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Molecular characterization and phylogenetic analysis of *Toxocara* species in dogs, cattle and buffalo in Egypt

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Summary

Toxocara canis of dogs and *Toxocara vitulorum* of cattle and buffalo are nematode parasites that cause serious economic and public health problems all over the world. This study aims to provide molecular data to identify and distinguish between *Toxocara* spp. from dogs, cattle and buffalo in Egypt. Moreover, constructing a phylogeny and phylogenetic relationships among these *Toxocara* spp. were performed through an analytic study of ATPase-6, a mitochondrial gene; 12S, small subunit ribosomal RNA gene and ITS-2, the second internal transcribed spacer nuclear ribosomal gene. *T. vitulorum* from cattle and buffalo were found to be almost identical. The ATPase- 6 and 12S regions showed 87.78 % and 90.38 % nucleotide similarity between *T. canis* and *T. vitulorum*, while for the ITS-2 region, only 78.38 % was found. Analysis of the three studied genes revealed that each *Toxocara* spp. has distinct molecular characteristics. Moreover, it was revealed that these genes, especially the ITS-2 gene, are useful and sensitive molecular markers for classifying and studying the phylogenetic analysis and relationships among closely related *Toxocara* spp. All sequences obtained in this study were registered in the GenBank under the accession numbers: MG214149 -MG214157.
Keywords: *Toxocara vitulorum; T. canis;* molecular characterization; PCR-mitochondrial gene- ribosomal gene; dogs; cattle; buffalo; Egypt

Introduction

Toxocara vitulorum is a nematode parasite of the small intestine of cattle and buffalo, particularly young calves that causes high morbidity and mortality Dorrny *et al*. (2015). These calves are infected when they suckle colostrum/milk contaminated with infective larvae from infected adult buffalo, Starke-Buzetti and Ferreira (2006). Moreover, *T. canis* is a zoonotic parasite that can cause serious diseases in dogs and humans. *T. canis* is usually found in 2 weeks to 2 months old puppies, which can be infected via the placenta (major route) or milk of the mother (minor route), Rahbarm *et al*. (2013). Migration of larvae through tissues produces severe inflammatory reactions and, consequently, a wide range of pathological and clinical manifestations, Joy *et al*. (2017).

The low economic status and lack of knowledge of farmers in tropical and subtropical countries that drink raw cattle or buffaloes' milk (Rast *et al*., 2013) are likely to accelerate the transmission of the parasite, which causes visceral larval migrans (VLM) in the human body. Additionally, poor hygiene, poor sanitation and close contact with dogs lead to the transmission of *T. canis*, Borecka *et al*. (2010). Coprological examination techniques fail to detect prepatent infections in adult buffalo and mild infections in young calves, Jyoti *et al*. (2011). Generally, there are limitations for diagnosing of *Toxocara* infections depending on morphological characters of its

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eggs or by using traditional serological methods. Nowadays, PCR considered being a valuable molecular diagnostic method that could be used for accurate identification of infection in different hosts. Therefore, it is critical to develop specific identification using molecular characterization methods, such as PCR-based techniques utilizing a range of genetic markers in the nuclear and mitochondrial genomes; these techniques are useful approaches to determine the genetic composition and phylogenetic relationships with high sensitivity, specificity, rapidity and utility, Chen *et al*. (2012). This aimed to assist ordinary diagnosis, understanding epidemiology and control of parasitic infections in the medical and veterinary sciences, Li *et al*. (2016). VLM diagnosed in Egypt still an open question as it caused by *Toxocara* species of canine or bovine origin, with the intimate morphological similarity between *T. vitulorum* extracted from cattle and buffalo, the disease is more common and dangerous in buffalo calves than in cattle one. According to the Egyptian conditions, the probability of infection by VLM in humans by *Toxocara* species from dogs or bovine sources is equal to the level of the rural area; the genomic study still required to investigate the source of infection to human. In this respect several authors, such as Zhu *et al*. (2000) in Australia, Wickramasinghe *et al*. (2009) in Sri Lanka, Jeeva *et al*. (2014) in India, and Oguz *et al*. (2018) in Turkey have investigated the value of using the sequences of ITS-1, ITS-2 and ATPase-6, 12S, 28S genes in the identification of *Toxocara* spp.

Furthermore, studies by Mikaeili *et al*. (2015) in Iran and Oguz (2018) in Turkey demonstrate the value of the mitochondrial cytochrome c oxidase subunit I (cox1) gene in the identification of *T. vitulorum* from cattle calves. Regarding Oguz *et al*. (2018), existing keys and PCR sequencing of the ITS-2 fragment were used to identify the phylogenetic tree of *T. canis* from stray dogs in Turkey. With significant variations in pathogenicity and destitution of *T. vitulorum* infection between cattle and buffalo calves in Egypt, no previous studies in this respect concerning the genomic difference between *T. vitulorum* extracted from two different hosts; cattle and buffalo as well as its computability or differences with that extracted from dogs. In Egypt, Sultan *et al*. (2015) in the mid-Delta region in Egypt demonstrate the amplified sequenced of *T. vitulorum* (cattle origin only) used ITS-1 and 18S genes of ribosomal DNA and constructed the phylogenetic trees of the obtained results without register to their sequenced products in GenBank.

Therefore, the objective of this study was to perform molecular characterization to identify and distinguish between the two closely related *Toxocara* spp. (*T. canis* and *T. vitulorum*) obtained from different definitive hosts (dogs, cattle and buffalo-calves) in Egypt. Through this study, ATPase-6, a mitochondrial gene, 12S, small subunit ribosomal RNA gene and ITS-2, the second internal transcribed spacer nuclear ribosomal gene were used. Moreover, phylogenetic analysis constructed the phylogenetic trees and compared them with nucleotide sequences for obtained *Toxocara* spp. The results of this study will provide new molecular data to support the study of the evolution and genetic diversity of *Toxocara* spp. concerning their different definitive hosts. The first published from Egypt of obtained sequences has been registered in the GenBank for future use by the scientific community.

Materials and Methods

All study steps and experimental procedures were approved by the Institutional Animal Care and Use Committee (CU-IACUC number II AS1516) of Cairo University.

Collection and preparation of samples

Intact, active 20 *Toxocara vitulorum* adult worms were collected from the intestine of freshly slaughtered buffalo and cattle calves from Cairo abattoir. While, seven adult *T. canis* worms were collected from naturally infected stray puppies (3weeks – 3months age), from Cairo governorate harbor eggs in their feces. The isolated ascarids nematodes specimens were washed extensively in phosphate buffered saline (PBS) to remove any debris and then were fixed in 70 % ethanol, cleared in lacto-phenol, permanent mount in glycerol and identified by microscopy as *T. canis* and *T. vitulorum* according to existing keys Soulsby (2012). After that, the parasites were fixed in 70 % ethanol. Other adults *Toxocara* species worms (n=3) from each *T. vitulorum* (cattle, buffalo) and *T.canis* of dogs origin were frozen at -20 °C until extraction of genomic DNA.

DNA extraction

DNA was extracted from a small portion of specimens of adult *T. vitulorum* and *T. canis* samples using QIA amp DNA Mini Kit (Qiagen, GmbH, Hilden, Germany) following the procedures recommended by the manufacturer.

DNA amplification

Amplification was performed using conventional PCR assays, according to the methods of Casiraghi *et al*. (2001).

The analysis was performed according to Wickramasinghe *et al*. (2009) using the listed primers' sequences in standard PCR procedures for amplification of the mitochondrial gene (ATPase-6, including the partial sequence of NAD1 and NAD2 genes), 12S gene (the small subunit ribosomal RNA) and ITS-2 nuclear ribosomal gene.

Primer	Sequence
nema ATPase-6F	5'-TWYCCWCGTTWTCGTTATGA-3'
nema ATPase-6R	5'-CTTAAAACAAATRCAYTTMT-3'
12SF	5'-GTTCCAGAATAATCGGCTA-3'
12SR	5'-ATTGACGGATGAGTTTGTACC-3'
Forward primer 3S	5'-CGGTGGATCACTCGGCTCGT-3'
Reverse primer 28A	5'- CCTGGTTAGTTTCTTTTCCTCCGC-3'

PCR was carried out in a final reaction volume of 25 µl. The amplifications were performed with 5 µl of DNA extract, 12.5 µl Taq® Green Master Mix, 5.5 µl of nuclease-free water and 1 µl of each forward and reverse primer. The amplification program was carried

out in MyCycler™ thermal cycler (BioRad, USA) with 3 min of initial denaturation at 94 °C; 31 cycles of 30 s at 94 °C; 30 s at 46 °C and 1 min at 72 °C; followed by a final 5 minextension at 72 °C for all primers except the 12S and ITS-2 primers, for which annealing was performed at 50 °C and 53 °C, respectively. The nine amplified PCR products were visualized via agarose gel electrophoresis and the molecular weight (M.W.) of each band was calculated using a 100 – 1500 bp DNA ladder. The obtained gel was examined under a transilluminator and photographed.

DNA sequencing

The PCR products of the three genes were purified with a QIA quick PCR purification column (Qiagen, GmbH, Hilden, Germany), and then the purified products were sequenced with an ABI 3730XL DNA sequencer using a Sequencing Clean-Up kit.

Sequence alignments and phylogenetic analysis

The obtained sequences were introduced into the NCBI Basic Local Alignment Search Tool (BLAST) for searching of sequence similarity for each one then; all sequences were aligned using BIOEDIT software and CLUSTAL W application. Phylogenetic analysis was performed using the MEGA program and the neighbor-joining method was performed to obtain phylogenetic trees which estimated by performing 1000 bootstrap replicates. The sequences were submitted to the GenBank following the provided instructions.

Results

The ascarids of both species *Toxocara* nematodes; *T. canis* and *T. vitulorum* were identified microscopically based on morphological features. *Toxocara vitulorum* is a large, robust worm up to 25 cm long with three large, prominent lips. The body was soft and translucent with clearly cuticle (Fig.1A – B). Male and females were ranged from 17.51 (15.54 – 19.00 cm) and 22.93 (19.32 – 29.11 cm) in average length, and 0.5 – 0.65 mm (mean 0.6 mm) in width, respectively. The three well defined lips; two subventral and one dorsal lip were determined from the worms (Fig.1D). The male worms had a posterior end curved ventrally (Fig.1B). The male posterior end exhibited two spicules, post cloacal papillae and the bell_ shaped projection were observed (Fig.1G). While, in female posterior end was distinguishable a straight-tailed (Fig.1F). *Toxocara canis* revealed a cephalic alae in the anterior region in both sex. The male worms had a posterior end coiled. The tail was conical and reached 0.89 – 1.3 mm (mean 1.1 mm) long. The spicules were equal, and reached 2.9 – 3.3 mm (mean 3.1 mm) long. In females, the cervical alae measured 3.2 – 3.9 mm in length and 0.22 – 0.32 mm (mean 0.34 mm) in width. In female worms, the uterus occupied 2/3 of the body, and contained many eggs. The eggs were measured 70 – 80 µm (mean 75.5 µm, n=10) in diameter, rounded in shape and brown in color. It contained a single-cell embryo, and had a thin shell with an aluminous surface which is finely mammilated.

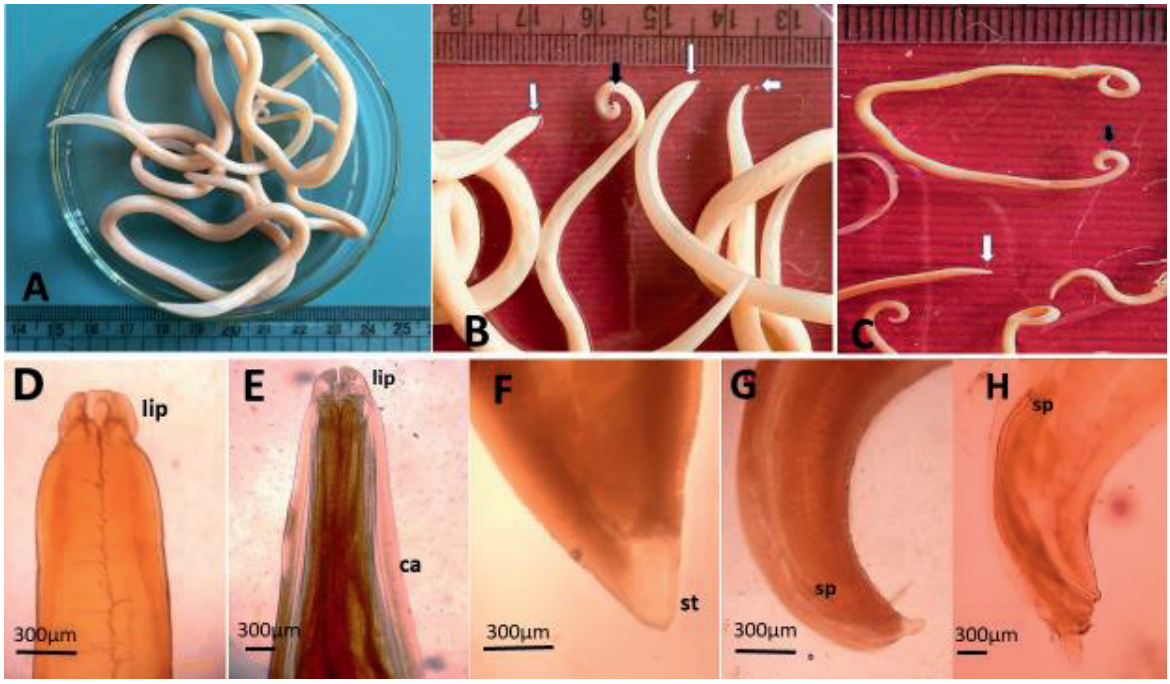


Fig. 1. A – B. *Toxocara vitulorum* collected from calves. B) Anterior end of worm showed lips (short white arrow), posterior end of male showed coiled tail (black arrow), and posterior end of female worm showed a straight tail end (long white arrow). D) Anterior end of *T.vitulorum* showed three lips (lip), F) posterior end of female showed short tail (st), G) posterior end of male showed two spicules (sp), C) *Toxocara canis* collected from street dogs; showed the coiled tail-end of male (black arrow), the straight tail-end of female (white arrow), E) Anterior end of *T. canis* showed the cephalic alae and lips, F) posterior end of male showed spicules.

