single hookworm species was confirmed. Digestion with *Hinf*I produced an identical restriction pattern in all samples, yielding three fragments of 50 bp, 219 bp and 301 bp, a profile characteristic of *Uncinaria stenocephala*. Before enzymatic digestion, the amplified PCR product measured 570 bp (Supplementary Figs S1 and S2). None of the tested samples showed evidence of co-infection, as no mixed band profiles indicative of *U. stenocephala* and *A. caninum* (33, 50, 84, 187 and 216 bp) were observed.

Uncinariasis was more frequently recorded in samples from northern and eastern Poland (11.3–12.6%) than in those from the southern and western parts of the country (2.6%). The results are presented in Table 1.

When assessing the extent of *U. stenocephala* invasion in dogs originating from different environments, the highest prevalence was recorded in rural areas (14.9%). Uncinariasis was least frequently detected in dogs living in urban areas. Differences in helminth infestation among dogs from different environments are presented in Table 2.

An analysis of the occurrence of *U. stenocephala* invasion over the annual cycle was conducted. The results are presented in Table 3.

In the dogs examined in Poland, invasion was most frequently recorded from June to August (13.6–19.8%). In the remaining months, the extent of infection did not exceed 10%. The lowest prevalence of hookworm infection was recorded in January.

Table 1. Extent of *Uncinaria stenocephala* invasion in dog faecal samples from different regions of Poland

Region of Poland	Shelter dog prev. (%)	City dog prev. (%)	Village dog prev. (%)	Overall prev. (%)
Northern	16.9 (n = 385)	5.4 (n = 202)	4.5 (n = 22)	12.6 (n = 609)
Southern	1.4 (n = 216)	3.5 (n = 115)	10 (n = 20)	2.6 (n = 351)
Eastern	11.8 (n = 373)	9.2 (n = 196)	19.2 (n = 26)	11.3 (n = 595)
Western	1.1 (n = 181)	2.9 (n = 69)	15 (n = 20)	2.6 (n = 270)

prev. – prevalence

Table 2. Extent of *Uncinaria stenocephala* invasion in dog faecal samples taken from different environments

Environment	Examined	Prevalence of *U. stenocephala* (%)
Shelter	1,155	114 (9.9)
City	596	35 (5.9)
Village	74	11 (14.9)
Total	1825	160 (8.8)

Table 3. Occurrence of *Uncinaria stenocephala* invasion over the annual cycle

Month	n	Positive samples (%)
I	21	0 (0)
II	29	2 (6.9)
III	73	2 (2.7)
IV	314	16 (5.1)
V	305	28 (9.2)
VI	198	27 (13.6)
VII	140	33 (23.6)
VIII	111	22 (19.8)
IX	250	19 (7.6)
X	264	8 (3.0)
XI	35	1 (2.8)
XII	85	2 (2.3)

Discussion

As shown by studies conducted over the last two decades in Europe, the prevalence of hookworm invasion in dogs ranges from 0.8% to 75%, depending on the region. It is most frequently recorded in the southern part of the continent (9.2–75%) (14, 25, 29, 37, 39). In Central Europe, the prevalence ranges from 0.8% to 26.8%, whereas in Northern Europe, it reaches 7.3–17.1% (1, 5, 11, 15, 22, 40, 45). *Uncinaria stenocephala* is among the hookworm species with the most northerly and most central geographical distribution. Coproscopic examinations conducted in samples from dogs in Poland indicate that hookworm nematodoses have been and remain a relevant problem, and are even considered some of the most frequently diagnosed parasitoses in dogs (6, 13, 34, 44). Hookworms are detected in this country with varying extents depending on the region: in Eastern Poland their prevalence was 12.5–26.8% (10, 17, 18), in western regions 4–21.6% (3, 27), in northern regions 7.7–16.0% (13, 31, 44), and in southern regions 3–15% (4, 24). The extent of these nematodoses in Poland has shown a marked decline, considering that in 1994 they were reported at levels as high as 75% (32). This downward trend persists, as confirmed by the present study conducted in 2021–2023, in which the extent of invasion decreased to 2.6% in the southern and western parts of the country, to 11.3% in the east and to 12.6% in the north. This phenomenon may result from continuous improvement of diagnostic methods and the use of new and effective preventive and therapeutic measures. Hookworm invasion is most frequently recorded in rural areas, where a proportion of dogs are still not subjected to parasitological diagnostics or preventive treatments, resulting in persistent environmental contamination with invasive stages. Similar conclusions were reached by Okulewicz *et al.* (32), who demonstrated that animals have the greatest contact with soil containing eggs and larvae in such environments. Lower extents of parasitisation have been observed in dogs from urban areas, and this trend, noted over the last 20 years, has persisted to the present day (3, 7, 43).

