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The grey wolf *(Canis lupus)* as a host of *Echinococcus multilocularis*, *E. granulosus s.l.* and other helminths – a new zoonotic threat in Poland

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Abstract

Introduction: The aim of this study was to estimate the occurrence of *Echinococcus* spp. and other helminth infections in grey wolves in south-eastern Poland. **Material and Methods:** Overall, 74 samples of wolf faeces were examined with a multiplex PCR and a system of real-time quantitative PCR methods to detect and identify *Echinococcus* spp. The faeces were additionally examined microscopically. Also, 20 samples of wolf intestines were examined with a sedimentation and counting technique (SCT). **Results:** *Echinococcus multilocularis* DNA was detected in 6.8% and *E. granulosus s.l.* (identified as *E. ortleppi*) in 4.1% of faeces samples. *Taenia* spp. DNA was found in 43.2% and *Mesocestoides* in 4.1%. Examination of the intestines by SCT showed *E. multilocularis* worms in 10%, *E. granulosus s.l.* (*E. ortleppi*) in10%, *Taenia* spp. in 100%, hookworms in 30%, *Alaria alata* in 20%, *Mesocestoides* sp. in 10%, *Trichuris vulpis* in 15%, *Molineus* sp. in 5% and *Euryhelmis* sp. in 5%. By coproscopy, Capillariidae eggs were found in 59% of faeces samples. Genetic analysis of *E. multilocularis* worms showed the presence of two European haplotypes previously described in Poland in red foxes and pigs. Sequences of *nad1* obtained from *E. ortleppi* worms shared full identity with a sequence from a human case in Poland. **Conclusion:** The study showed the presence of *E. multilocularis* in wolves for the first time in Poland and confirmed our earlier observations on *E. ortleppi*. This double threat from *Echinococcus* in this wolf population should be taken into account when assessing the epidemiological risk. The study enriched the knowledge of other helminths found in wolves, also those (*Euryhelmis*) that were recorded for the first time in this species.

Keywords: *Echinococcus*, helminths, wolf, *Canis lupus*, Poland.

Introduction

The grey wolf (*Canis lupus*) is a large canine native to Eurasia and North America. In Europe, its importance as a large predator has been increasing in recent decades. In the period 2013–2018, wolves were observed in 21 European Union (EU) countries, and their population numbered 11,000–17,000 individuals. In 2023, they were reported in 23 EU member states and the population was estimated at 20,300 individuals (6, 7).

In Poland the grey wolf is one of the three large autochthonous predators, along with the brown bear (*Ursus arctos*) and the lynx (*Lynx lynx*). All are species protected by Polish law. Before wolves were protected, their range was mainly limited to the regions of south-eastern Poland. Since these animals were recognised as a protected species (56), the population size has constantly been increasing and their range is expanding (54, 56). According to data from Statistics Poland (69), since the year 2000 the population of this species has increased by approximately 300%. In 2022 the wolf population in Poland numbered 4,328 individuals (69), of which approximately one third was located in the south-eastern part of the country. It is one of the largest populations in Europe.

Although the growth of the population of these animals is good from the point of view of environmental protection, it may also pose threats to human health and the economy. In addition to direct threats to farm animals through predation – in Poland in 2022 and 2023, over 2,700 domestic animals were reported to have been attacked by wolves, most of which were sheep and dogs (70) – it should be remembered that wolves may also be a source of infection with dangerous zoonotic parasites. There are many helminth species reported in wolves (4, 13, 15, 18, 20, 52). However, the most important from the point of view of human health are tapeworms

of the *Echinococcus* genus. The larvae of members of this genus cause an extremely dangerous disease in humans which takes one of two forms depending on the species: alveolar echinococcosis or cystic echinococcosis. The *Echinococcus* genus includes several species differing in morphology and life cycles (60) for which the grey wolf may act as the definitive host: *Echinococcus multilocularis* and species grouped in the *E. granulosus s.l.* complex. *Echinococcus multilocularis* occurs in animals only in the northern hemisphere, there infecting its typical definitive host, the red fox. The definitive host can also be *i.a.* the raccoon dog, wolf or dog and the intermediate hosts are rodents. *Echinococcus granulosus s.l.* occurs all over the world and when it parasitises domestic animals, the typical final host is the dog and the intermediate host is domestic ungulates. However, in the sylvatic life cycle, the most common definitive hosts are wolves and intermediate hosts are wild ungulates (46, 60). Investigations on *Echinococcus* tapeworms in wolves were conducted in various regions of the world, reporting *E. granulosus s.l.* in Asia with prevalence of 4.2–19.5%) (1, 26), revealing it in North America with prevalence of 63% (21) and numerous describing them in Europe with prevalence of 3–26% depending on the country (24, 27, 48, 51, 52). *Echinococcus multilocularis* was also reported in wolves in Asia (3, 26), North America (65) and Europe (4, 27, 49, 76).