Table 1. Characterization of the PCR products of the selected genes in the tested *Toxocara* spp. showing the M. W. of the bands for each gene.

Gene	M.W. (bp)		
	T. canis	*T. vitulorum* from cattle	*T. vitulorum* from buffalo
ATPase-6	804	828	828
12S	572	576	572
ITS-2	592	625	625

The data in Table 1 and Plate 1 show the results of agarose gel electrophoresis of the nine PCR products of the three worms from each tested *Toxocara* spp.; *T. canis* (dogs) and *T. vitulorum* (cattle and buffalo-calves). The ATPase-6 gene of *T. canis* produced a 804 bp band, while that of *T. vitulorum* from both cattle and buffalo calves produced 828 band bp. Electrophoretic analysis of the 12S gene showed bands of M.W.572, 576 and 572 bp for *T. canis, T. vitulorum* from cattle and *T. vitulorum* from buffalo respectively. Moreover, analysis of the ITS-2 gene revealed a band of M.W. 592 bp for *T. canis*. While a band of 625 bp was detected for *T. vitulorum* from both cattle and buffalo-calves.

ATPase-6 gene

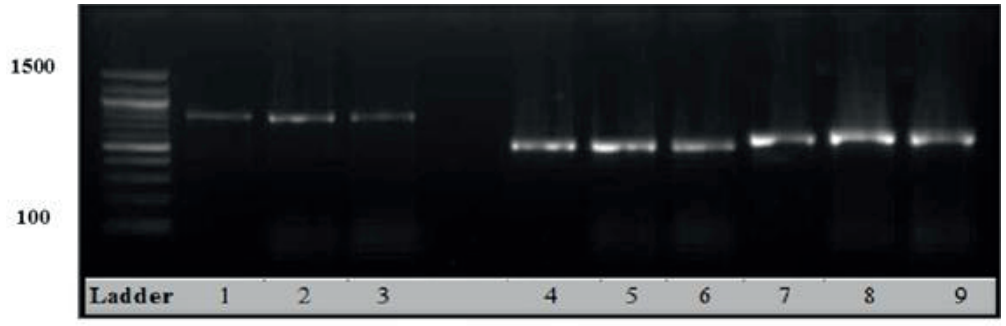
Analysis of the ATPase-6 gene revealed that the sequences of this gene in *T. canis* of dogs and *T. vitulorum* from cattle and buffalo-calves were G/T rich. Moreover, the length of the nucleotide sequence in *T. canis* was 600 bp, while that in *T. vitulorum* from both cattle and buffalo-calves was 598 bp.

A phylogenetic tree constructed by the neighbor-joining method for the ATPase-6 region. There was 99.16 % identity in this gene between the tested *T. vitulorum* specimens from cattle and buffalo-calves in the present study, while 87.78 % identity was detected between the tested *T. canis* and *T. vitulorum* specimens from Egypt. Additionally, it was revealed that the *T. vitulorum* from buffalo-cattle calves (MG214154.1) in the current study was closely related to *T. vitulorum* from in India (KJ777176.1), with 99.50 % identity to that in Sri Lanka (FJ418793.1) with 99.33 % identity. Moreover, the sequence for *T. canis* in the current study was 100 % identity to that *T. canis* isolated in India (KJ777174.1) While, with 99.67 % identity to *T. canis* isolated in Sri Lanka (FJ418787.1), Plate 2.

12S rRNA gene

The data showed that the lengths of the 12S gene sequences obtained from *T. canis* (dogs), *T. vitulorum* (cattle-calves) and *T. vitulorum* (buffalo-calves) were 493, 464 and 465 bp, respectively. Moreover, the sequences of this gene in the tested *Toxocara* spp. were found to be A/T rich.

Concerning the phylogenetic tree constructed for the 12S gene, 98. 01 % identity was observed between the tested *T. vitulorum* from buffalo origin and cattle origin in the present study, while 90.38 % identity was detected between the tested *T. canis* and *T. vitulorum* isolated in Egypt. Also, *T. vitulorum* from buffalo (MG214157.1) in Egypt was found to be closely related to *T. vitulorum* isolated in Sri Lanka (FJ418789.1), with 97.22 % identity. The gene sequence in *T. vitulorum* from cattle (MG214156.1) in the current study was 99.35 % identity to *T. vitulorum* isolated in India (KJ777171.1 – KJ777172.1). Moreover, the sequence in *T. canis* (MG214155) from Egypt in the current study was closely related 100 % identity to that from Sri Lanka (FJ418782.1), China (JN256964.1) and in India (KJ777168.1) and Plate 3.



- ATPase-6 primer:
1) *T. canis* from dogs
2) *T. vitulorum* from cattle
3) *T. vitulorum* from buffalo

- 12S primer:
4) *T. canis* from dogs
5) *T. vitulorum* from cattle
6) *T. vitulorum* from buffalo

- ITS-2 primer:
7) *T. canis* from dogs
8) *T. vitulorum* from cattle
9) *T. vitulorum* from buffalo

Plate 1. Results of agarose gel electrophoresis of the PCR products of the selected genes in *Toxocara* spp. showing the M. W. of each gene band.

Plate 2. Phylogenetic tree constructed in MEGA by neighbor- joining method for the ATPase-6 region of the tested *Toxocara* spp.

ITS-2 gene

Analysis of the ITS-2 gene showed that the lengths of nucleotide sequences were 503, 532 and 537bp for *T. canis*, *T. vitulorum* cattle and buffalo, respectively. Moreover, the sequences of this gene in the tested *Toxocara* spp. were found to be A/T rich. Regarding the phylogenetic tree constructed for the ITS-2 region, cleared that *T. vitulorum* of cattle origin and buffalo origin calves was 95.50 % identity. While they were detected 78.38.0 % identity between *T. canis* and *T. vitulorum* buffalo origin and 80.79 % identity between *T. canis* and *T. vitulorum* from cattle origin in Egypt. This result indicates that the ITS-2 gene appeared to be a good sensitive tool for the identification of adult worms of *Toxocara* spp. The phylogenetic tree distinctly separated the ITS-2 sequences gene of *T. vitulorum* group from that of *T. canis* into two major lineages.

It was revealed that *T. vitulorum* of cattle origin (MG214151.1) was found to be closely related to that in Canada (JQ083352.1) with 99.77 % identity. Concerning had 95.5 % identity with buffalo in Egypt (MG214152.1) and Sri Lanka (FJ418784.1). *Toxocara vitulorum* isolated from cattle in India (KJ777159.1) and Scotland (EU189085.1) with 97.38 % and 99.43 % identity.

Moreover, *T. canis* in Egypt (MG214150.1) was found with 100 % identity to that in India (KJ777155.1 & MK728992.1), in Japan

Plate 3. Phylogenetic tree constructed in MEGA by neighbor-joining method for the 12S region of the tested *Toxocara* spp.

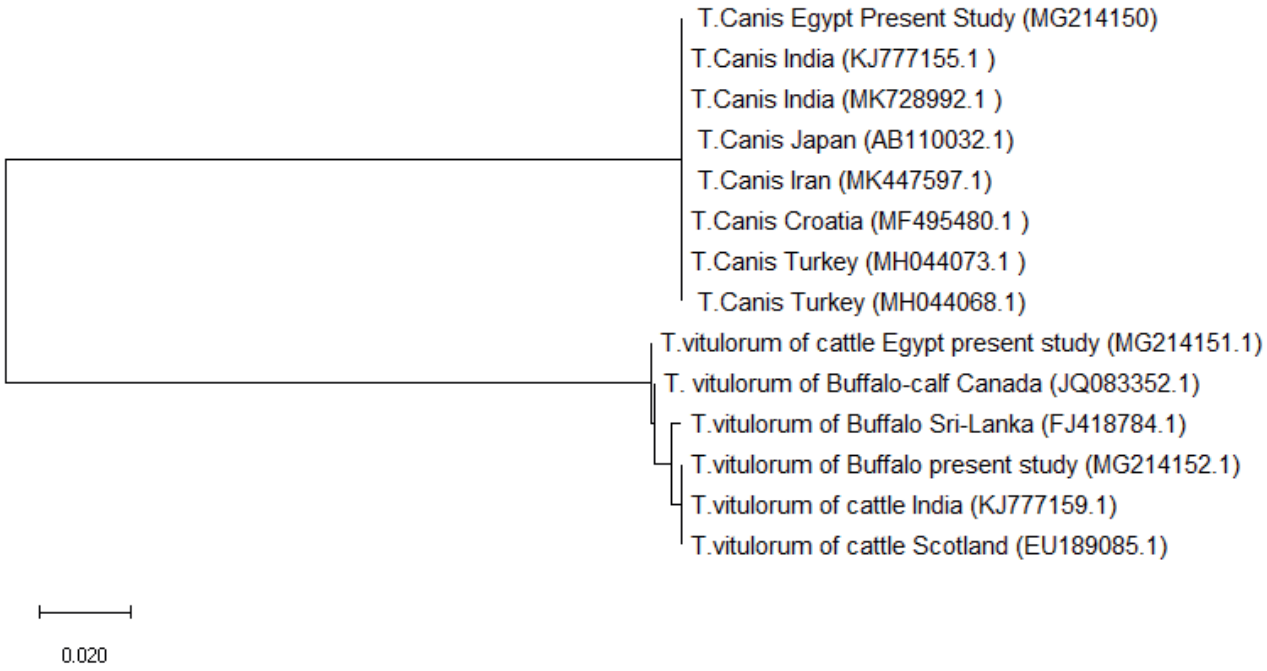


Plate 4. Phylogenetic tree constructed in MEGA by neighbor- joining method for the ITS-2 region of the tested *Toxocara* spp.

(AB110032.1), in Iran (MK447597.1), in Croatia (MF495480.1), in Turkey (MH044073.1 & MH044068.1), Plate 4. All generated sequences were registered in the GenBank database for the first time in Egypt with accession numbers including, MG214149 – MG214157.

Discussion

T. canis and *T. vitulorum* are nematode parasites that cause serious problems in dogs, cattle and buffalo, Strub *et al*. (2013). With the grate morphological difference between canine and bovine species, Soulsby (2012), the morphological characters of their diagnostic stages (their eggs) are similar. In a time where no clear morphological differences between *T. vitulorum* extracted from infected cattle or buffalo calves, the incidence and pathogenicity of them considered to be dangerous in buffalo than in cattle on the level of the same localities (Fagiolo *et al*. 2007). With the presence of recorded cases of VLM in humans who are in close contact with these animals, no accurate determination to genotypic data support the ability of *Toxocara* of bovine origin to cause problems similar to that previously caused by *Toxocara* of dog origin from the aspect of VLM in human. Due to the limitations of traditional (morphological and corpological) approaches for the identification of *Toxocara* spp. and the diagnosis of related infections, it was essential to develop specific identification criteria using molecular characterization techniques to determine genetic compositions and phylogenetic relationships with high sensitivity, specificity, rapidity and utility, Jyoti *et al*. (2011) and Chen *et al*. (2012).

Two common species of *Toxocara* present in Egypt (*T. canis* and *T. vitulorum*) infect three widely distributed hosts (dogs, cattle & buffalo). Its eggs and larval stages were disseminated in feces and milk and considered to be easily transferred to humans. With the known data about the ability of *T. canis* larvae to cause VLM in humans, are the other species (*T. vitulorum*) that can do the same. With difficulty in proving this on the human level, the present study directed toward determine molecular and phylogenetic compatibility between them. The recent studies in Turkey by Oguz and Oguz *et al*. (2018) demonstrate the value of the mitochondrial cytochrome c oxidase subunit I (cox1) gene of *T. vitulorum* from cattle calves and ITS-2 fragment of *T. canis* from dogs were used to identify the morphological gene in identification, respectively. In this respect, DNA-based approaches have been applied to identify the nematode species, mainly using ITS fragments (rDNA) Li *et al*. (2006) and Fogt-Wyrwas *et al*. (2013). Sultan *et al*. (2015) in the mid-Delta region in Egypt demonstrate the amplified sequenced of *T. vitulorum* (cattle origin only*)* used ITS-1 and 18S genes of ribosomal DNA and constructed the phylogenetic trees of the obtained results without register to their sequenced products in GenBank.

Therefore, in Egypt, the present study was extracted the first genomic DNA of *Toxocara* species from dogs, cattle and buffalo-calves using mitochondrial ATPase-6 and ribosomal gene 12S and the nuclear ribosomal gene ITS-2 regions were analyzed amplified and all obtained sequences were registered in GenBank. The mitochondrial ATPase-6, rather than single gene, to better account for genetic variability in these genomes across different regions of two closely related nematodes; *T. canis* of dog origin and *T. vitulorum* of cattle, buffalo-calves in Egypt. It is worth noting that the present study in Egypt, is the first to compare between

T. vitulorum from cattle and buffalo calves in addition to *T. canis* to search for any molecular differences between them that may occur due to the presence of the adult worm in the intestine of two different ruminant host.

The sequences of ATPase-6 gene of *T. canis* and *T. vitulorum* from cattle and buffalo were found to be G/T rich. While the sequences of the 12S gene and ITS-2 region were found to be rich with A/T . Similar results were reported for *Toxocara* spp. in Sri Lanka by Wickramasinghe *et al*. (2009). On the other hand, Hu and Gasser (2006) stated that all mitochondrial genes sequenced in *Toxocara* spp. are A/T rich.