As mentioned previously, it is increasingly recognised that routine diagnostics based on flotation techniques and identification of hookworm species according to the morphometric characteristics of eggs do not allow reliable differentiation between *Ancylostoma caninum* and *Uncinaria stenocephala*, the two hookworm species reported so far in dogs in Poland (16, 18, 21, 23, 33, 43). This problem arises from the marked morphological similarity of the eggs of these nematodes, which at the time of excretion are oval and thin-shelled, and differ only slightly in size and in the number of blastomeres (4−16 for *A. caninum* and 8−16 for *U. stenocephala*) (2, 12). Differentiation of the eggs of these species on the basis of morphometric features is problematic because data available in the literature sometimes differ considerably (2, 8, 12, 20, 24, 26, 27, 33), and the shape and dimensions of these dispersal stages vary depending on their developmental stage (21). In addition, the transformation of the larva within the egg is a highly dynamic process, particularly during the summer period. For this reason, the currently recommended diagnostic investigations using molecular methods may resolve the problem of distinguishing the two species. Palmer *et al.* (33) demonstrated that the restriction enzymes *Hinf*I and *Rsa*I can be used to differentiate *Uncinaria stenocephala, Ancylostoma caninum*, *A. ceylanicum*, and *A. tubaeforme* based on their distinct restriction profiles. In this study, we employed only *Hinf*I, as literature suggests that only the *U. stenocephala and A. caninum* canine hookworm species pair are present in Poland. It is important to note that PCR-RFLP may fail to detect hookworm species present at very low abundance in mixed infections. Therefore, low-level *Ancylostoma caninum* co-infections cannot be completely excluded. Nevertheless, all tested samples produced clear RFLP patterns consistent with *U. stenocephala*, and successful amplification was obtained in every case, suggesting that single-species *A. caninum* infections would have been detected by the applied method.

Genetic studies on the species differentiation of hookworms have so far been conducted in dogs from the central-eastern part of Poland (10, 18). The results of these studies unequivocally indicate *Uncinaria stenocephala* as the only hookworm species molecularly confirmed in these areas, both in dogs and in foxes, which serve as reservoirs of these nematodes. This finding is not surprising and is well justified. *Uncinaria stenocephala* prefers the cooler climate characteristic of Eastern Poland, and a larval developmental temperature range of 7–30°C (optimally 20°C). For this reason, it is one of the most frequently noted nematodes in canids in cooler regions of Europe (2, 36). Such success in cooler European regions is aided by the ability of invasive larvae to survive the winter months. Unfavourable environmental conditions do not kill *U. stenocephala* larvae because of the retained moulting sheath from the second ecdysis, which under favourable temperature and humidity conditions enables larvae to remain invasive for up to four of the coldest months (12, 35). However, despite the relative resistance of this species to low temperatures, *U. stenocephala* exhibits variability in invasivity depending on the sampling

