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Folia Parasitologica 2016, 63: 018
doi: 10.14411/fp.2016.018

Folia Parasitologica
http://folia.paru.cas.cz

Research Article OPEN ACCESS

First detection of *Echinococcus multilocularis* in dogs in a highly endemic area of Poland

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Abstract: The aim of the investigation was to estimate the epizootic situation concerning infection by the cestode *Echinococcus multilocularis* Leuckart, 1863 in dogs (*Canis lupus familiaris* Linnaeus) from a Polish region where this parasite is highly prevalent in red foxes. Faecal samples (n = 148) were collected from rural dogs in Podkarpackie Province. Samples were examined through nested PCR (for *E. multilocularis*), multiplex PCR (*E. multilocularis*, species of *Taenia* Linnaeus, 1758) and PCR [*E. granulosus* (Batsch, 1786)]. Specific products were sequenced. Faeces were also examined coproscopically. In samples from two dogs (1.4%), there were positive PCR results for *E. multilocularis*. *Taenia*-specific PCR products were found in nine dogs (6.1%). Sequencing identified *Taenia serialis* (Gervais, 1847), *T. hydatigena* Pallas, 1766, *T. pisiformis* (Bloch, 1780) and *Hydatigera taeniaeformis* (Batsch, 1786). One sample (0.7%) was identified as *Mesocestoides litteratus* (Batsch, 1786). All samples were negative for *E. granulosus* with PCR. Taking into account coproscopic and PCR results, 28% of dogs were infected with helminths (8% with tapeworms). It should be stressed that one of the infected with *E. multilocularis* dogs shed eggs of the *Taenia* type and had a habit of preying on rodents. This investigation revealed the presence of *E. multilocularis* in dogs for the first time in Poland.

Keywords: alveolar echinococcosis, *Echinococcus granulosus*, *Taenia*, PCR, faeces

Echinococcus multilocularis Leuckart, 1863 is a tapeworm, the larval forms of which cause alveolar echinococcosis in humans. One of the most dangerous zoonotic diseases, it can be fatal if untreated. Humans only play the role of a non-specific accidental intermediate host, certain rodent species being specific intermediate hosts in the parasite's life cycle. The principal final host is the red fox, *Vulpes vulpes* (Linnaeus). *Echinococcus multilocularis* is widespread in the red fox population, so this species is mainly responsible for environmental contamination by spreading the parasite's infective eggs via their faeces (Hegglin and Deplazes 2013).

Among domesticated animals, this function can also be carried out by dogs (*Canis lupus familiaris* Linnaeus) and even cats (*Felis catus* Linnaeus). Unlike cats, in which infection is very limited (Kapel et al. 2006, Thompson et al. 2006, Umhang et al. 2015), the dog is an adequate host for the development of the mature forms of *E. multilocularis*: the time and intensity of the excretion of tapeworm eggs by infected dogs were comparable to the results obtained for foxes and raccoon dogs (Kapel et al. 2006).

The role of dogs as potential hosts of *E. multilocularis* is increasing as globalisation develops, facilitating movements over long distances. It creates new opportunities for spreading this infection to areas where it is almost impossible for the parasite to be brought through free-living hosts, such as islands. In order to control this threat, the EC Commission adopted Regulation No. 1152/2011, which defines rules on transporting dogs to countries known to be free of this infection (currently four Member States, namely Ireland, the United Kingdom, Malta and Finland).

However, with the exception of a single study (Machnicka-Rowińska et al. 2002) that gave negative results, investigations into the prevalence of this infection in dogs have not been conducted in Poland, a country where *E. multilocularis* is highly prevalent in foxes. In 2009–2013, the mean prevalence rate was 17%, but there were areas in the east of the country where prevalence reached 50% (Karamon et al. 2014). It was shown to have been persisting at a high level for several years (Karamon et al. 2015), which indicates a high risk for people living in these areas. This is why Podkarpackie Province – which is characterised by one of

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the highest percentages of infected foxes (47%) – was selected to study the infection of dogs with *E. multilocularis*. The aim was to evaluate the prevalence of *E. multilocularis* in dogs from areas where the parasite is highly prevalent in red foxes.