In Poland, several studies were carried out in wolves to detect intestinal parasites (8, 22, 41) failed to detect *Echinococcus* spp. Recently, however, our research on several animals showed the presence of *E. ortleppi* in a wolf in south-eastern Poland (34). This was a sign for us to continue investigations for *Echinococcus* in this area, which is the region of Poland most populated by wolves. The aim of the investigations was to estimate the occurrence of infections with *Echinococcus* spp. and other helminths in wolves in south-eastern Poland with the use of different diagnostic methods.

Material and Methods

Samples. Samples from wolves were collected in south-eastern Poland (NUTS PL821). Details concerning the distribution of sample collections are presented in Fig. 1. Overall, 74 samples of wolf faeces were collected. Fifty-five samples were taken from the environment by forest workers experienced in recognising wild animal faeces. Additionally, faeces were obtained from the rectum of 19 wolves (described below). The study on intestinal helminthofauna was conducted additionally on the intestines of 20 wolves officially sampled and necropsied by regional veterinary officers. Eleven of these wolves were found dead and nine were officially shot under permit by the Polish General Director of Environmental Protection. Thirteen of these animals (including one *E. ortleppi*-positive wolf) were described previously in the study related only to *Echinococcus* worm detection (34). There were 10 male, 9 female and 1 of which the sex was not determined, and they were aged from 0.5 to more than 15 years. All samples were frozen for at least 7 days at −80°C before examination for safety reasons.

Microscopic examination. Three equal parts of the small intestine (anterior, middle and posterior) and the large intestine were separately examined using the sedimentation and counting technique (SCT) (25, 78) to detect intestinal parasites. Detected *Echinococcus* spp. tapeworms were isolated during the SCT procedure and preserved in 70% ethanol for further molecular identification. Faecal samples were examined by flotation (the McMaster method with Raynaud's modification) (59) to detect the parasites' eggs and oocysts.

Molecular examination. Extraction of DNA from all wolves' faeces was undertaken using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol for larger volumes of stool. The extracted DNA was examined using the following methods: 1) multiplex PCR for the detection of *E. multilocularis*, *E. granulosus* and other cestodes (mainly *Taenia* spp.) (74); 2) a real-time quantitative PCR (qPCR) for detection of *E. multilocularis* (42) (with following modifications: in a final volume of 20 µL there were used 30 pmol of each primer and 4 pmol of rrn-Em probe; moreover, rrn-Em reverse primer (SEQ-PCR) 5'- GGGGTCAATCACAACAACCC -3' was used directly instead of standard rr-Em reverse primer); 3) four separate qPCRs for detection of *E. granulosus s.l.* (genotypes: G1–3), *E. equinus* (G4), *E. ortleppi* (G5) and *E. canadensis* (G6–8,10) (47).

Extraction of DNA from the adult *Echinococcus* worms from the wolves' intestines for genetic analysis was achieved using a QIAamp DNA Mini Kit (Qiagen) following the manufacturer's protocol. Before extraction, the worms were thoroughly washed in a 0.9% NaCl solution in a Petri dish. Three tapeworms from each infected animal were prepared and used for analysis. *Echinococcus granulosus s.l.* worms were analysed by amplification of the fragments of two mitochondrial genes: the reduced nicotinamide adenine diphosphate dehydrogenase subunit 1 gene (*nad1*) and the cytochrome c oxidase subunit 1 gene (*cox1*). A PCR was performed according to the procedure by Bowles and McManus (11) for *nad1* amplification. *Cox1* was amplified with a PCR specified by Casulli *et al.* (14). *Echinococcus multilocularis* worms were analysed by amplification of the *cox1* and *nad2* genes following the protocol provided by Nakao *et al.* (55) and using the modifications of Santoro *et al.* (62). The PCR products were separated by horizontal electrophoresis in a 1.5% agarose gel stained by Simply Safe (EURx, Gdańsk, Poland). The selected PCR products were sequenced by standard Sanger sequencing at a commercial company (Genomed, Warsaw, Poland). The sequences obtained were compared to the GenBank collection using the National Center for Biotechnology Information basic local alignment search tool.