In the present study, the analysis of the ATPase-6, 12S and ITS-2 genes sequences revealed that the length of the nucleotide sequence in *T. canis* (600 bp, 493 bp.,503 bp., in *T. vitulorum* cattle origin; 598 bp, 464 bp, 532 bp. and in *T. vitulorum* buffalo origin, 598 bp., 465 bp, 537 bp. respectively. These results were nearly similar to those reported for specimens from Wickramasinghe *et al*. (2009), who stated that the length of the nucleotide sequence of ATPase-6 gene in *T. canis* and *T. vitulorum* was 597 bp. However, the present results are inconsistent with those of Zhu *et al*. (2000), who stated that the ITS-2 region ranged from 240 – 348 bp long in *T. canis* and *T. vitulorum* from Australia.

In the present study, after alignment of the sequences of the three analyzed genes/regions (ATPase-6, 12S and ITS-2) in *T. canis* and *T. vitulorum* from cattle and buffalo in Egypt and in *Toxocara* spp. from other countries as well as subsequent analysis and construction of a neighbor-joining phylogenetic tree, it was observed that *T. vitulorum* from both cattle and buffalo formed a single group, as both hosts are ruminants. In contrast, *T. canis* formed a separate group. Each group consisted of several sister clades for each species analyzed, each of which represented a different definitive host. These findings are consistent with those obtained by Li *et al*. (2006) in China. In addition, they present results stated that the ATPase-6, 12S and ITS-2 regions were more conserved 87.78 %, 90.38 % and 78.38 %, nucleotide similarity between *T. canis* and *T. vitulorum.* While, revealed that the previous regions was more conserved 99.16 %, 98.01 % and 95.5 % nucleotide similarity between *T. vitulorum* from cattle and buffalo-calves in Egypt. This result can provide a foundation for accurate identification of *T. canis* and *T. vitulorum* using PCR characterization of 12S and ITS-2 region with nucleotide similarity (90.0 % and 78.38 %).

This result agrees with Wickramasinghe *et al*. (2009) reported that the nucleotide identity between *T. canis* and *T. vitulorum* from Sri Lanka was 88.0 % and 85.0 % for the 12S and ITS-2 gene, respectively.

T. vitulorum were almost genetically identical to those isolated in other countries. These data are consistent with those of Sultan *et al*. (2015) who amplified, sequenced of *T. vitulorum* (cattle origin) using ITS- and 18S genes had 99.0 % identity with *T. vitulorum* (buffalo origin) registered by Wickramasinghe *et al*. (2009) in Sri Lanka.

Moreover, the obtained presently sequenced concluded that *T. vitulorum* isolated from cattle, buffalo calves using the ITS-2 sequences and those other sequences of the same nematode species were previously registered in GenBank were genetically identical to those isolated from beef calf such as Wickramasinghe *et al*. (2009) in Sri Lanka, Woodbury *et al*. (2012) in Canada, Jeeva *et al*. (2014) in India and Redman *et al*. (2007) in Scotland. Furthermore, presently concluded genetically identical had 100 % identity of *T. canis* from dogs using the ITS-2 sequences and those other sequences of the same nematode species were previously register in GenBank such as Ishiwata *et al*. (2004) in Japan, Jeeva *et al*. (2014) in India, Valizadeh and Tahvildar (2019) in Iran, and Oguz *et al*. (2018) in Turkey.

Concerning, the ATPase-6 and 12S genes were found to be more conserved (87.9 % nucleotide similarity between *T. canis* and *T. vitulorum*) than the ITS-2 region (78.38 %). This result is consistent with that recorded by Durant *et al*. (2012), who stated that analysis of the ITS-2 gene appeared to be a sensitive tool for the identification of adult worms of *Toxocara* spp.

Conclusion

This current study was comparing the molecular characterization and phylogenetic analysis of the relationship between closely related species of *Toxocara* species from three different hosts (dogs, cattle and buffalo) in Egypt. The characterization of the ITS-2 region can provide a foundation for accurate identification of *Toxocara* species using PCR than ATPase-6, 12S regions. Concerning, the ITS-2 gene appeared to be a sensitive tool for 78.38 % nucleotide similarity between *T. canis* and *T. vitulorum* when compared with ATPase-6, 12S regions were found to be more conserved (87.78 % and 90.38 % nucleotide similarity). In the present study, the first genomic DNA was extracted using three different genes amplified and register in GenBank for the first time in Egypt.

Conflict of Interest

The authors have declared no conflict of interest

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A pilot comparative study between serological and genetic investigations in relationship to clinical outcomes on patients with cystic echinococcosis

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Summary

The aim of this study was to investigate whether Enzyme-Linked Immunosorbent Assays (ELISA) and Western Blotting (WB) methods could contribute to the assessment of clinical outcomes in genotype-defined cystic echinococcosis (CE) patients. Twenty-nine human isolates and blood samples have been taken from patients who underwent surgery or percutaneous aspiration (PAIR) for therapeutic purposes at Ege University and Manisa Celal Bayar University Hospitals. All sera of patients were screened for the presence of *E. granulosus* IgG antibodies using in-house approved ELISA and WB methods. According to the ELISA results, five patients had high, thirteen patients had medium and eight patients had low specific antibody level response which ranged 1/640 -1/5000. Despite confirmed WB positivity three patients were found to be negative by ELISA. Immunoblot analysis of EgAg showed many protein bands with size of 8, 12, 20, 22, 24, 36, 75 and 90 kDa. Among of them, 8 – 12 kDa bands (90 %), 20 – 22 kDa and 36 kDa bands presented strong reactivity against human serum specimens. No serum samples from healthy control reacted with EgAg. Phylogenetic analysis of resulting COX1 and NAD1sequences has revealed that all patients in our study were infected with the *E. granulosus* G1-G3 genotype. There was no consistent correlation between results of ELISA and WB, the number or size of cysts and genotype. Our study brings a unique contribution in terms of relationship between serological investigation, disease genotypes and clinical outcomes.
Keywords: Cystic echinococcosis; ELISA; western blotting; serology; sequence analysis

Introduction

Cystic echinococcosis (CE) is a parasitic disease spread worldwide. It establishes a major public health problem in the regions where sheep and cattle are bred and used for the consumption. The CE is endemic and occurs frequently in Mediterranean regions, Middle East, Asia, North and East Africa, Australia and South America, and some European countries (McManus *et al*., 2003). In Turkey, the CE has a negative impact on the national economy as well as the health, and still remains one of the most important and serious helminthic diseases. It is quite common due to the wide prevalence of stray dogs and the lack of necessary sanitary precautions (Altintas, 2003; Šnábel, 2009).

In humans, CE may be represented by wide spectrum of clinical manifestations. Clinical signs and symptoms of the disease are not specific and depend on the location of the cyst. The growth rates of cysts may vary between cysts in the same organ or within the same individual and between individuals in various regions. Early diagnosis and prompt treatment are essential to treat disease efficiently. Both, imaging techniques (US, CT) and serology provide useful and complementary information about the character of the cyst that may be relevant for therapeutic intervention. Sero-

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logical tests provide an extremely important information related to the diagnosis and prognosis of disease (Yolasigmaz *et al*., 2006; Manzano-Roman *et al*., 2015).

The CE genotype could affect the biological outcome and disease management In human G1 genotype is the most common (88.44 %) while the G6/G7 genotypes which are closely related strains are less frequent it (11.07 %). On the other hand the G5, G8, and G10 genotypes occur in human rarely (Alvarez Rojas *et al*., 2014). So far only one human case with G4 genotype was identified (Kim *et al*., 2017). However, for many genotypes of *E. granulosus* sensu lato we still have insufficient information regarding e.g. geographical distribution, host specificity, morphology and infectivity. In a study which compared the cysts locations, their size and other features isolated from various strains (*E.granulosus* G1, *E.ortleppi* and *E.canadensis* G6) was noted that G6 genotype showed accelerated growth rate (Guarnera *et al*. 2004, Alvarez Rojas *et al*., 2014).

Studies on the genotyping of *E. granulosus* isolates obtained from different geographic regions of Turkey and intermediate hosts (sheep, cattle, goat, camel, buffalo, horses, mules, and mouflon) confirmed the occurrence of G1-G3, G4 genotypes. Meanwhile in another group of intermediate hosts (sheep, cattle, goat, camel, buffalo, horses, mules) and human regarding the *E. granulosus* G1,G3 and G7 genotypes were confirmed (Simsek & Eroksuz 2009; Šnábel *et al*., 2009; Eryıldız *et al*., 2012; Altintas *et al*., 2013; Utuk *et al*., 2013; Gokpinar *et al*., 2017).

These reports also showed that both, the early CE diagnosis and appropriate hospitalization are very important for lessening the number of fatal cases and serious health outcome (Khachatryan, 2017). In addition, a good symptoms distinction and adequate information about the disease play an important role in death cases prevention (Belhassen-García, M, 2014). The, molecular identification of human CE cases should be suggested for better understanding of pathology, the disease outcome and epidemiology. The essential question which needs to be addressed is whether the link between clinical outcomes and distinct CE genotypes exists.

The aim of this study was to investigate the association between antigenic presentation and antibody response in CE genotype defined patients where the clinical outcomes based CE genotype were compared and analyzed.

Materials and Methods

Patient samples

Twenty-nine human isolates (germinal layer and/or protoscoleces) and blood samples have been taken from CE patients just before the surgery (22 patients) and the application of puncture-aspiration-injection-reaspiration (PAIR) (7 patients) for diagnostic purposes at Ege University Hospital and Celal Bayar University Hospital were collected. The livers cysts were classified according to the classification determined by WHO Informal Working Group on Echinococcosis (WHO-IWGE). According the USG results eight patients were classified as CE1, six patients were CE2, four patients were CE3 and three patients were CE4/CE5 by USG. Remaining cysts were determined by CT. In total, 29 hepatic CE cyst fluid and germinal layer isolates were obtained and examined under microscope for the presence of protoscoleces or hooklets. All samples were kept at -20 °C until further used. The details of demographic and clinical data obtained from the patients (age, sex, geographical area, cyst type, cyst location, size of cyst) were recorded. Regarding the control group, only the blood serum from healthy individuals of which the fecal examinations for the other parasitic infections including the CE were negative was used.

Serological Analysis

All serum samples of patients were screened for the presence of *E. granulosus* IgG antibodies using in-house approved ELISA and WB tests. In ELISA and WB tests, sheep hydatid fluid (HF) was used as the antigen. HF was collected from fertile cysts obtained at slaughterhouse in city Izmir, Turkey. After centrifugation at 10,000 g for 30 min at 4 °C, the antigen was concentrated by Amicon ultrafiltration with YM2 membrane (Amicon Corp., Danvers, MA, USA) and kept at -20 °C for subsequent use. Protein concentrations were determined by the Bradford protein assay kit (Bio-Rad) and bovine albumin used as a standard. Based on the results of ELISA tests, patient outcomes were interpreted taking into account the negative and positive serum readouts and cut-off values.

Enzyme-Linked Immunosorbent Assays (ELISA)

ELISA was carried out on polystyrene microtiter plates with 96 wells (F-Form; Maxisorp, Nunc, Fisher Scientific, USA) coated with 100 µl/well (at a concentration of 5 µg of proteins per well) of HF diluted in phosphate-buffered saline (PBS) buffer and incubated at +4 °C overnight. Plates were washed three times in 0.5 % PBS with Tween 20 (PBS-T) and blocked with milk with PBS-T for 1 h at room temperature. Serum samples (100 µl) diluted 1:640 in 5 % non-fat milk with PBS-T were added and incubated for 1 hour at RT. After washing, plates were treated for 1 hour with alkaline-phosphatase anti-human IgG (Sigma) conjugate diluted at 1:5.000. After repeated washing, the reaction was stopped after about 20 min of incubation in dark by 100 µl of 1µg/ml p-Nitrophenyl Phosphate (pNPP) in dietanolamine buffer (DEAB). The optical density at 405 nm (OD405) was determined by ELISA plate reader (Thermo Labsystems Opsys MR, USA). Cut off values were determined by taking the average OD of negative control sera plus 3 standard deviations (SD).

Western Blotting Assay (WB)

Patients blood serum positive for the CE determined by ELISA were also confirmed by Western-blot technique. Electrophoresis (ELFO-SDS PAGE) was performed with Bio-Rad Mini Protein Slab Cell (Bio-Rad Laboratories, CA, USA) on a 12 % SDS-polyacrylamide gel and 4 % stacking gel under reducing conditions (Laemmli,

Table 1. Oligonucleotide primers used in PCR and DNA Sequencing for typing of *Echinococcus granulosus*.

Primers	Gene Regions	Nucleotide Sequences	Sources
MS1	NAD1	CGTAGGTATGTTGGTTTGTTTGGT	Sharbatkhori *et al*., 2009
MS2	NAD1	CATAATCAAATGGCGTACGAT	Sharbatkhori *et al*., 2009
JB3	CO1	TTTTTTGGGCATCCTGAGGTTTAT	Utuk *et al*., 2008
JB4.5	CO1	TAAAGAAAGAACATAATGAAAATG	Utuk *et al*., 2008
BD1	ITS-1	GTCGTAACAAGGTTTCCGTA	Bowless & McManus, 1993
4S	ITS-1	TCTAGATGCGTTCGAA(G/A)TGTCGATG	Bowless & McManus, 1993

1970). Antigens were electrophoresed at 60 V for approx. 2 h at room temperature. Low molecular weight markers (prestained SDS-PAGE standards, Bio-Rad) were included into each electrophoretic run. Following electrophoresis, proteins were transferred on nitrocellulose (NC) membrane in Tris-glycine buffer (pH 8.8) for 1h using a Bio-Rad Trans-Blot Cell. After blotting, the NC membrane was cut into 2 mm wide strips and blocked with 5 % (w/v) dry milk in Tris-Borate-Saline solution containing 0.1 % Tween 20 (TBS-T) (pH 7.2) for 1 h at room temperature. All serum samples of patients were diluted 1:100 with 0.5 % (w/v) dry milk in TBS-T and incubated in shaker for 1 h RT. The strips were than washed three times with TBS-T and reacted with alkaline-phosphatase-conjugated anti-human IgG (Sigma) at dilution 1:5000 for 1 hour at RT. Subsequently, the strips were washed again three times in TBS-T, and bands were visualized by incubating 5 min with 33 µl 5-bromo-4-chloro-3-indolyl phosphate and 330 µl nitro blue tetrazolium chloride (BCIP/NBT) in 10 ml alkaline phosphatase (ALP) buffer distributed evenly (1 ml) to the wells.