MATERIALS AND METHODS

Dogs. Samples of faeces were collected from 148 rural dogs originating from four districts in Podkarpackie Province (south-eastern Poland) between March and May 2015. Most of the samples were obtained from Strzyżów District (n = 125), and the others from three bordering districts: Krosno (n = 11), Rzeszów (n = 9) and Ropczyce-Sędziszów (n = 3). Samples were obtained from pet and guard dogs in villages, farms and rural areas of small towns. Fresh samples of faeces were collected by veterinarians during their visits to individual locations for an anti-rabies vaccination campaign in cooperation with dog owners who provided the samples. Up to 48 h after collection, each sample was first frozen (-20 °C) by veterinarians and then sent in batches to the National Veterinary Research Institute (NVRI). Owners were asked to fill in questionnaires including information on deworming (last treatment, kind of drug) and their dogs' habits as to preying on rodents.

In the NVRI laboratory, faeces were frozen for at least seven days at -80°C before examination for safety reasons. DNA from faecal samples was extracted with the QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol for larger volumes of stool. In this protocol, 1 g of sample was first diluted (1 : 10) in lysing buffer and homogenised. Next, 2 ml of mixture was used in the following extraction stages. DNA samples were examined by multiplex PCR with the use of primers to detect *E. multilocularis* and species of *Taenia* Linnaeus, 1758 (Trachsel et al. 2007). Additionally, each sample was tested by nested PCR as described by Dinkel et al. (1998) with some modifications (Karamon et al. 2012) to identify *E. multilocularis*. Moreover, samples were examined to detect *Echinococcus granulosus* (Batsch, 1786) by PCR according to Abbasi et al. (2003) with the use of only one pair of primers, Eg1121a and Eg1122a. Each DNA sample was examined by PCR using two variants – undiluted and 1 : 10 diluted – to minimise the possibility of inhibition (Karamon 2014).

The *E. multilocularis* and *Taenia* spp. positive PCR products were sequenced. Samples for sequencing were purified using Sephadex G-50 columns. Sequencing was performed using a BigDye™ Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, USA) on an ABI3730xl Genetic Analyzer (Applied Biosystems). The sequenced data were analysed and compared to the GenBank collection using BLAST searchers.

A part of each fecal sample (1–2 g) was examined by flotation (McMaster method according to Raynaud's modification – Raynaud 1970) to detect parasite eggs and oocysts.

People. Members of a family whose dog was infected with *E. multilocularis* were referred by family doctor to a serological diagnostic tests due to the potential risk of infection. Two IgG ELISA tests (Bordier Affinity Products S.A., Crissier, Switzerland) for the serological diagnosis of human echinococcosis were used: an *E. granulosus* ELISA and an *E. multilocularis* ELISA. The tests were carried out according to the manufacturer's instructions in the Diagnostics Laboratory of the Department of Medical Parasitology NIPH–NIH, Warsaw.

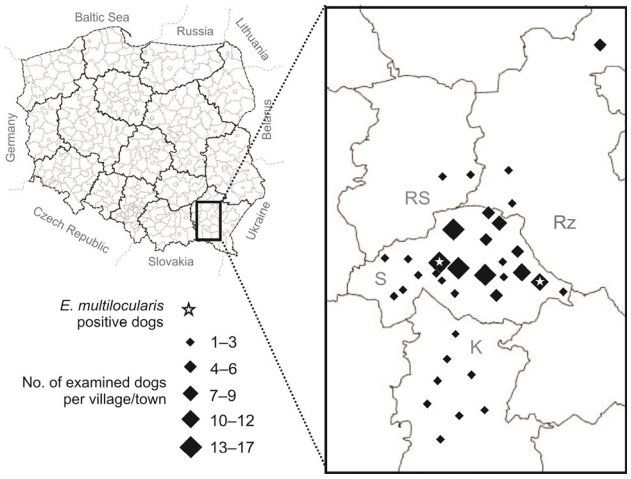

Fig. 1. Distribution of the location of collected faecal samples and location of dogs (*Canis lupus familiaris* Linnaeus) positive for *E. multilocularis* Leuckart, 1863. (Districts: K – Krosno, RS – Ropczyce-Sędziszów, Rz – Rzeszów, S – Strzyżów,)

Red foxes. In the area where the dogs were sampled, the prevalence of *E. multilocularis* in foxes was estimated by necropsy to confirm the high prevalence observed previously. Fifty nine foxes were shot by hunters from October 2014 to January 2015 in the area covering most of the locations of the dogs studied, i.e. Strzyżów and Krosno districts. The small intestines were examined after being frozen for at least seven days at -80 °C before examination for safety reasons. All the samples of intestines were examined using the sedimentation and counting technique (Hofer et al. 2000, OIE 2008).