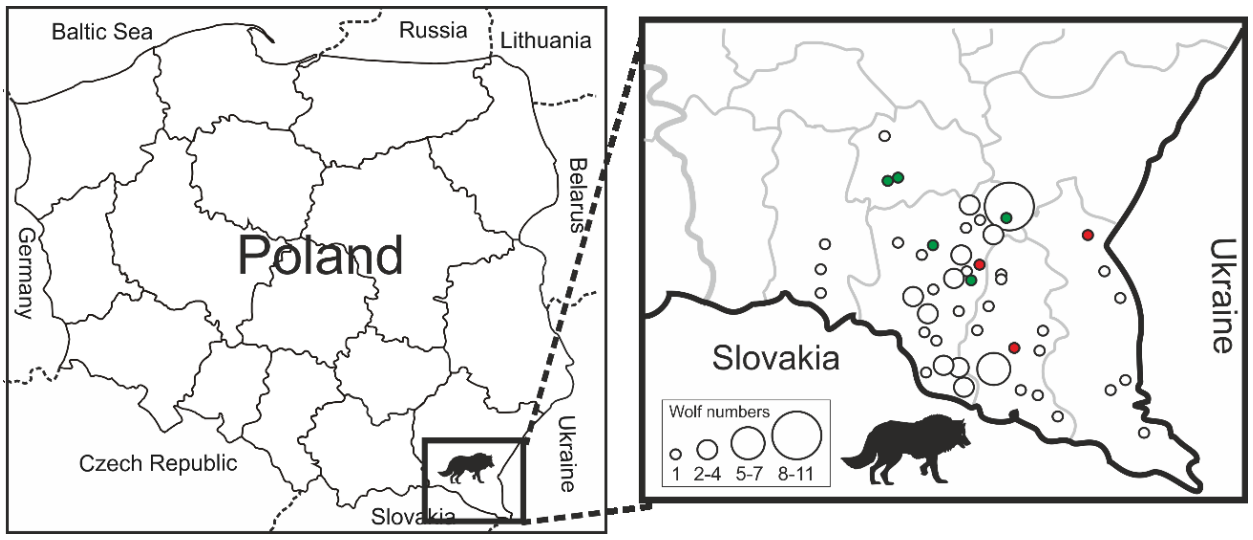

Fig. 1. Geographical location of wolf faecal samples collected for the study. Numbers of samples obtained in individual locations are represented by circles of different sizes. Green circles – *Echinococcus multilocularis*-positive samples; red circles – *E. ortleppi*-positive samples; white circles – *Echinococcus* spp.-negative samples