Molecular Analysis

For molecular evaluation the DNA was extracted from both, germinal layer and protoscoleces. The total genomic DNA was extracted with RTA-DNA Isolation Kit (Gebze/Kocaeli, Turkey) according to the manufacturer instructions. The amount of DNA in samples was determined in ng/µl by a spectrophotometry (NanoDrop ND-1000 Spectrophotometer) at a wavelength ratio of 260/280 nm. Kit-isolated DNAs were PCR-primed with primer sets specific for NAD1, COX1 and ITS-1 (Table 1). The extracted DNA was kept at -20 ° C until further analysis. All PCR products were run on a gel and gel images were imaged and photographed with UV gel imaging system (SYNGENE, Cambridge, UK) located at the Molecular Biology Laboratory of Medical Biology Department Faculty of Medicine of Manisa Celal Bayar University. After the PCR treatment, the resulting products were run on 3 % agarose gel and amplified with PCR using primer sets specific for typing of *E. granulosus*. Post-PCR specimens were cut with RsaI (Fermentas), MspI (Fermentas), and CfoI (Fermentas) ve AluI (Fermentas) enzyme for the determination of ribosomal ITS's-1 gene (PCR-RFLP). For genetical characterization of all isolates these procedures were applied to all patients and control group of individuals. Subsequently ribosomal ITS-1 gene region in all samples were digested with restriction endonucleases and deoxyribonucleic acid sequencing of the mi-

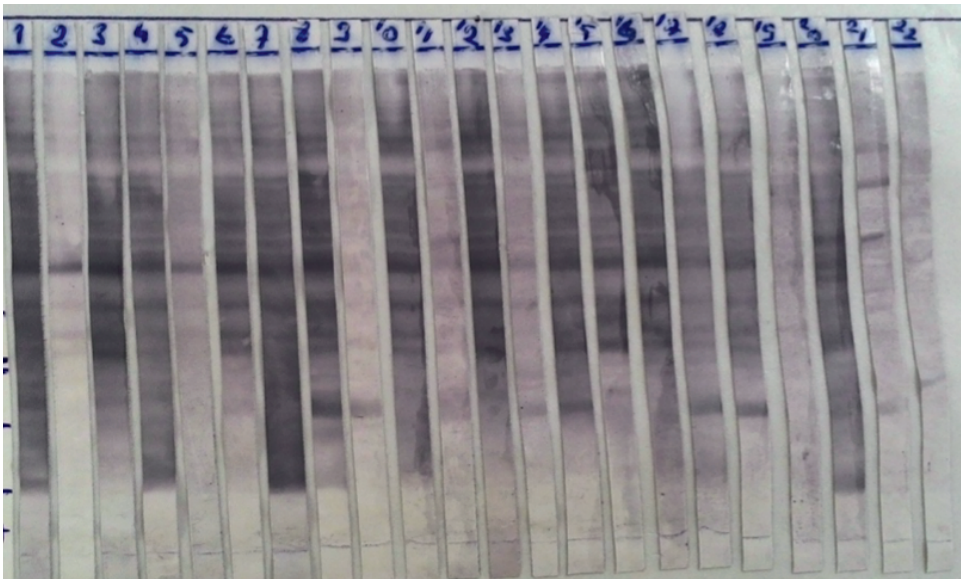

Fig. 1. Western Blot analysis of patient sera using HF (HF-WB). The lines represent: 1-Positive Control, 2-Negative Control, 3-22 Sera with confirmed CE.

Table 2. Gender and age, ELISA and Western Blot results, organ localization, molecular identification and clinical symptoms, drug used and dog owner informations of the 29 CE cases.

No of patient	Gender	Age	Province	ELISA Od value / Evaluation	WB bands (kDa)	Organ localisation	Genotype of *E. granulosus s.s.*	Drug Used /NA	Dog owner	Clinical symptoms
1	M	29	Kütahya	2,403/H	12, 20-22, 36, 75, 90	Liver right lobe	*E. granulosus s.s.* (G1-G3)	Y	Y	Mild pain
2	M	36	İzmir	2,859/H	8 12, 20-22, 36, 75, 90	Liver right lobe seg. 6-7	*E. granulosus s.s.* (G1-G3)	Y	N	Pain, palpable mass, headache
3	M	12	Izmir	1,047/M	8, 12, 20-22, 36, 75, 90	Liver right lobe posterior	*E. granulosus s.s.* (G1-G3)	Y	N	Pain
4	F	31	Bornova/İzmir	0,792/M	12, 20-22, 36, 75, 90	Liver right lobe seg. 6-7	*E. granulosus s.s.* (G1-G3)	Y	N	Pain, vomiting
5	M	36	Bergama/İzmir	0,865/M	8, 12, 20-22, 36, 75, 90	Liver left lobe seg. 7	*E. granulosus s.s.* (G1-G3)	Y	Y	Palpable mass, pain, nausea, vomiting,
6	M	63	Karabağlar/İzmir	2,069/H	8, 12, 20-22, 36, 75, 90	Liver right 4A-B	*E. granulosus s.s.* (G1-G3)	Y	N	Pain
7	M	53	Izmir	0,498/L	12, 36 (low), 75, 90	Liver seg. 4-5-6	*E. granulosus s.s.* (G1-G3)	Y	N	Palpable mass, pain, weakness, nausea
8	M	23	Manisa	0,974/M	20-22, 36, 75, 90	Liver right lobe	*E. granulosus s.s.* (G1-G3)	Y	Y	Palpable mass, pain
9	M	42	İzmir	0,469/L	12, 20-22, 36 (low), 75, 90	Liver right 4A-B	*E. granulosus s.s.* (G1-G3)	Y	N	Pain
10	M	40	Buca/İzmir	1,032/M	20-22, 36, 75, 90	Liver hilum	*E. granulosus s.s.* (G1-G3)	N	Y	Pain
11	F	30	İzmir	0,831/M	12, 36 (low), 90	Liver right 4B	*E. granulosus s.s.* (G1-G3)	Y	Y	Pain, nausea, vomiting
12	F	13	Merkez/Aydın	1,569/H	12, 20-22, 36, 75, 90	Liver	*E. granulosus s.s.* (G1-G3)	Y	N	Pain, nausea, vomiting
13	F	61	Alaşehir/Manisa	0,552/L	20-22, 36, 75, 90	Liver right lobe	*E. granulosus s.s.* (G1-G3)	N	Y	Ağrı,çarpıntı
14	F	49	Denizli	1,058/M	8, 20-22, 36, 75, 90	Liver	*E. granulosus s.s.* (G1-G3)	N	Y	Pain, nausea
15	M	49	Söke/Aydın	0,936/M	8, 12, 20-22, 36	Liver	*E. granulosus s.s.* (G1-G3)	Y	N	Pain, palpitations
16	M	10	Gömeç/Balıkesir	0,099/N	12, 20-22 (low)	Liver	*E. granulosus s.s.* (G1-G3)	Y	Y	Palpable mass, pain, hepatomegaly, dizziness
17	F	62	Balıkesir	0,454/L	12, 20-22, 36, 75, 90	Liver	*E. granulosus s.s.* (G1-G3)	Y	Y	Severe pain, nausea, vomiting, jaundice
18	F	19	Aydın	0,142/N	20-22 (low)	Liver	*E. granulosus s.s.* (G1-G3)	Y	Y	Palpable mass, severe pain
19	M	59	Akhisar/Manisa	0,944/M	36, 75, 90	Liver	*E. granulosus s.s.* (G1-G3)	Y	Y	Back stiffness, severe pain, hepatomegaly
20	M	30	Balıkesir	1,018/M	20-22, 36, 75, 90	Liver	*E. granulosus s.s.* (G1-G3)	Y	N	Severe pain, nausea, vomiting, jaundice
21	F	15	Bornova/İzmir	0,243/N	12, 36, 75	Liver	*E. granulosus s.s.* (G1-G3)	Y	N	Severe pain, nausea
22	M	15	Izmir	0,551/L	8, 20-22, 36, 75, 90	Liver	*E. granulosus s.s.* (G1-G3)	Y	N	Palpable mass, pain
23	F	52	Menemen/İzmir	1,063/M	8, 20-22, 36, 75, 90	Liver	*E. granulosus s.s.* (G1-G3)	Y	Y	Pain
24	M	8	Ayvalık/Balıkesir	0,649/L	8, 12, 20-22, 36, 75, 90	Liver	*E. granulosus s.s.* (G1-G3)	Y	Y	Palpable mass, fever, vomiting
25	M	26	Söke/Aydın	0,649/L	8, 12, 20-22, 36, 75, 90	Liver	*E. granulosus s.s.* (G1-G3)	Y	Y	Severe pain, swelling
26	M	19	Akhisar/Manisa	1,066/M	8, 12, 20-22, 36, 75, 90	Liver	*E. granulosus s.s.* (G1-G3)	Y	N	Pain
27	F	10	Manisa	0,498/L	12, 20-22	Liver	*E. granulosus s.s.* (G1-G3)	Y	N	Pain, loss of appetite, anemia
28	M	48	Muğla	1,262/H	8, 12, 20-22, 36, 75, 90	Liver	*E. granulosus s.s.* (G1-G3)	Y	Y	Vomiting, weakness, hepatomegaly
29	M	10	Izmir	1,047/M	8, 12, 20-22, 36, 75, 90	Liver	*E. granulosus s.s.* (G1-G3)	Y	Y	Palpable mass, nausea, vomiting

ELISA: H: High, M: Medium, L: Low, N: Negative. Cut off value: 0,382
Drug used and dog owner: Y: Yes, N: No.
Drug used: NA (duration is not available)

tochondrial COX1 and NAD1 genes with ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Forward and reverse sequences of amplicons were examined with Sequencing Analysis software and their alignment analyses were performed with SeqScape V2.6 software (Applied Biosystems, Foster City, CA). Finally, the alignment analyses of all samples were accomplished with NCBI BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to compare the other *E. granulosus* sequence data. Phylogenetic analysis of strains was done by Genius software (Biomatters, New Zealand).

Statistical Analysis

Sensitivity, specificity, positive and negative predictive values were calculated using SPSS program (IBM Corporation, Chicago, USA).

Ethical Approval and/or Informed Consent

Informed written consent was obtained from each participant. The study was approved by the local Clinical Research Ethical Committee.

Results

Antibody responses caused by E. granulosus

All patients had from 1 to 3 of hydatid cysts in the liver (100 %). According to the ELISA results the samples of these patients were grouped as negative (-), low positive (+), medium positive (++) and high positive (+++). Five patients had high specific antibody response, thirteen patients had medium specific antibody response, and eight patients had low level of specific antibody. The response ranges were between 1/640 and1/5000. Three patients were found to be specific antibody negative. However, those three patients which were negative by ELISA were found to be positive by Western Blotting (Table 2). Immunoblot analysis of EgAg showed protein bands of 8, 12, 20, 22, 24, 36, 75 and 90 kDa size. Among of them, 8 – 12 kDa bands, 20 – 22 kDa and 36 kDa bands displayed strong reactivity against human serum specimens. No serum samples from healthy control reacted with EgAg (Fig.1 and Table 2). The most common clinical manifestation in case of hepatic cyst was abdominal pain which was present in 96 % of patients. The other complaints were palpable abdominal mass, hepatomegaly, nausea, vomiting etc. In all cases the USG and/or CT examinations confirmed the CE.

Molecular identification of Echinococcus species causing CE

Using the DNA Sequence Analysis Technique of CE patients, it was determined that the patients were infected with *Echinococcus granulosus s.s.* (Genotype G1-G3) (Fig. 2). Nineteen of the 29 cases were male (65.5 %) and 10 were female (34.5 %). Their ages ranged from 8 to 63. When the gender and age range of the patients were taken into account, CE occurrence and clinical outcomes were found to be in accordance with the literature information that can be seen in both genders and all age groups (Table 3). Most of them lived in rural areas without significant differences ($P > 0.05$) or kept a dog (55 %) for a long time. Data on patient gender, age and residence is available in Figure 3.