season, as shown in Table 3. The mean extent of hookworm infection in Eastern Poland during the period of highest annual activity of *U. stenocephala* (May–August) in the years 2016–2023 amounted to 17.5–28.5% (10 and the authors' own unpublished data). Similar values were recorded during the analogous period in North-western Poland (11.11–24.07%) (44), whereas in the southern part of the country, the highest extents of hookworm infection were noted in March and July (10–14%) (24). The theory commonly accepted in Poland is of the dominance of *U. stenocephala* in the eastern, cooler part of the country, with the Vistula River constituting the boundary. West of the Vistula, *A. caninum* is presumed to predominate, which is associated with the milder climate prevailing in this part of Poland. Indeed, the distribution of nematodes of the genus *Ancylostoma* encompasses regions with relatively high temperatures reaching 15–37°C (spanning its optimal 30°C) (2, 12). As early as 1975, Mark (28) stated that low temperatures and precipitation favour the elimination of larvae of this species from the environment. For this reason, a higher extent of *A. caninum* invasion is commonly recorded in Southern than in Northern Europe. Likewise, the milder climate of Western Poland could suggest that any invasion with this species would have been detected in the present investigation precisely there, as they have been reported previously nationwide with extents ranging from 0.8% to 12% (3, 27, 32, 34, 36, 38, 44). However, all of the cases in this literature were identified exclusively by coproscopic methods; therefore, it is difficult to determine whether *A. caninum* actually occurs in Poland at all because of coproscopy's poor differentiation of *U. stenocephala* from *A. caninum*. Considering the biology of both hookworm species and the difficulties in their correct differentiation based on morphometric parameters of their eggs, it may be assumed that some of the diagnosed cases of *A. caninum* invasion in Poland were in fact infections with *U. stenocephala*. This assumption is supported by more advanced methods, such as molecular analyses and examinations based on coproculture of hatched L1 larvae, having exclusively found the occurrence of *U. stenocephala* in the central-eastern region of Poland where such studies were performed (10, 17, 18, 43). These data are also confirmed by the results of the present study, in which, irrespective of the region of sample collection, the PCR unequivocally indicated invasion by *U. stenocephala* and in no case by *A. caninum*.

Conclusion

The available literature suggests that standard coproscopic examination methods are insufficient for unequivocal diagnosis of invasions by the family *Ancylostomatidae* members *U. stenocephala* and *A. caninum*. The present study demonstrates a clear dominance of *U. stenocephala* in dogs in Poland. If *A. caninum* occurs in the country at all, this nematodosis is most likely at a very low extent and results primarily from the importation of carrier animals from other climatic zones. It may be assumed that the increasingly widespread use of molecular methods in parasitological diagnostics will gradually update information on the distribution of invasions by both hookworm species in dogs in Poland as well as in other European countries. Such knowledge will make it possible to accurately adapt a dog deworming programme to the current prevalence in a given country. New diagnostic methods will also enable a realistic assessment of the zoonotic risk posed by *A. caninum* as a component of the canine parasitofauna, which is particularly important in the context of the 'One Health' concept.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: This work was funded by the National Centre for Research and Development of Poland under the Intelligent Development Operational Programme 2014–2020 (project No. POIR.01.01.01-00-1715/20).

Animal Rights Statement: None required.

CRediT Authorship Contribution Statement: Marta Demkowska-Kutrzepa: research concept and design, collection and assembly of data, data analysis and interpretation, writing the article, final approval of the article. **Monika Roczeń-Karczmarz:** collection and assembly of data, data analysis and interpretation, writing the article, critical revision of the article. **Izabella Rząd:** collection and assembly of data, final approval of the article. **Alicja Laska-Modzelewska:** collection and assembly of data, data analysis and interpretation, writing the article, final approval of the article. **Mirosława Skupińska:** research concept and design, data analysis and interpretation, critical revision of the article, final approval of the article. **Agnieszka Belter:** research concept and design, data analysis and interpretation, critical revision of the article, final approval of the article. **Ezequiel Palumbo:** collection and assembly of data.

Krzysztof Tomczuk: critical revision of the article, final approval of the article.

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