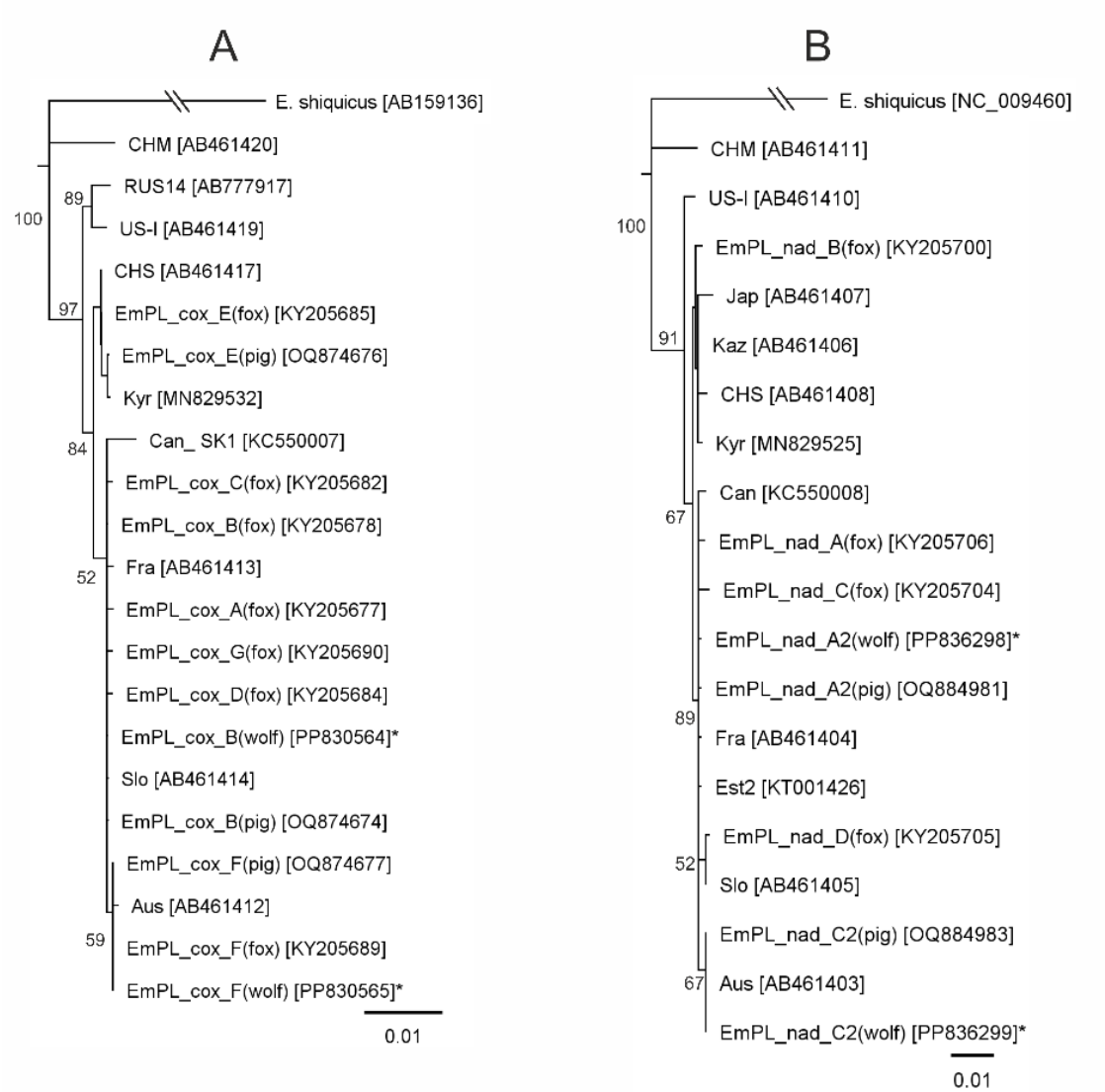

Fig. 2. Phylogenetic trees of *Echinococcus multilocularis* based on the *cox1* gene (A) and *nad2* gene (B). EmPL_cox_A–EmPL_cox_G and EmPL_nad_A–EmPL_nad_D – Polish haplotypes (* – sequences of this study); Aus – Austria; Can/Can_SK1 – Canada; CHM – China (Inner Mongolia); CHS – China (Sichuan); Est2 – Estonia; Fra – France; Jap – Japan, Kaz – Kazakhstan; Kyr – Kyrgyzstan; RUS14 – Russia; Slo – Slovakia; US-I – USA (Indiana). Values on the tree nodes are bootstrap proportions (%)

Phylogenetic analysis. The sequenced fragments of *cox1* and *nad1* (*E. ortleppi*) and *cox1* and *nad2* (*E. multilocularis*) were edited and analysed in Geneious R11 (39). Previously trimmed sequences were aligned according to ClustalW using the following parameters: gap-opening penalty 10 and gap-extension penalty 0.2. For the phylogenetic trees, a Tamura–Nei genetic distance model and the neighbour-joining method were used in Geneious R11. One thousand nonparametric bootstrap inferences were performed. The nucleotide sequence data reported in this paper are available in the GenBank database under the following accession numbers: PP830564, PP830565, PP836298 and PP836299 (for *E. multilocularis*) and PP833027 and PP836300 (for *E. ortleppi*). To estimate the phylogenetic position of the isolates, homologous mitochondrial DNA sequences logged in GenBank (2, 10, 11, 19, 31, 34, 37, 55, 67) were retrieved and used in the analyses. The accession numbers of the homologous sequences are given in Fig. 2.

Results

The PCR results are presented in Table 1.

Table 1. Combined results of multiplex and quantitative PCR analysis of wolf faeces samples (n = 74) to detect helminth DNA

	Positive results	
	n	% (95% CI)
Echinococcus multilocularis	5	6.8 (2.9–14.9)
Echinocccus granulosus s.l.[a]	3	4.1 (1.4–11.3)
Taenia spp.[b]	32	43.2 (32.6–54.6)
Mesocestoides litteratus	3	4.1 (1.4–11.3)

Sequencing identification: [a] – *E. ortleppi* (genotype G5); [b] – *Taenia serialis* (n = 25, 33.8%), *T. hydatigena* (n = 6, 8.1%), *Hydatigera taeniaeformis* (n = 1, 1.4%). CI – confidence interval

In total, *E. multilocularis* DNA was detected in five wolf faeces samples, which constitute 6.8%. In four samples, positive results were obtained using both the multiplex PCR (74) and qPCR (42), while in one sample positive results were only obtained in the qPCR (42). The PCR products were sequenced and their correspondence with the *E. multilocularis* sequences deposited in the GenBank database was confirmed. *Echinococcus granulosus s.l.* DNA was detected in three stool samples (4.1%). In two samples, positive results were obtained both in the multiplex PCR (74) and qPCR for the sensu lato species *E. ortleppi* and *E. canadensis* (47). However, in the other sample, a positive result was only obtained using qPCRs for these two species (47). The PCR products obtained from faeces (and additionally from adult tapeworms from the intestines which were also examined) were sequenced and their consistency with the *E. ortleppi* sequences deposited in the GenBank database was confirmed. The geographical origins of positive samples are presented in Fig. 1.

By multiplex PCR followed by sequencing of the obtained amplicons, *Taenia* spp. DNA was detected in 32 (43.2%) faecal samples: *Taenia serialis* in 25 (33.8%), *T. hydatigena* in 6 (8.1%) and *Hydatigera taeniaeformis* in 1 (1.4%). Additionally, *Mesocestoides litteratus* DNA was found in 3 samples (4.1%). Examination of the faeces by PCR also showed the occurrence of co-infections with tapeworms of various species: *E. ortleppi* with *T. serialis* in two wolves (2.7%), *E. multilocularis* with *T. serialis* in two wolves (2.7%) and *E. multilocularis* with *M. litteratus* in one wolf (1.4%).