The NADH1 gene sequence profiles of all samples were detected as the same and compatible with sequence data with the GenBank

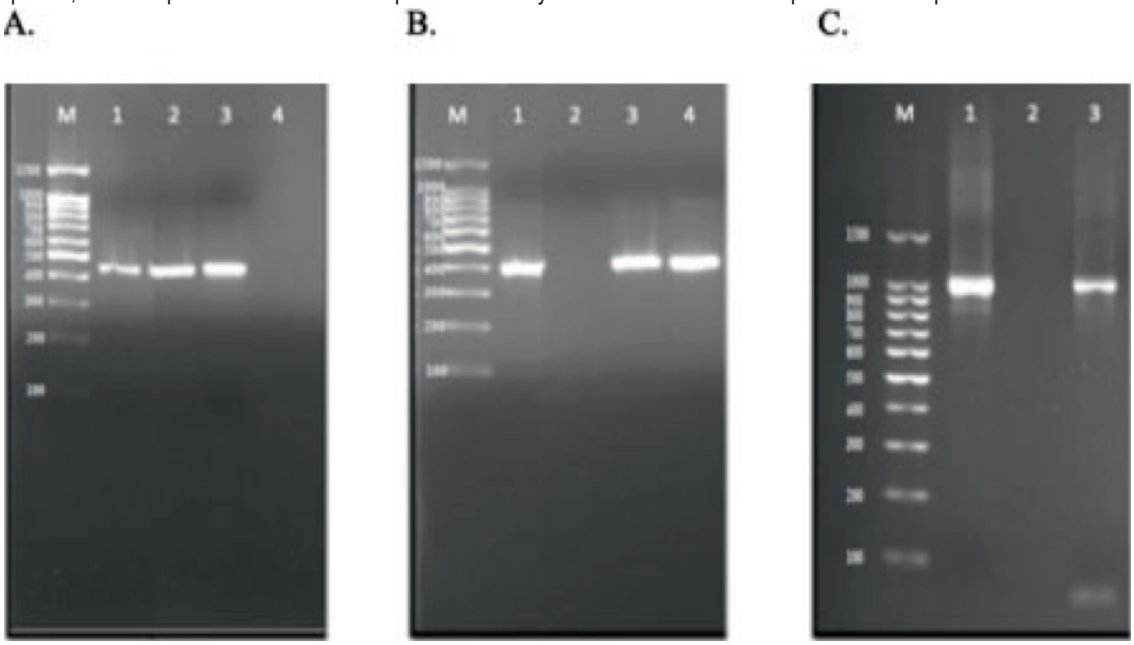

Fig. 2. CO1, NAD1 and ITS-1 Gene Amplicons. A. CO1 gene amplicons (446 bp). M:DNA Marker, 1:Positive Control, 2-3:Human Isolates, 4:Negative Control (Distilled water) B. NAD1 gene amplicons (378 bp). M:DNA Marker, 1:Positive Control, 2:Negative Control (Distilled water), 3-4:Human Isolates. C. ITS-1 gene amplicons. M:DNA Marker, 1:Positive Control, 2:Negative Control (Distilled water), 3:Human.

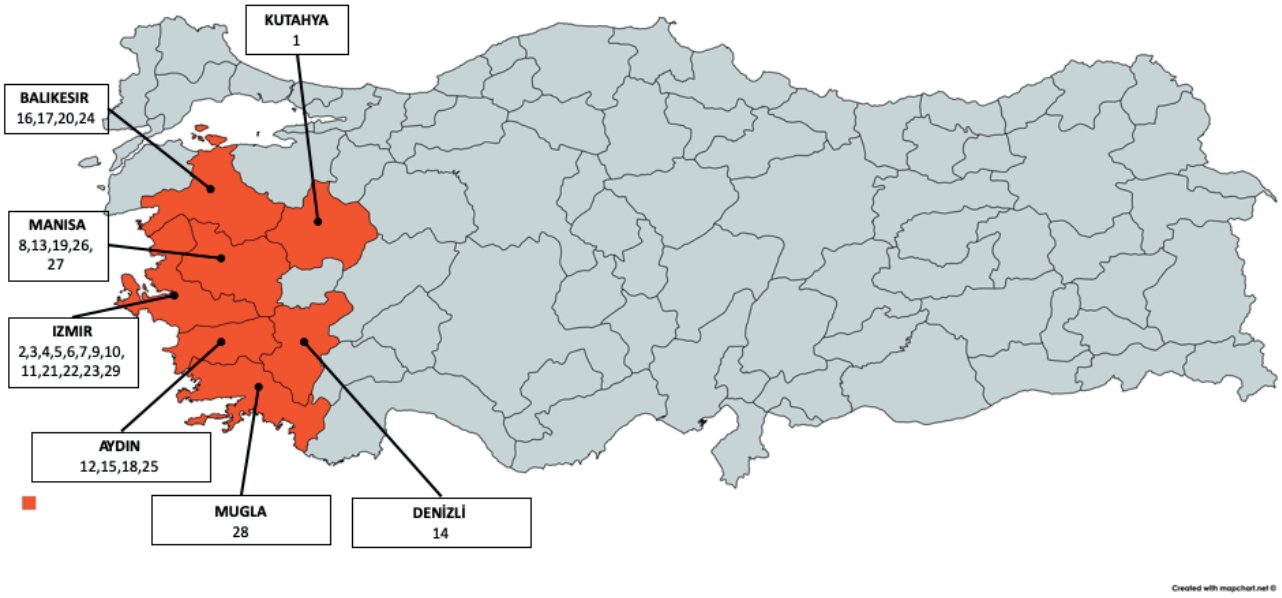

Fig. 3. A map of Turkey showing the distribution of 29 CE cases province caused by *E. granulosus*.

accession number MN270000, and accordingly, COX1 gene sequence profiles of all samples were compatible with KT001403 GenBank accession number (both sequences are attributable to *E. granulosus s.s.* G1-G3).

The forward and reverse sequences of all samples were analyzed and compared with the BLAST program. As a result of our study all patients found to be infected with the *E. granulosus s.s.* (G1-G3) genotype. There was no consistent correlation between results of ELISA and Western Blotting, the number or size of cysts and genotype.

Discussion

Human CE caused by the tapeworms *Echinococcus granulosus s.s* is among the most pathogenic helminthic zoonoses. The larval stages of the tapeworm *Echinococcus granulosus sensu lato* are the causative agent of CE, one of the most important cestodes infections responsible for the morbidity and mortality in humans and significant economic losses in livestock (Salamatin *et al.,* 2017). Around one million or more people are currently suffering from CE globally. The financial burden with up to $2 billion lost annually on the livestock industry is substantial (Torgerson & Macpherson, 2011).

In Turkey, as determined by DNA sequence analysis of COX1 region, the G1 is the dominant genotype in human and the other intermediate hosts (Utuk & Simsek, 2008). The current study which supported this interpretation examined the CE agents responsible for causing 29 cases of liver CE and all were found to be G1. It would also be interesting to see and compare results from different localities and CE patients with different genotypes and with clinical outcomes from various Turkey regions.

For clinical practice it should be noted that the ELISA utilizing crude hydatid cyst fluid has a high sensitivity (over 95 %) but its specificity is often unsatisfactory. It should be remembered that approximately 10 to 20 % of patients with hepatic cysts and about 40 % with pulmonary cysts do not produce detectable specific serum antibodies (IgG) and therefore give false-negative results

Table 3. Age and gender distribution of CE patients.

Patient Age	Male	Female	Total
0 – 12	4	1	5
13 – 20	2	3	5
21 – 35	3	2	5
36 +	10	4	14
Total	19	10	29

(Pawlowski *et al*., 2001). Cysts in the brain, bone, or eye and calcified cysts often induce no or low antibody responses. In routine laboratory practice, usually at least two different tests should be used to obtain the most accurate results (Eckert & Deplazes, 2004). So, in this study ELISA and WB tests were used together to get reliable results. Three patients which were negative by ELISA were found to be positive by Western Blotting in which the USG and CT examinations were also positive.

Study covering Turkey's east and southeast Anatolian 179 sheep, 19 cattle and 7 goats were examined by the PCR-RFLP method for 205 ribosomal ITS-1 gene region of the *E. granulosus* isolate. It has been reported that all isolates were of G1 genotype (Utuk *et al.,* 2008). Another study in the western region of Turkey in which 22 *E. granulosus* isolates (12 sheep, 10 humans) were analyzed by DNA sequencing methods for mitochondrial COX1 and NAD1, and found that G1 genotype were confirmed in 17 isolates, while G1-G3 strain was found in sheep isolate. In Turkey, for the first time G7 strains were detected in two sheep and one human isolate (Šnábel *et al*., 2009). Analysis of 58 samples (42 human, 13 cattle, and 3 sheep) in Çukurova region of Adana found that the active genotype is solely G1 strain (Eroglu *et al*., 2016).

The purpose of our study was to determine the cysts genotype obtained from 29 individuals who were diagnosed with CE and compared them with ELISA and Western Blot results. In our recent study, we found that common genotype in human is *E. granulosus s.s.* G1-G3. There was no consistent correlation between ELISA and Western Blotting results, the number or size of cysts and CE genotype.

Regarding molecular analysis, the COX1 and NAD1 genes and the ITS-1 gene region were amplified in all isolates obtained. When the amplicons of the COX1 and NAD1 genes (COX1:446 bp and NAD1:378 bp) were electrophoresed on agarose gel, a single band was observed. There was no difference in the size of the amplicon's bands obtained from cysts taken from the same host. The amplicons of the COX1, NAD1 and ITS-1 genes were similar to those obtained from previous studies (Xue *et. al* 1993; Mwambete *et al.,* 2004; Utuk *et al.,* 2008; Ergin *et al.,* 2010; Eryildiz & Sakru, 2012; Parsa *et al.,* 2011; Mogoye *et al.,* 2013; Adwan, 2013; Yan *et al.,* 2013; Ahmed *et al.,* 2013; Altintas *et al.,* 2013). According to the ELISA results, antibody titers varied broadly and were found low in some patients or very high in others. Immunoblot analysis of EgAg showed many protein bands of with size 8, 12, 20, 22, 24, 36, 75 and 90 kDa. Among of them, 8 – 12 kDa bands, 20 – 22 kDa and 36 kDa bands presented strong reactivity with human serum specimens. Obviously none of the blood serum samples from healthy individuals reacted with EgAg. All patients in our study were found to be infected with the *E. granulosus* G1-G3 genotype. In conclusion; this was the first study that investigates the correlation between clinical outcomes with specific species or genotypes and serological results using CE parasitic material derived from Turkish human hosts. Our results showed that the *E. granulosus* s.s. (G1-G3) is predominant in the Aegean Region of Turkey where G7 strain is also present as it was determined in the study published earlier (Šnábel *et al.,* 2009). In order to investigate whether the specific immunoreactions in CE patients is related to the species by DNA sequence analysis and with ELISA and WB results it is necessary to compare different types or genotypes with infected patients antigen and antibody outcomes. In our study it was not possible because all our patients were infected with *E. granulosus s.s.* (G1-G3 genotype). Unfortunately, the limitation of this study was that there is limited number of patients to search for different genotypes. As reported by Grubor *et al*., (2017), future studies will give us opportunity to investigate the role of determined genotypes on immunomodulatory effects on parasite infections in humans. However, it might be difficult to understand the host-parasite relationship because of development of CE takes long-time. These findings are extremely important for the better development and improvement of CE diagnosis and treatment, as well as for control strategies and vaccine development.

For many of the genotypes we still have insufficient information. In particular regarding geographical distribution, host relationships in humans and animals, clinical outcomes and pathology. Therefore, these areas of interest need to be investigated more comprehensively on larger groups of patients and hosts. It will be interesting to compare results from different Turkey regions where *E. granulosus* is quite common. We understand that further studies and collaborations utilizing larger sample sizes are required to examine the links between different genotypes (if possible), localizations, cyst stages as well as clinical and immune parameters outcomes.

Conflict of Interest

The authors do not have a conflict of interest.

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Prevalence and age distribution of enterobiasis in North-Eastern Bulgaria

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Article info

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Summary

Enterobiasis is a worldwide distributed helminthiasis that mainly affects children. Bulgarian health-care legislation mandates annual tests for *E. vermicularis* in large part of the population.
This study aimed to establish the prevalence of enterobiasis in children and adults in Varna district, in North-eastern (NE) Bulgaria.
A retrospective analysis of 71,308 laboratory results from patients tested for *E. vermicularis* between 2009 and 2018 was performed. The overall prevalence of enterobiasis was 0.91 % with a significantly higher rate in children (1.49 %) than in adults (0.25 %). An increased tendency of the annual prevalence rates (0.45 %/2009 – 1.45 %/2018) was demonstrated solely due to the propagation of this disease in children population. Detailed analysis in different groups clustered by age and attendance of children's collectives showed that the lowest level of enterobiasis (0.36 %) was found in children younger than 36 months and afterwards an increased level (1.58 %) in preschool kids (3 – 6 y) was detected. The highest occurrence (9.57 %) was observed in school-age children (7 – 17 y) where no prophylactic measures by law are required or performed.
The prevalence of the enterobiasis observed in Varna District corresponds with the official reports in the country and reflects the actual situation of this disease in Bulgaria. The regional and countrywide data demonstrate that the levels are significantly lower than those reported in the other European countries. The main reason for that is the implementation of an extensive and effective system for the surveillance, prevention and control of *E. vermicularis*-infection in Bulgaria.
Keywords: enterobiasis; *E. vermicularis*; age distribution; Bulgaria; Europe; parasitic diseases

Introduction

Enterobiasis (pinworm infection) is caused by the small intestinal nematode *E. vermicularis.* It has a worldwide distribution and is probably the most common helminth infection with prevalence rates reaching up to 30 – 50 % (Burkhart & Burkhart, 2005; Wendt *et al*., 2019). Children older than two years, especially those visiting childcare establishments and primary schools are the most affected group (Remm, 2006; Wendt *et al*., 2019). Adults contract enterobiasis usually from infected children by domestic or occupational exposure. Consequently, the prevalence in adults is low and thus seldom researched (Burkhart & Burkhart, 2005).
Hands contamination with the parasite's eggs plays the most critical role for infection and autoinfection. It is the primary factor for the circulation of the disease in the population (Herrström *et al*., 2001; Wendt *et al*., 2019). The facilitated person-to-person transmission

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exempts enterobiasis from the principle that intestinal parasites are uncommon in developed regions. Infection can be observed in children of all strata regardless of socioeconomic level, culture or race (Boas *et al*., 2012; Wendt *et al*., 2019).