Statistical analysis

Differences in prevalence between dogs grouped according to questionnaire parameters (dewormed/untreated or preying/not preying) were tested using V-square tests ($P < 0.05$) with Statistica 9.1 (StatSoft Inc., Tulsa, USA).

RESULTS

Dogs. In samples from two dogs (from Strzyżów District), PCR results were positive for *E. multilocularis* (prevalence 1.4%) (Fig. 1). In one positive dog, a specific product was detected by both PCRs (nested PCR and multiplex PCR) only in diluted DNA (1 : 10 dilution). However, a positive result was found in the other dog only by using nested PCR and only in an undiluted isolate. The amplicons obtained were sequenced and then compared to the GenBank database, confirming that the causative agent was *E. multilocularis* in both cases.

Multiplex PCR products of 267 bp were found in ten dogs. A comparison of sequences with those in the GenBank database identified nine samples (6.1%) as *Taenia* spp.: four samples contained *Taenia serialis* (Gervais, 1847), two *T. hydatigena* Pallas, 1766, two *T. pisiformis* (Bloch 1780), one *Hydatigera taeniaeformis* (Batsch, 1786) and one sample *Mesocestoides litteratus* (Batsch, 1786). None of the dogs were coinfected with *E. multiloc-*

Table 1. *Echinococcus multilocularis* Leuckart, 1863 and other parasites in dog (*Canis lupus familiaris* Linnaeus) faeces detected by PCR and coproscopy.

	PCR				Coproscopy						Coproscopy and PCR	
	E. multilocularis Leuckart, 1863	*E. granulosus* (Batsch, 1786)	*Taenia* Linnaeus, 1758	*Mesocestoides litteratus* (Batsch, 1786)	Taeniidae Ludwig, 1886	*Trichuris* Roederer, 1761 / *Capillaria* Zeder, 1800	*Toxocara* Stiles et Hassal, 1905	*Toxascaris leonina* (von Linstow 1902)	*Cystoisospora* Frenkel, 1977	Helminths together	Tapeworms	Helminths together
Number of positive	2	0	9	1	4	26	22	3	6	36	12	41
Prevalence % (95% CI)	1.4 (0.4–4.8)	0.0 (0.0–2.5)	6.1 (3.2–11.2)	0.7 (0.1–3.7)	2.7 (1.1–6.7)	17.6 (12.3–24.5)	14.9 (10.0–21.5)	2.0 (0.7–5.8)	4.1 (1.9–8.6)	24.3 (18.1–31.8)	8.1 (4.7–13.6)	27.7 (21.1–35.4)
Mean EPG/OPG* (CV)	-	-	-	-	90 (82%)	1 898 (349%)	1 201 (222%)	82 (39%)	137 (169%)	-	-	-

* EPG/OPG – eggs/oocysts per 1 g of faeces.

Table 2. The prevalence of helminth infections in dogs (*Canis lupus familiaris* Linnaeus) taking into account questionnaire results concerning deworming and preying on rodents.

Questionnaire answers		Number of dogs	% of infected dogs (CI 95%)*	
			Tapeworms	Helminths together
Preying on rodents	Yes	43[1]	9.3 (3.7–21.6)	23.3 (13.2–37.8)
	No	58	6.9 (2.7–16.4)	25.9 (16.3–38.4)
Deworming	Yes	90[1]	7.8 (3.8–15.2)	23.3 (15.8–33.1)
	Yes (<3 m)**	22	9.1 (2.5–27.8)	22.7 (10.1–43.4)
	No	58[1]	8.6 (3.7–18.6)	34.5 (23.6–47.3)

* flotation and PCR results were analysed together; **only dogs dewormed no longer than three months before sampling; [1] shows that one dog infected with *E. multilocularis* Leuckart, 1863 is included in this group.

ularis and *Taenia* spp. Moreover, all the samples were negative for *E. granulosus* by PCR.

The results of coproscopic examination are presented in Table 1. Overall, helminth eggs were detected in 36 samples (24.3%). Taeniid eggs (morphologically identical with those of species of *Taenia* and *Echinococcus*) were detected in four dogs (2.7%). One of these was the dog positive for *E. multilocularis* by PCR (only by nested PCR and only in undiluted DNA). The other three samples with taeniid eggs were identified by PCR and sequencing as *T. pisiformis* (one sample) and *T. serialis* (two samples). Two of these three samples were positive by PCR both in undiluted and 1 : 10 diluted DNA, and one only in undiluted DNA.