The results of intestinal helminth content screening are presented in Table 2. The presence of *E. multilocularis* tapeworms was found in two wolves – in one, 6 adult tapeworms were detected, and in the other 55,660. This species of tapeworm was found only in the anterior and middle parts. In the wolf infected with the higher number of tapeworms, it was found that the vast majority of parasites were located in the middle part (n = 52,000), the remaining 3,660 individuals being in the anterior part. In neither of the two infected wolves was *E. multilocularis* detected in the posterior part of the small intestine, and only single individuals were found in the large intestine. *Echinococcus granulosus s.l.* tapeworms were found in two other wolves with counts of 64 and 436. Most of them were located in the anterior part, where the mean content was 184, and the middle part, where it was 61. Very low numbers of tapeworms of this species were also found in the posterior part of the small intestine and in the large intestine.

In addition, infections with *Taenia* spp. tapeworms, which occurred in all 20 wolves examined with SCT, were detected at a relatively high intensity (an average of 33 tapeworms per intestine, with the highest intensity in the middle part of the small intestine). Tapeworms of the genus *Mesocestoides* were found in the intestines of two wolves (10%) in similar numbers in the anterior and middle parts. *Alaria alata* flukes were found in four wolves and had only infected the anterior part of the small intestine. *Euryhelmis* sp. trematodes were identified in only one animal and were located only in the posterior part of the small intestine. Hookworms were identified in six wolves and had mainly colonised the middle part of the small intestine, although a few were also found in other parts and in the large intestine. Nematodes of the *Molineus* genus were found most abundantly in the posterior part of the small intestine in one wolf. One parasite was found only in the large intestine, and it was *Trichuris vulpis*, single individuals of which were seen in three wolves.

The results of microscopic examination of stools for parasite eggs and oocysts are presented in Table 3. Coproscopic examination was performed on 63 samples. The most frequently found were *Capillaria*-like eggs (58%) and tapeworm eggs from the Taeniidae family (27%). The average numbers of eggs per gram of faeces of these parasites were relatively high and respectively 316 and 399. In single samples and in low numbers, eggs

of flukes, hookworms, *Trichuris*, *Toxocara* and coccidia oocysts were found.

The PCRs for *cox1* and *nad2* obtained from the six *E. multilocularis* worms isolated from two wolves in this study gave positive results. The analysis of the *cox1* and *nad2* genes showed that worms isolated from one wolf all belonged to the same haplotype and a different haplotype to that of the worms from the other wolf. Two haplotypes of the *cox1* gene found in the present investigation (PP830564 and PP830565) (Fig. 2) corresponded to the haplotypes EmPL_cox_B and EmPL_cox_F described earlier in red foxes and pigs in Poland (GenBank records KY205678, KY205689, OQ874674 and OQ874677) (31, 37). Similarly, two haplotypes of the *nad2* gene resulting from the present research (PP836298 and PP836298) corresponded to haplotypes described earlier in Poland in pigs (31) – EmPL_nad_A2 and EmPL_nad_C2 (OQ884981 and OQ884983) – and in red foxes (with a difference of only one nucleotide) – EmPL_nad_A, EmPL_nad_C (KY205706 and KY205704) (37). All identified haplotypes were grouped in the European clade.

The PCRs for *cox1* and *nad1* obtained from all three *E. granulosus s.l.* tapeworms isolated from the wolf in this study showed specific products. Comparison of the new PP836300 and PP833027 sequences with others previously logged in the GenBank database showed similarity to the *E. ortleppi* (G5) sequences. It showed 100% identity with the *E. ortleppi* nucleotide sequences MZ322608 and MZ322609 isolated from another positive wolf which was previously described separately as the first Polish case (34). Full identity was observed in the *nad1* sequence in relation to larvae from a human case of echinococcosis in Poland (MH492788) (19). Sequences were also similar to those of larvae isolated from a Philippine spotted deer in a zoo in the United Kingdom (JX068638) (10) and cattle in the Netherlands (AJ237636) (11).

Table 2. Occurrence of helminths in wolf intestines (n = 20) estimated using sedimentation and counting technique taking into account the location and distribution of parasites in individual parts of the intestines

Helminth	Entire intestines (small + large)		Small intestine						Large intestine	
			Anterior part		Middle part		Posterior part			
	% Positive (95% CI)	Mean intensity (range) [CV]	% Positive (95% CI)	Mean intensity (range) [CV]	% Positive (95% CI)	Mean intensity (range) [CV]	% Positive (95% CI)	Mean intensity (range) [CV]	% Positive (95% CI)	Mean intensity (range) [CV]
Echinococcus multilocularis	10 (3–30)	27,833 (6–55,660) [141%]	10 (3–30)	1,832 (3–3,660) [141%]	10 (3–30)	26,002 (3–52,000) [141%]	0	-	5 (1–24)	8
E. granulosus s.l.[a]	10 (3–30)	250 (64–436) [105%]	10 (3–30)	184 (42–326) [109%]	10 (3–30)	61 (14–108) [109%]	10 (3–30)	3 (1–5) [94%]	10 (3–30)	2 (1–3) [71%]
Taenia spp.	100	33 (1–208) [135%]	90 (70–97)	5 (1–30) [178%]	90 (70–97)	24 (1–150) [140%]	80 (58–92)	8 (1–30) [121%]	35	1.3 (1–3) [59%]
Mesocestoides spp.	10 (3–30)	15 (1–28) [131%]	5 (1–24)	1	5 (1–24)	28	0	-	0	-
Euryhelmis spp.	5 (1–24)	477	0	-	0	-	5 (1–24)	477	0	-
Alaria alata	20 (8–40)	34 (1–135) [190%]	20 (8–40)	34 (1–135) [190%]	0	-	0	-	0	-
Uncinaria/ Ancylostoma	30 (15–50)	45 (1–219) [188%]	10 (3–30)	1	30 (15–50)	32 (1–148) [183%]	15 (5–36)	26 (1–70) [144%]	5 (1–24)	2
Molineus spp.	5 (1–24)	47	5 (1–24)	1	5 (1–24)	1	5 (1–24)	45	0	-
Trichuris vulpis	15 (5–36)	1	0	-	0	-	0	-	15 (5–36)	1
Total (all parasites)	100	-	90 (70–97)	-	90 (70–97)	-	85 (64–95)	-	55 (34–74)	-