Despite its wide dissemination on the European continent, enterobiasis is rarely a subject of in-depth epidemiological inquiries. Due to its mild or asymptomatic manifestation, it is sometimes considered more as a nuisance than an actual health problem. The most recent data on prevalence rates of the infection in Europe vary depending on the scope of the research and the investigated population. The reported prevalence rates are 37 % in allergic and 23 % non-allergic children in Sweden (Herrström *et al*., 2001), 32.4 % in Polish children and adults (Heciak, 2006), 24.4 % in Estonian nurseries (Remm, 2006), 22 % in hospitalised children in Denmark (Lacroix & Sørensen, 2000), 18.2 % in children in Norway (Boas *et al*., 2012), 17.4 % in the population of Berlin area, Germany (Friesen *et al*., 2019), 7.73 % in children of central Greece (Patsantara *et al*., 2016), and 3.59 % in pre-school and school-aged children in the eastern Slovakia (Dudlová *et al*., 2018).

The aim of the current study was to establish the enterobiasis prevalence and its age distribution in Varna district population, North-eastern (NE) Bulgaria within 2009 – 2018 period and compare the results with the official countrywide data available for the same period.

Material and Methods

Data source, study design and patient population

Varna District is one of the largest administrative areas in the Republic of Bulgaria. It is located in the North-Eastern region on the Black Sea coast. The administrative centre is Varna-city, and with a population of 336 505 for 2018, it is the third-largest town in Bulgaria (NSI, 2019).

Bulgarian healthcare legislation regulates the monitoring and prevention of local parasitic diseases. Testing for intestinal parasites, including *E. vermicularis,* is mandatory as follows:

- Once yearly in children at age two to seven;
- During the enrollment and after a prolonged leave of absence in nurseries (children 0 – 3 years), in kindergartens and preschools (children 3 to 7 years);
- In adults engaged in professions with an impact on public health.

This retrospective study was conducted in one of the largest Specialized Medical Diagnostic Laboratories in Varna, Bulgaria, where the Parasitology department carries out more than 10,000 diagnostic tests for the detection of human parasites annually.

The investigation encompassed 71 308 patients examined for *E. vermicularis* between 01/01/2009 and 12/31/2018. The primary criterion for inclusion was a current residence within the municipality of Varna. Patients outside of those parameters were excluded from the research.

Of all 71 308 samples, 37 886 (53.1 %) belonged to children (<18 years of age), and 33 422 (46.9 %) to adults. Male to female ratio was 39.2 % (27 916) to 60.8 % (43 392).

Patient's data were grouped by age in 2 categories – adults and children. The children's group was further stratified according to age and child care facilities attendance:

Group 1: adults – all patients at the age of 18 years and older;
Group 2: infants attending nurseries – at age 1 – 3 years;
Group 3: preschool children between 3 and 6 years, attending kindergartens and mandatory (at five years) preschool education;
Group 4: school attending children and adolescents at age 7 – 17 years.

Sample collection and microscopic examination

E. vermicularis eggs in the perianal area were detected microscopically using Graham's adhesive-tape test in concordance with the standards for parasitological testing in Bulgaria. The patients or their parents/legal guardians executed the perianal tests following the explicit instructions from the laboratory personnel. All samples were collected and screened microscopically within 48 hours of submission. Tests containing *E. vermicularis* ova or adult parasites were considered positive and entered as such in the laboratory information system. Due to the low sensitivity of the adhesive-tape test (Boas *et al*., 2012; Friesen *et al*., 2019; Wendt *et al*., 2019) the patients (especially in cases with strong clinical suspicion, direct contact with enterobiasis or during treatment monitoring) were advised to submit at least three probes from 3 consecutive days. All results from a single patient (irrespective of the number examined slides) were treated as a unique data record in the analysis. All patients diagnosed with enterobiasis received standardized etiological therapy, followed by control tests at day 10 and 20. All family members and the close contacts were invited for testing and treated if needed.

Data analysis

The data were processed and analysed with the R language and environment for statistical computing (R Core Team, 2018; Devleesschauwer *et al*., 2015). All observations are reported within the 99.9 % confidence interval (CI) calculated by the Clopper-Pearson's exact method for binomial proportions. Groups were compared using Pearson's χ^2-test (in 2-by-2 analyses) or likelihood ratio χ^2-test (in binomial logit models). The size of the observed differences is represented by odds ratios (OR). Each OR is accompanied by its 99.9 % asymptotic CI and the value of the exact (in 2by2 analyses) or asymptotic (in binomial logit model) probability (p-value) for the OR being equal to 1.

Ethical Approval and/or Informed Consent

The study was designed, conducted and reported in agreement with the Declaration of Helsinki (rev. 2013). Each patient or their legal representative gave informed consent for the laboratory testing as it is mandatory for all tests performed on an outpatient basis in clinical laboratories in Bulgaria. All data regarding the testing

Table 1. Enterobiasis prevalence and distribution by age and sex, Varna District, NE Bulgaria, 2009 – 2018

Age	Sex	Positive	Negative	Prevalence % (CI)	OR (CI)	p
Children	Female	277	18 057	1.51 (1.23 – 1.83)	1	–
Children	Male	289	19 263	1.48 (1.21 – 1.78)	0.98# (0.74 – 1.29)	0.80
Children	**Total**	**566**	**37 320**	**1.49 (1.30 – 1.71)**	**5.95* (4.05 – 8.73)**	**$2.2x10^{-16}$**
Adults	Female	58	25 000	0.23 (0.14 – 0.35)	1	–
Adults	Male	27	8 337	0.32 (0.16 – 0.58)	1,40# (0.65 – 3.01)	0.17
Adults	**Total**	**85**	**33 337**	**0.25 (0.17 – 0.36)**	**1**	
Total	**Female**	**335**	**43 057**	**0.77 (0.64 – 0.92)**	**1**	**–**
Total	**Male**	**316**	**27 600**	**1.13 (0.94 – 1.36)**	**1.47# (1.13 – 1.90)**	**$1.21x10^{-6}$**

OR male vs female; * OR children vs adults

and detection of *E. vermicularis* performed between January 2009 and December 2018 were extracted retrospectively from the laboratory information system. Only study-relevant patient information (age, sex, residence, year of sample reception) was collected for the purpose of this research. The data was presented in the form of frequency tables containing combinations of factor levels and the corresponding number of positive and negative results. This data format makes it impossible to reconstruct the initial source completely and to identify the patients. Given the retrospective nature of the study and the preserved anonymity, no further ethical approval was required and requested.

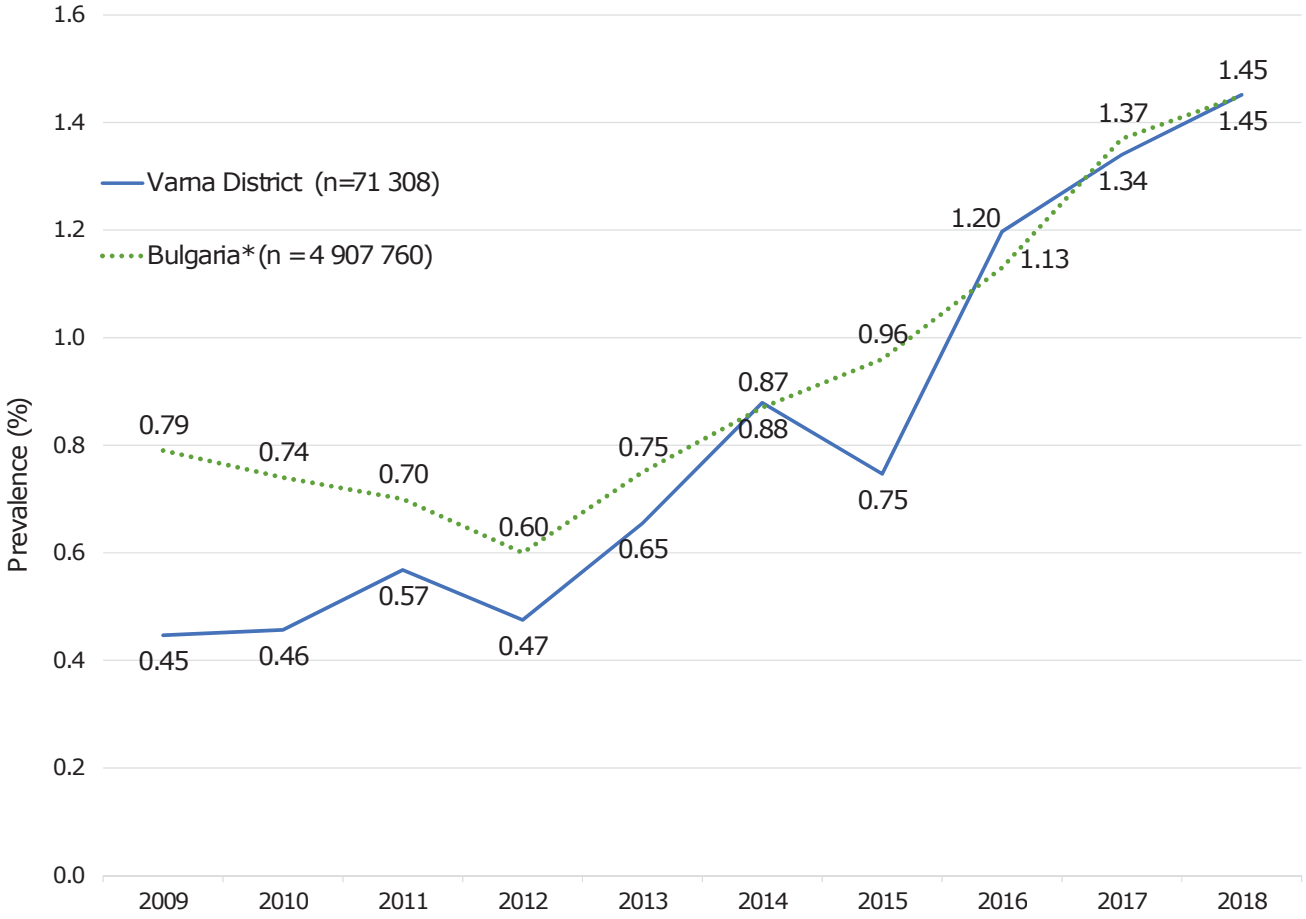

Fig. 1. Annual prevalence of enterobiasis in Varna District and Bulgaria, 2009 – 2018. Overall annual prevalence of enterobiasis in Varna district (blue line) compared to observed levels for the entire country (green dotted line). * Data from the official analyses of the NCIPD (Rajnova *et al*., 2018, 2019; Stoyanova, 2019).

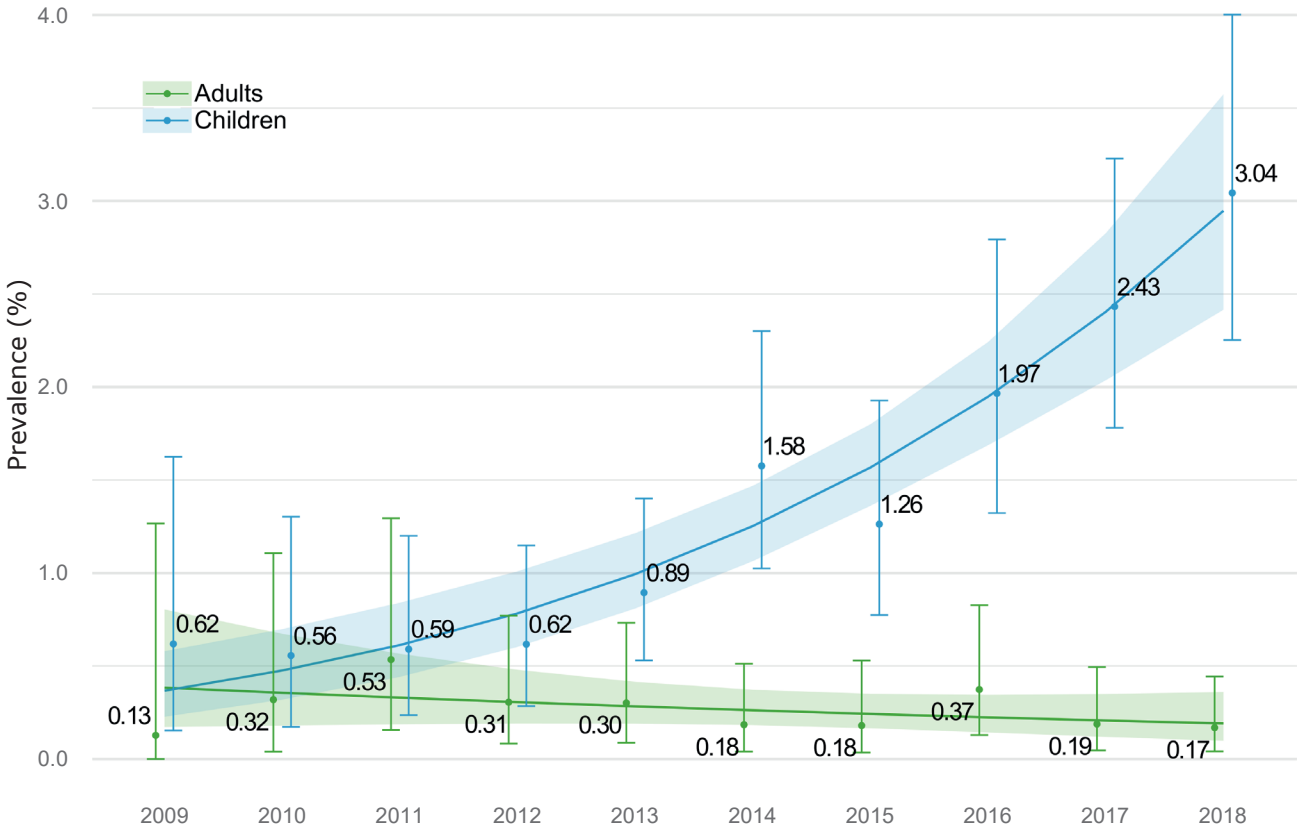

Fig. 2. Annual dynamics of the prevalence of enterobiasis in children and adults in Varna District, NE Bulgaria, 2009 – 2018. The observed annual prevalence (labelled points) of enterobiasis in children (blue) and adults (green) is depicted within the exact Clopper-Pearson CI (error bars). The predictions of the binomial linear model and CI are presented by continuous lines and shaded areas correspondingly. The model confirms the observed significant 5 – 6-fold increase in the prevalence of enterobiasis in children during the observed period as compared to the relatively constant levels in adults.