Besides tapeworms, eggs of nematodes of the following genera were detected: *Toxocara* Stiles et Hassal, 1905, *Toxascaris leonina* (von Linstow, 1902) and *Trichuris* Roederer, 1761 or *Capillaria* Zeder, 1800. Eggs of *Trichuris* and *Capillaria* could not distinguished because of their similar morphology and were counted together. Moreover, oocysts of species of *Cystoisospora* Frenkel, 1977 were found in six dogs (4.1%).

According to owners' declarations, 90 of the examined dogs (61%) were dewormed (Table 2). Seventy four dogs (82% of dewormed dogs) were treated with combined anthelmintics containing praziquantel, two dogs were treated with a drug not recommended for tapeworms and in 13 cases, no information about the content of anthelmintics was available. Twenty two dogs were treated in the three months prior to sample collection and 68 dogs 3–12 months before sampling. According to owners, 43 dogs had a habit of preying on rodents (58 dogs did not prey and the owners of 47 dogs declared that they did not know). One *E. multilocularis*-positive dog was dewormed (ten months before sampling) and the second one was not treated at all. The same untreated dog was also included in the group of dogs preying on rodents (the owners of the other dog declared that they did not know their dog's preying habits).

Although some differences in prevalence between dewormed and untreated dogs are visible in the general results concerning all helminths, no statistically significant differences were observed.

People. One of the two families whose dogs were *E. multilocularis*-positive decided to undergo a serological test. Eight persons were examined: four adults (44, 40, 22 and 23 years) and four children (2, 7, 12 and 14 years). None were positive for the presence of IgG antibodies to *Echinococcus* spp.

Red foxes. An examination of the intestines of red foxes shot in the area of investigation showed the presence of *E. multilocularis* in 46% of these animals. The mean intensity of infection was 1 687 tapeworms per fox (range 1–23 150, SD = 4 535).

DISCUSSION

Two *Echinoccocus multilocularis*-positive dogs were found in this study. This is the first report of such an infection in dogs living in Poland. An attempt to detect this parasite in dogs was conducted in Poland early in the 21st century with no positive results (Machnicka-Rowińska et al. 2002). However, these dogs originated from northern and southeastern Polish regions where the prevalence of

E. multilocularis in red foxes was relatively low (reported to be about 6% at the time). It can be assumed that the large increase – up to around 50% according to Karamon et al. (2014) – in the prevalence of *E. multilocularis* in foxes observed from 2000 to 2013 in the eastern part of Poland may have an impact on prevalence in dogs. This would easily explain the discovery of two positive dogs in this study in contrast to the absence of any cases ten years earlier.

The relatively low values of prevalence in our study are similar to those obtained in other endemic regions in Europe. The broadest cross-sectional study on infection of *E. multilocularis* (along with *E. granulosus* and *Taenia* spp.) in dogs in Europe was conducted by Dyachenko et al. (2008). The authors examined more than 21 000 faecal samples from dogs in Germany and some other European countries. They found *E. multilocularis*-positive dogs only in Germany, with a prevalence of 0.24%. Of the 289 dogs examined in Slovakia, 2.8% were found to be infected by *E. multilocularis* – Antolová et al. (2009). Most of the Slovak dogs examined originated from the eastern regions directly bordering our study area. Lithuania is another country bordering Poland where *E. multilocularis* infected dogs were found: 0.8% (2/240) of village dogs were positive by PCR (Bruzinskaite et al. 2009). Moreover, the major study covering more than 800 dogs in two highly endemic regions of historical focus in eastern France revealed infection by *E. multilocularis* of 0.5% of the examined dogs (Umhang et al. 2014). Some of the regions where this infection was investigated in dogs gave no positive results, such as the Netherlands (Maas et al. 2014) and northeastern France, where where 142 and 493 dogs were examined, respectively (Umhang et al. 2012).