[a] – molecularly identified as *Echinococcus ortleppi* (genotype G5); CV– coefficient of variation; CI – confidence interval

Table 3. Results of microscopic examination (flotation) of wolf faeces for helminths (n = 63)

	% of positive samples (95% CI)	Mean EPG/OPG (range) (CV)
Taeniidae	27.0 (17.6–39.0)	399 (15–3,000) (186%)
Trematoda	3.2 (0.9–10.9)	26 (15–50) (76%)
Capillariidae	58.7 (46.4–70.0)	316 (7–2,250) (173%)
Uncinaria/Ancylostoma	1.6 (0.2–8.5)	15
Trichuris vulpis	1.6 (0.2–8.5)	15
Toxocara sp.	1.6 (0.2–8.5)	15
Coccidia	1.6 (0.2–8.5)	300

EPG – eggs per gram; OPG – oocysts per gram; CV– coefficient of variation; CI – confidence interval

Discussion

Our investigations conducted on wolves from south-eastern Poland showed the presence of several species of helminths. Particularly noteworthy is the detection of tapeworms of the *Echinococcus* genus, both from the *Echinococcus granulosus s.l.* group and *E. multilocularis*. To the best of our knowledge, this is the first case of *E. multilocularis* infection recorded in wolves in Poland. Despite the common occurrence of this parasite in Poland in its typical definitive host (the red fox) (30), as well as its detection in dogs and cats (32, 36), so far attempts to detect these tapeworms in wolves in Poland have not yielded positive results (8, 22, 41). The presence of *E. multilocularis* is not heavy and the almost 7% carriage in the examined animals is not a high percentage. However, it should be taken into account that wolves (like dogs or raccoon dogs) are not the typical definitive host of this tapeworm and the percentage of infected animals in endemic areas is usually significantly lower than the percentage found in red foxes. A prevalence of over 40% of *E. multilocularis* was previously reported in red foxes in this area (30). Data from other countries indicate that this detected presence of *E. multilocularis* in wolves in Poland is not an isolated case. In Europe, detection rates of this infection ranged from 0.3% in France (76) and 5.9% in Latvia (4) to 35% in Slovakia (27). Studies from other continents also confirmed the presence of this tapeworm in wolves: publications from Iran (5), Mongolia (26) and eastern Turkey (3) cite its distribution in Asian populations and one from Canada demonstrates that North American wolves can be infested (64). The last two studies are particularly interesting because of co-infections of *E. multilocularis* with *E. granulosus s.l.* in the same individuals, which we did not find in our studies.

However, the distribution of *E. multilocularis* tapeworms in the intestine indicated that the highest intensity of infection was observed in the middle part of the small intestine, a slightly lower one was in the anterior part, while in the posterior part they were not recorded at all. This is a different distribution to that found in the typical definitive host, where the posterior part of the small intestine is the predilection site for *E. multilocularis* (35, 75). It is worth noting that half of the samples positive for *Echinococcus* spp. were also positive for *Taenia* spp. However, this is probably due to the very high prevalence of *Taenia* spp. in the investigated wolf population.

Phylogenetic studies of isolated tapeworms based on both the *cox1* and *nad2* genes indicated the presence of two *E. multilocularis* haplotypes previously described in Poland in foxes and pigs (31, 37). Both of these haplotypes belong to the European clade. One of them corresponds to the haplotype seen to occur over most of Poland (EmPL_cox_B for *cox1* and EmPL_nad_A for *nad2*) (44, 62). The second haplotype is characteristic of the southern regions of Poland (EmPL_cox_F for *cox1* and EmPL_nad_C2 for *nad2*), as well as regions of Europe located south of Poland, specifically Slovakia and Austria (55, 62). This confirms the characteristic distribution of haplotypes in Poland previously described in red foxes and pigs (31, 37).