Results

The overall prevalence of enterobiasis

From the total number of 71 308 individuals, examined with Graham's adhesive test, eggs and/or adult forms of *E. vermicularis* were detected in 651 patients. The youngest diagnosed person was a 16 months old infant, and the oldest was a 73-years old male. In all cases, the patient or the patient's legal representative received personalised advice from a specialist in clinical parasitology regarding the treatment, personal hygiene, and the obligatory follow up of the treatment effectiveness.

The overall prevalence of enterobiasis for the period 2009 – 2018 was 0.91 % (CI:0.80 – 1.04). In children enterobiasis was significantly more prevalent – 1.49 % (CI:1.30-1.71), than in adults – 0.25 % (CI:0.17-0.36), with OR=5.95 (CI:4.05-8.73; $p<2.2*10^{-16}$).

Distribution of enterobiasis by sex

Table 1 demonstrates the distribution of enterobiasis by sex and age. The disease was more prevalent in males – 1.13 % (CI:0.94-1.36) than in females – 0.77 % (CI:0.64-0.92). The difference between the sexes, however, becomes negligible when the prevalence was analysed in children and adults separately.

Annual dynamics of enterobiasis

In the investigated period, we observed a steady, substantial increase in absolute numbers of positive samples through the years – n=10 in 2009 to n=140 in 2018. The total increment corresponded with the growing trend in the overall yearly prevalence. Fig. 1 compares the results in Varna District with the annual prevalence rate of enterobiasis in Bulgaria as reported by the National Centre of Infectious and Parasitic Diseases (NCIPD) (Harizanov *et al*., 2020; Rainova *et al*., 2019, 2018).

Initially, the prevalence of the disease followed a relatively flat trend. Starting from 2012, however, a steady increase was evident in both datasets. For the additional investigation, we compared the average rates for the periods – 2009 – 2012 and 2013 – 2018. The

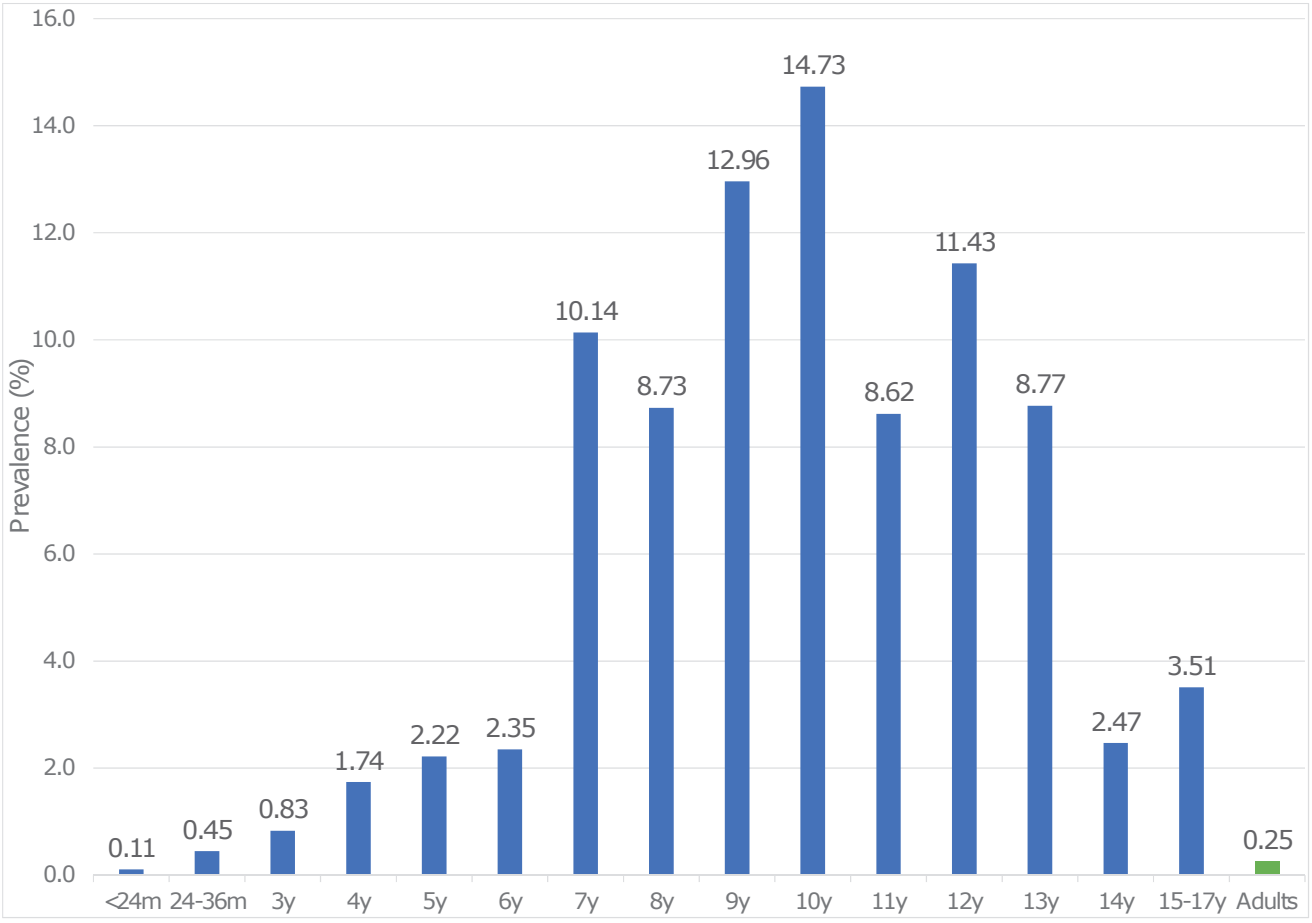

Fig. 3. Average ten-year prevalence of enterobiasis distributed by age, Varna District, NE Bulgaria, 2009 – 2018.

total prevalence before 2012 was 0.49 % (CI:0.34-0.69) whereas after 2012 it rised to 1.06 % (CI:0.92-1.21). This observation corresponded to an approximately two-fold increase in the odds of detecting the parasite in the studied population (OR=2.15; CI:1.72-2.69; χ^2=47.55; p=3.3*10^{-13}).

Since there is a significant difference in the rates between children and adults, where the age has the potential to play a major confounding role, we stratified the analysis further. We fitted the data for *E. vermicularis*-infection to a binomial logit model with age category ("children" or "adults"), year of detection and their interaction as independent predictors.

Fig. 2 shows the model's predictions and the observed annual point prevalence. The model was significantly better at explaining the observed data than the hypothetical model of random variance (χ^2=513.24; p<2.2x10^{-16}) and the model without interaction (χ^2=42.3; p=7.85x10^{-11}). The rates of *E. vermicularis* in adults remain relatively constant throughout the entire decade. In children, the model predicted 1.26-fold (CI:1.18-1.34) annual increment in the odds of contracting enterobiasis. This finding confirmed that the observed significant increase in enterobiasis between 2012 and 2018 is predominantly due to the facilitated disease circulation in children's population.

Age distribution of enterobiasis is demonstrated on Fig. 3. The lowest levels were recorded in children younger than 24 months (0.11 %; CI:0.01-0.50; n=3/2 794) with an subsequent increase (0.45 %; CI:0.24-0.75; n=36/8 000) at age of 24 – 36 months. The prevalence continued to grow throughout the preschool age until it peeked in group of 6-year old children (2.34 %; CI:1.63-3.25; n=92/3 925). At school age, we observed a sudden jump in the prevalence rates which reached the highest values in 9- (12.96 %; CI:5.81-23.64; n=21/162) and 10-year old (14.73 %; CI:6.32-27.34; n=19/129) children.

To reduce the bias introduced through the unbalanced number of test subjects, we stratified the children in the groups above and compared the average prevalence in each group with the adult population (Fig. 4). The prevalence in 0 – 2-years old kids was the lowest (0.36 %; CI:0.20-0.59) and comparable with the rate found in the adults (OR=1.42; CI:0.73-2.64; p=0.07). In children at kindergarten and preschool age enterobiasis affected 1.58 % (CI:1.34-1.86) of the population (OR=6.32; CI:4.33-9.53; p=2.2x10^{-16}) while in 7 – 18 years old children reached its peak at 9.57 % (CI:7.01-12.63) with OR=41.49; CI:25.78-67.54; p=2.2x10^{-16}).

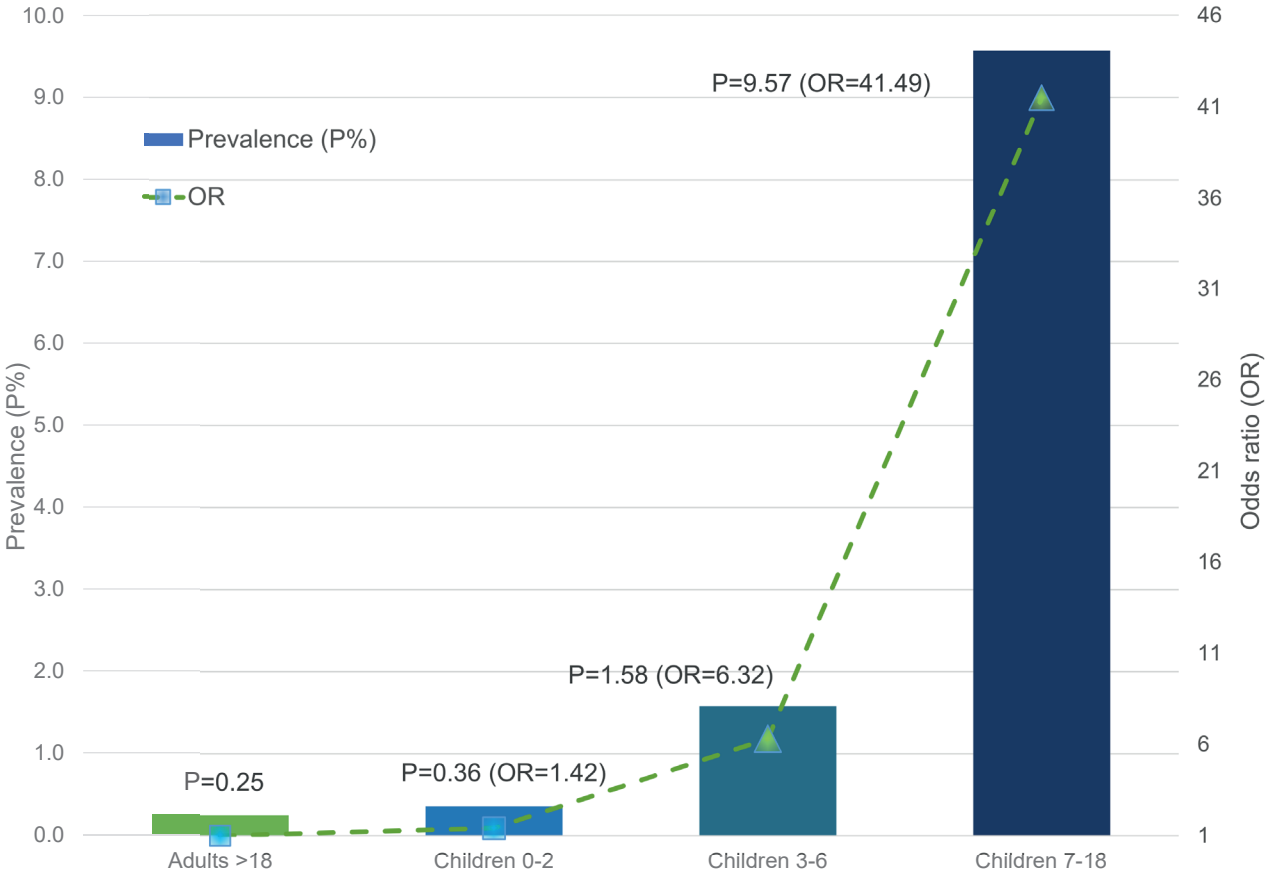

Fig. 4. Average ten-year prevalence and odds ratio of enterobiasis in different age groups, Varna District, NE Bulgaria, 2009 – 2018**.** Average prevalence (columns; left axis) for the observed decade in adults and children 0 – 2, 2 – 6 and 7 – 18-year-old. Odds ratios comparing the relative risk of infection to the risk in the adult population are presented with labelled dots and projected on the right axis. Odds ratios significantly different from 1 are presented with a triangular shape.

Discussion

With an average prevalence of 0.91 %, *E. vermicularis* was the most common intestinal parasite in Varna District for the last decade when compared with all other intestinal helminths and protozoa during an overlapping period (2007 – 2016) (Stoyanova, 2019). The rates in the NE region are equivalent to the average prevalence of enterobiasis in Bulgaria (0.90 %; n=4 4115/4 907 760) as reported by NCIPD (Harizanov *et al*., 2020; Rainova *et al*., 2018, 2018). It is important to note that the real prevalence might be even higher. The Bulgarian healthcare regulations do not require the adhesive tape tests to be carried out by a specialist personal, and this might increase the rate of false-negative results. Our results about the sex distribution of enterobiasis showed higher prevalence in males than females. Although the overall odds ratio suggested elevated risk in males (OR=1.47), further analysis demonstrated that sex differences are insignificant in adults (OR=1.40) as well as in children (OR=0.98). Thus, similarly with other researchers (Boas *et al*., 2012; Herrström *et al*., 2001), we conclude that the observed small discrepancies are not part of a regular pattern but arise from random variance and inhomogeneities within the tested populations.