Echinococcus granulosus was not detected in any of the dogs examined during this study. Similarly, no tapeworms were found by Dyachenko et al. (2008) in Europe despite examining comprehensive number of samples. Studies in France (Umhang et al. 2014) and the Netherlands (Maas et al. 2014) did not find this tapeworm either. However, in Lithuania, *E. granulosus* was detected in 3.8% of dogs (Bruzinskaite et al. 2009). In the present study, one of two *E. multilocularis*-positive dogs shed taeniid eggs. We were able to assert that these were eggs of *E. multilocularis* because *Taenia* spp. was not detected by PCR in this sample. However, the *E. multilocularis* DNA was detected in the second positive dog (having no detected eggs in the sample) using DNA isolated directly from a faecal sample.

Moreover, this study used a specific approach for detecting *E. multilocularis* in that we systematically tested copro-DNA samples by two different PCR techniques (one multiplex and one nested) directly on the extracted DNA but also with a 1 : 10 dilution. One *E. multilocularis*-positive sample was identified due to the better sensitivity of nested PCR since it was only detected without dilution and not in the multiplex assay. Furthermore, the other *E. multilocularis*-positive sample was only identified with the 1 : 10 dilution in both PCR assays, like two other taeniid-positive samples during the *Taenia* PCR assay. It appears important to test diluted copro-DNA samples because the presence of PCR inhibitors are frequently reported when analysing such samples. This particular PCR protocol succeeded in increasing the sensitivity of detection, which is of particular interest when focusing on infection with *E. multilocularis* of dogs where a low prevalence is generally expected. The real-time PCR recently developed by Knapp et al. (2014) may constitute a relevant approach in order to overcome the lack of sensitivity of conventional PCR and enable identification of the potential presence of inhibitors by using an internal control.

The percentage of dogs from the dewormed group that were infected with helminths in general (as well as tapeworms, which were analysed separately) was slightly higher than in the untreated group, but not statistically significant, even though we analysed only dogs dewormed within a three-month-period prior to sampling. This could indicate ineffective drug administration and a potential lack of information for those administering the anthelmintics, whether veterinarians or owners. The inefficacy of deworming dogs was also shown by Sager et al. (2006). The authors revealed that in spite of regular treatment (every three months), the yearly incidence of helminth eggs was relatively high. They showed that there was no significant positive effect of anthelmintic treatment more than twice a year in reducing infections, with the exception of hookworms. Likewise, no or only slight differences were noted by Umhang et al. (2012) between dewormed and untreated dogs. The authors observed a significant decrease in parasitic infection in only one of the two analysed locations and only in dogs treated more than twice a year. Deworming recommendations based on the life cycle of *E. multilocularis* suggest deworming predisposed dogs (especially those preying on rodents) monthly (Eckert and Deplazes 1999), but such a frequency is difficult for owners to accept.

One of the *E. multilocularis*-positive dogs in our investigation had a habit of catching rodents. Predation behaviour was estimated as a risk factor of infection with *E. multilocularis* in the study conducted in Slovakia (Antolová et al. 2009). Umhang et al. (2014) showed the significantly higher prevalence of tapeworm infection in hunting dogs, which have easier access to rodents, resulting in a higher risk of infection.

Owning a dog that killed game or roamed outdoors unattended was identified as a risk factor for alveolar echinococcosis (AE) in humans (Kern et al. 2004). In our study, no positive serological results were found in eight individuals exposed to the infection with *E. multilocularis*. However, it should be mentioned that due to long incubation period of alveolar echinococcosis in humans, negative results of serological investigations do not definitely mean absence of the disease. Persons that were in direct contact with infected dog should be examined repeatedly to confirm negativity and thus absence of developing disease. Especially because the problem of human AE in Poland is present. From 1990–2011, more than 120 human cases of AE were detected (Nahorski et al. 2013) and new cases have been registered since then (Gołąb and Czarkowski 2014).

This study revealed infection with *E. multilocularis* of dogs for the first time in Poland. It again shows that the ru-

ral environment close to human houses in an endemic area of Poland may be contaminated by the eggs of *E. multilocularis*. The parasite had been previously detected directly in soil (Szostakowska et al. 2014) and indirectly using pigs as an indicator (Karamon et al. 2012). Despite the low prevalence of *E. multilocularis* in rural dogs, they may be an important source of infection for humans due to their close contact.

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Received 16 March 2016 Accepted 27 April 2016 Published online 2 June 2016

Cite this article as: Karamon J., Samorek–Pieróg M., Kochanowski M., Dąbrowska J., Sroka J., Gołąb E., Umhang G., Cencek T. 2016: First detection of *Echinococcus multilocularis* in dogs in a highly endemic area of Poland. Folia Parasitol. 63: 018.