The second species of the *Echinococcus* genus found in 4% of wolves was *E. ortleppi*. This species is part of the taxonomic complex known as *E. granulosus s.l.* We have already described the first case of this species infecting a grey wolf in Poland (34), which was confirmed morphologically by the observation of adult worms in the intestines and corroborated molecularly. The research described in the present article, which was a continuation and extension of those earlier studies, confirmed the presence of this species in the wolf population in south-eastern Poland. Some years ago, DNA of *E. ortleppi* was also detected in wolf faeces in the south-western Italian Alps (together with that of *E. multilocularis*). So far, the most frequently identified species in the world in the group *E. granulosus s.l.* were *E. canadensis* and *E. granulosus s.s.* (12, 26, 45, 52, 57). There are studies in which species within the *E. granulosus s.l.* complex were not distinguished and which reported these cases as *E. granulosus* because of the methodology or old nomenclature. Therefore, it cannot be excluded that *E. ortleppi* was also among them (1, 21, 23, 24, 38, 68).

Echinococcus ortleppi tapeworms were located in similar numbers in the anterior and middle parts of the small intestine, but only a quarter as many were found in the posterior part. This is a typical distribution for *E. granulosus*. *Echinococcus ortleppi* is considered a species with a typical life cycle, colonising dogs as the definitive host and cattle as the intermediate host (60). Cattle as a source of infection for wolves cannot be completely excluded. However, the fact that all cases of *E. granulosus s.l.* detected in our study were *E. ortleppi* may suggest that the intermediate hosts were cervids. Cattle constitute a small part of the diet of wolves in Poland (they represent approximately 5% of the food biomass eaten by these predators), unlike deer or other cervids which are their main prey (28). The cervid prey preference of wolves and detection in south-eastern Polish wolves of *E. ortleppi* recommend the extension of testing for *Echinococcus* to the wild deer population in this area. Also, the zoonotic potential of this species in Poland should be emphasised, not least because an identical *nad1* haplotype to that found in wolves was previously isolated from a human cystic echinococcosis case (19).

It should be stressed that in all faeces samples obtained from the intestines where *Echinococcus* spp. tapeworms were detected by SCT, positive results were obtained in molecular tests. However, differences in efficiency were found between the multiplex PCR and qPCR methods. Analysing the results, we can notice the higher sensitivity of the qPCR in the finding of one sample positive for *E. multilocularis* by this qPCR (as well as by SCT) but the finding of that sample negative by the multiplex PCR. This may correlate with the very low intensity of infection found in the intestine (six tapeworms). Also, another sample that was positive

by the qPCRs for *E. granulosus s.l.* (*E. ortleppi*/*E. canadensis*) was negative by the multiplex PCR. Interestingly, for all three samples positive for *E. granulosus s.l.* in the set of qPCR methods set out by Maksimov *et al.* (47), an amplification product was found in qPCRs for both *E. ortleppi* and *E. canadensis* (ultimately, sequencing confirmed that it was *E. ortleppi*). Such a cross-reaction may result from the close relationship of both species, which are sometimes called "sister species"; the author of the method also noted this kinship when writing about possible cross-reactivity (47).

The most frequently detected helminths in our investigation were tapeworms of the *Taenia* genus: PCR tests showed 43% of wolves to be positive, and all of the 20 wolves of which the intestines were examined were infected with these tapeworms. In many studies where *Taenia* was identified, the prevalence was also high (36–100%) (1, 9, 17, 18). In our investigation, *T. serialis* was most frequently identified (three times more often than *T. hydatigena*). A similarly high percentage of this species in wolves was noted in central Italy (17). Surprisingly, it may suggest that lagomorphs (typical intermediate hosts of this tapeworm) are frequent prey of wolves. However, according to recent findings (53), European roe deer can be intermediate hosts of *T. serialis,* which suggests a more probable source of infection for the wolves in our investigations. In other studies, the dominant species was *T. multiceps* or *T. hydatigena* (4, 18, 52). In Poland, previous allied studies were based on microscopic examination of faeces. Taeniidae eggs were found in a relatively low percentage of samples (1.4–11%) (8, 22, 58) or were not detected at all (41, 72). The real percentage of animals infected with *Taenia* was probably much higher, because coproscopic examination is characterised by relatively low effectiveness in detecting *Taenia*-like eggs. In our studies, Taeniidae eggs were found only in half of the samples which were positive for Taeniidae (*Taenia* or *Echinococcus*) in the PCRs and SCT. An unrepresentatively low prevalence probably also resulted from the limitation of coproscopic methods in our earlier studies in red foxes and dogs, where the percentage of samples with *Taenia*-like eggs was significantly, and even several times, lower than it was in SCT or PCR results (29, 32, 36).

Other tapeworms found in our investigations were *Mesocestoides litteratus*. They occurred in a relatively low percentage (4%). A similar prevalence of *Mesocestoides* spp. in wolves was found in Italy (18), Latvia (4) and Spain (66), and a slightly higher one (12%) in Estonia (52) and Kazakhstan (1). Red foxes in the same area (south-eastern Poland) yielded a much higher prevalence of these tapeworms (92%) (29). The prevalence difference between *Mesocestoides* spp. in wolves and in foxes, as in the case of *E. multilocularis*, is probably mainly due to the different diet of red foxes to that of wolves: foxes prey on the small mammals that are the intermediate hosts of *Mesocestoides* much more often than wolves.

In our study, *Toxocara* nematodes were extremely rare – they were not detected in any of the examined intestines, and *Toxocara* eggs were found in only one stool sample (1.4%). Our earlier findings in red foxes and stray dogs in the same area showed *Toxocara* spp. in 20% and 24% of these animals, respectively. This may indicate that wolves are less susceptible to this infection. In other studies conducted on wolves in Poland, this percentage was higher than ours and ranged from 5.6% to 13% (8, 41, 58). A similarly low percentage of *Toxocara*-positive wolves (0.2–2.1%) was found in studies conducted in Canada (13, 71); however, studies outside Poland in other European countries showed from 3.9% to 9.5% (4, 15, 18, 52, 66), and equivalent research in Asia and Africa indicated from 3.6% to 36% (1, 5, 77).

Hookworms were detected in 30% of wolves in SCT examination. Most of these nematodes were detected in the middle and posterior parts of the small intestine, which indicates a distribution similar to that in foxes (35). A similar percentage of wolves infected with hookworms was found in the north-eastern part of Poland (41). Because of the specificity of the method employed, hookworms were not identified to species level; however, based on data from this part of Europe, it can be assumed that the majority were in the *Uncinaria* genus. In other studies conducted in southern Poland, *Uncinaria* was found in 37% of wolves (58). In other countries, *Uncinaria* was also detected in high percentages ranging from 26 to 77% (4, 18, 20, 52, 66). However, *Ancylostoma* infection was recorded in significantly lower percentages ranging from 3% to 8.5% (4, 18, 66).

Adult *Molineus* spp. nematodes were found in one wolf. It is a rarely recorded nematode in wolves, which the small percentage (2.4%) detected in wolves in north-western Italy bears out (18). *Molineus* occurs mainly in mustelids, where the prevalence reached 85% (43, 73). However, in our other study conducted in red foxes and raccoon dogs, these nematodes were detected only in raccoon dogs and in a surprisingly high percentage (41%) (33).

Capillariidae eggs were found in a significant percentage of faeces samples (58.7%). It may be assumed that these were eggs of the lungworm *Eucoleus aerophilus*. Relatively high percentages of samples with eggs of this type were previously found in other regions of Poland in wolves (58, 72). Examples in the indicate that this parasite is often found in wolves throughout the world (4, 13, 18, 52, 61).

Another nematode found in wolves was *Trichuris vulpis*. In our study, *T. vulpis* adult worms were detected in 15% of wolves in the predilection site, the large intestine. During microscopic examination of faeces, eggs of this parasite were detected in 1.6% of samples. This low percentage obtained in the coproscopic examination differs from the results obtained earlier in Poland (14–38%) (8, 41). In other countries, whipworm infections have been reported in varying rates from 0.2% to 22.3% (1, 13). When analysing coproscopic results,

the similarity of *Capillaria* and *Trichuris* eggs should be taken into account, because sometimes it may cause false results.

In our study, *Alaria alata* flukes were found in 20% of wolves of which the intestines were examined using the SCT method. Studies from Poland and other countries show wide variations in prevalence, from 2% to 89% (4, 52, 58, 66, 71, 72). Prevalence differences between regions are due to the life cycle of *A. alata* – this parasite requires intermediate hosts associated with the aquatic environment, which predisposes animals living in areas with many bodies of water to infection. In our research on red foxes, such a relationship was noted (30) in red foxes from north-eastern regions with many lakes: this population showed 90% prevalence of *A. alata* while red foxes in the south-eastern region showed only 20% (29), similar to the percentage in wolves in the current study.

An interesting element in our study was the detection of *Euryhelmis* spp. flukes in the intestine of one wolf. These are flukes with a complex life cycle, where the intermediate hosts are often snails and amphibians. However, the most common definitive hosts are mustelid mammals (50, 63, 73), raccoons (16) or raccoon dogs (40). To the best of our knowledge, our study presents the first case of detection of this type of fluke in the grey wolf. Because small animals are a marginal part of the wolf's diet, infection with these parasites is probably not a significant health problem for these animals. In Poland, there is no data on this parasite in other animal species, so it is advisable to develop research in this direction.

Conclusion

Our study showed the presence of *E. multilocularis* in wolves for the first time in Poland and confirmed our earlier observations regarding the occurrence of *E. ortleppi*. This is important information from an epidemiological point of view and this double zoonotic threat from *Echinococcus* in the south-eastern wolf population (the largest in Poland) should be taken into account when assessing the risk of infection to humans. In addition, the research enriched knowledge of other helminths found in wolves, including zoonotic ones, but also those (*Euryhelmis*) that were recorded for the first time in this species.

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