We have found an alarming tendency for rising of enterobiasis after 2012 (Fig. 1). The average prevalence in Varna District tripled in the decade under observation and in 2018 (1.45 %) exceeded levels detected in the region at the end of the last century (1.11 % in 1989 and 1.20 % in 1990) (Ruseva & Popova, 1992). A similar pattern was observed throughout the whole country, as reported by NCIPD (Fig. 1).

The statistical models of the annual prevalence rates in children and adults (Fig. 2) clearly demonstrated that the increase in enterobiasis is specific and solely due to the elevated infection rates in childhood. The annual differences in prevalence were typical for the entire 10-year interval but did rise sharply in the second half of the decade.

Several factors may have contributed to this tendency. Regionally and countrywide, there is continuous overcrowding of child care facilities accompanied by a deficit of qualified and skilled person-

nel (Stoyanova, 2019; Stoyanova & Cvetkova, 2018). Additionally, during various periods in 2014, 2015 and 2016, anthelminthic drugs were absent from the pharmacy network in Bulgaria. This fluctuation in the availability of the medication and the complete absence of an appropriate administration form for younger children (suspension or syrup) lead to a delay of etiological therapy and prolonged the period in which the infected persons were epidemiologically active sources of infection (Stoyanova, 2019; Stoyanova *et al*., 2017).

Since the last wide-scale examination in Varna District, the levels of enterobiasis in children dropped approximately ten times from 12.4 % in 1975 – 1977 (Kovchazov, 1979) to 1.49 %. The same did not apply for the adult population, where the average prevalence diminished only by half – from 0.50 % (1975 – 1977) to 0.25 % (2009 – 2018). The prevalence levels for the adults have not changed substantially in the last 40 years as concurred by the annual model (Fig. 2). This finding indicates that there is a stable background level of enterobiasis in the adult population, and it can be used as a base level for comparison with other age groups.

The enterobiasis in adults is rarely researched in other European countries. One epidemiological study in Poland reported 2.59 % prevalence in children and 0.43 % in adults (Kasprzak *et al*., 2017). Another older survey in Lithuania revealed rates of 18.4 % and 1.3 % in children and adults, respectively (Mazhilene, 1991).

Fig. 3 demonstrates the growing rate of enterobiasis in children by age which is facilitated by increased exposure. In the older adolescent (>14 years), the level dropped and reached the background rates observed in the adult population. Despite the significantly higher prevalence, similar distributions are reported in different European countries. Investigation in healthy Norwegian children shows 2 – 3 years old kids as least affected (7.3 %), what is followed by 4 – 5 years-old group (14.1 %), and the highest prevalence (34.4 %) was observed in 5 – 14 years old children (Boas *et al*., 2012). Parallel age dependency was observed in Estonia where the prevalence was 16.2 % in 1 – 3 years-old kids, 16.0 % in 4-year-olds, 29.9 % in 5 and 6-year-olds and 42.0 % in 7 – 8 years old group (Remm, 2006).

The attendance in children's collectives is a significant risk factor for the propagation of enterobiasis in Bulgaria (Genov *et al*., 1975; Rainova *et al*., 2018; Ruseva & Popova, 1992; Stoyanova, 2019). The age-dependent partitioning of the prevalence that was apparent in Fig. 3 also corresponded with the main types of facilities for child care and education. Infants attending nurseries (0 – 2-years old) are less exposed and less affected. Their primary contact with the disease is probably via infected family member – parent or older sibling, and thus the average level of the infection (0.36 %) is similar and insignificantly higher than in adults.

The disease rate increased substantially to 1.58 % in the age group attending kindergartens and preschool (2 – 6-year-olds) as the odds of contracting the disease here are about six times higher than in adults (OR=6.32).

These results also showed the encouraging trend that over the past 40 years, the enterobiasis prevalence in the childcare facilities in Varna District has decreased about 3 times in both age groups – from 1.1 % (1971 – 1975) (Kovchazov, 1979) to 0.36 % (2009 – 2018) in the nurseries and from 3.6 % to 1.58 % in the kindergartens.

Almost the same rate in reduction of enterobiasis was observed in the children in school age. It decreased from 35.6 % (1971 – 1975) (Kovchazov, 1979) to 9.57 % in our current study. Nevertheless, students remain the most affected by enterobiasis. The significant differences from younger age-groups can be attributed to the absence of any preventive measures that should focus on the children or on their close environment (schools). In contrast, there is an effective system for the prevention in nurseries, kindergartens and preschools. The higher prevalence detected at school-age must be interpreted cautiously. This group is not subjected to preventative screening and is comprised of a population tested for *E. vermicularis* either on clinical (manifested symptoms) or on epidemiological (contact with an infected) ground. Therefore, the observed rates are biased upwards, and the actual prevalence is probably lower. Still, there is a six-fold increase in odds as compared with kindergarten age group (OR=6.57; CI:4.59-9.41) and 41-fold in comparison with adults. This finding requires detailed proactive surveillance in this group and the implementation of focused preventive measures to limit disease circulation in schools.

Similarly to our findings, higher rates of enterobiasis in older children were found in Denmark (29 % in 5 – 12 years old kids) (Lacroix & Sørensen, 2000), and in children from neighbouring Turkey where 10.1 % of the pupils in the primary schools in South-Western (Aksoy *et al*., 2007) and 18.2 % of the 7 – 14 year-old students in Western region (Okyay *et al*., 2004) were affected.

Conclusion

This research identifies an alarming upward trend in the annual enterobiasis prevalence in children within NE Bulgaria. The infection is most frequent in preadolescent and early adolescent age where targeted preventive measures should be introduced and enforced.

Our results about the prevalence of enterobiasis in Varna District and its rates in children and adults reflected the overall epidemic process of this helminthiasis in Bulgaria. The levels of *E. vermicularis* infection (regionally and countrywide) were significantly lower than the prevalence data reported from most of the European countries. Two factors were responsible for the observed discrepancy. Our pool of subjects included a vast number of children and adults, tested annually on prophylactic indications which rarely are an object of epidemiological studies available. More importantly, the Bulgarian proactive surveillance system for intestinal parasites serves as an effective approach to screen for such mild or generally asymptomatic infections. Consequently, the majority of the 'hidden' disease carriers are revealed, and subsequently treated and eradicated as potential sources of further transmission.

Conflict of Interest

Authors have no potential conflict of interest pertaining this submission to Helminthologia.

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Soil and sand contamination with canine intestinal parasite eggs as a risk factor for human health in public parks in Niš (Serbia)

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Summary

Regarding geographical distribution and clinical relevance, the most common canine geohelminths are *Toxocara canis*, ancylostomatids, and *Trichuris vulpis*. Canine intestinal parasites from the soil and sand present an important potential serious human health hazard, especially for the children preschool and school – age. This paper aimed to establish the degree of contamination of soil and sand with zoonotic parasites from the canine feces and the degree of risk they could pose for human health in public places and playgrounds in the city of Niš. Our parasitological study involved 200 soil samples and 50 sand samples from the public parks in the city of Niš in southeastern Serbia (43°19'15"N, 21°53'45" E). From several locations, about 100 g of soil and sand was collected based on the bioclimatic indices. Parasitological diagnosis was performed using conventional qualitative and quantitative coprological methods, abiding by the recommendations about the diagnosis of parasitic diseases. In 38 – 46 % of soil samples and 40 % of sand samples seven species of endoparasites were diagnosed. In the samples of soil, a medium and high degree of contamination with the ascarid *T. canis* (14 – 22 %) was detected, as well as a low and medium degree of contamination with ancylostomatids (4 – 12 %), and in the samples of sand, a variable degree of contamination with the helminths *T. canis* (26 %) and *A. alata* (16 %) was found. A statistically significant difference was found in the contamination with *A. alata* eggs between the samples of sand and samples of soil. The studied public surfaces represent the reservoir of zoonotic parasites, which is a public health problem requiring a synergistic action of several factors to be successfully resolved, i.e. the implementation of prevention, surveillance, and control measures.

Keywords: intestinal parasites; dog; contamination; public spaces; public health

Introduction

More than 2 billion people worldwide are exposed to a risk for geohelminth infections. Regarding geographical distribution and clinical relevance, the most common canine helminths are *Toxocara canis*, *Ancylostomatidae*, and *Trichuris vulpis*, but the significance of these pathogens has been often underestimated by both veterinary and human medical professionals, as well as the general public (Traversa *et al*., 2014).

Human contact with the soil is one of the possible ways of spread-

* – corresponding author

ing the canine intestinal parasites. These pathogens reach the soil via canine and human feces and can survive in this environment for a long period, making it a potential reservoir of infection (Tudor, 2015). The developing forms of geohelminths can survive the longest and enter the organism of susceptible individuals via skin or wounds in the visible mucosa (*Ancylostoma caninum*, *Uncinaria stenocephala*, *Strongyloides stercoralis*), causing a cutaneous larva migrans (Heukelbach & Feldmeier, 2008; Jaleta *et al*., 2017), or by ingestion (*T. canis*), when a visceral or ocular larva migrans is formed, which may persist in human tissues even for as long as several years (Aydenizöz-Özkayhana *et al*., 2008**;** Overgaauw & van Knapen, 2013). The infections such as these are mainly sporadic, but in extraordinary situations (natural disasters and wars), they can also occur in epidemic scales (Tudor, 2015).

According to the information from the available literature, different degrees of soil contamination with helminth eggs and larvae from canine feces have been established. In Poland, the percentage was between 7.9 % and 10.6 % (Blaszkovska *et al*., 2012) and 18.6 % (Bojar & Kłapeć, 2012); in Italy, 24 % (Habluetzel *et al*., 2003); in Portugal, 63.3 % (Otero *et al*., 2014); in Slovakia, 79.2 % (Rudohradska *et al*., 2011); in India, 12.84 % (Sudhakar *et al*., 2013); in Spain, 71.33 % (Martínez-Moreno *et al*., 2007); and in Romania, 22.22 % (Tudor, 2015).

The studies performed in public parks in several Serbian cities, such as Belgrade (Pavlović *et al*., 2015), Požarevac, Kostolac (Pavlović *et al*., 2015) and Kruševac (Raičević & Pavlović, 2019), have demonstrated a degree of contamination of urban green spaces, soil, and sand ponds in day-care facilities for pre-school children with zoonotic protozoa, nematodes, and cestodes.

This paper aimed to establish the degree of soil contamination and sand with zoonotic parasites from the canine feces and the degree of risk they could pose for human health in public places and playgrounds in the city of Niš.

Material and Methods

Study design

In the parasitologic examination performed in the period February – May 2019, we collected and analyzed 200 samples of soil and 50 samples of sand. The soil was sampled in three public parks in Niš: Tvrđava (Park 1) – 50 samples; Čair (Park 2) – 50 samples; Sveti Sava (Park 3) – 50 samples; and „Pet park" (Park 4) – 50 samples (representing a special, isolated group of sampled material), enclosed and situated within the Čair park. The sand was sampled only from the „free" part of Čair park (50 samples), since in other parks of interest for the study there were no sandpits.

Study area

The city of Niš (43°19'15" N, 21°53'45" E) is the largest in southeastern Serbia and is the seat of the Nišava administrative district. It is situated at an altitude of 194 m and occupies an area of around 596.73 km^2. According to the European NUTS (Nomenclature of territorial units for statistics) classification, it belongs to the NUTS3 category. Two important European transport corridors (Corridors X and VII) directly or indirectly connect the city with the border European surroundings. The city has a moderate continental climate (warm summers and moderately cold winters), with an average yearly temperature of 12.08°C, average precipitation of 577.79 mm/m^2 (the highest in October; the lowest in February), and average air humidity of 70.4 % (the highest in January; the lowest in August) (source: Spatial plan of the Niš City Council).

The parks of interest for this study are situated in urban parts of the city, with a high population density and considerable fluctuation of pet and stray dogs. The Sveti Sava park is in one of the newer and largest communities in Niš, covering the area of 4.31 hectares. The Čair park is the largest in Niš situated in the city core, covering the area of 16.4 hectares. In 2016, an enclosed pet park was open within the Čair park, with the area of 5.000 m^2, in which pet owners may walk or train their pets without leashes. The Tvrđava park is a complex cultural monument and an urbanistic hallmark of the central part of Niš. It is situated on the right bank of the Nišava river and occupies the area of 22.1 hectares.

Samples

From each of the above four locations, about 100g of soil and 100g of sand, for each sample was collected with a small shovel from the square area of 25 x 25 cm and depth of 10 cm. The material was collected from the sites without any vegetation (grass) to avoid more intense draining of a grass-covered soil. The sampling was performed based on the bioclimatic conditions indexes, abiding by the method of bioclimatogram by Uvarov (1931) and taking into account the parameters of average temperatures and humidity values for the studied area.

The number of soil and sand samples was determined by the location size and degree of apparent contamination with canine feces. The samples were packed in PVC bags and appropriately labeled (with date of sampling, location, number, and type of samples). After that, the samples were stored in a handheld refrigerator at +4 °C and transported to the laboratory of the Department of Parasitology, Faculty of Veterinary Medicine, University of Belgrade. Parasitological diagnosis was performed in the first 24 – 48 hours after the sample receipt.

Parasitological examination

The diagnosis of parasitic sample contamination was performed using the qualitative method without any concentration of parasitic elements – native examination by Pataki (Soulsby, 1986) and qualitative methods with the concentration of parasitic elements – the method of gravity flotation with saturated NaCl solution, specific weight 1,200 and saturated $ZnSO_4$ solution, specific weight 1,400, at 20 °C (Urquhart *et al*., 1996), sedimentation method (Hansen and Perry, 1994), the method by Fülleborn (Euzeby, 1982), and sedimentation-flotation method for soil and sand (Pavlović, 2017).

In all examined samples of soil/sand, using the method by McMas-

ter (Kochanowski *et al*., 2013), we were able to diagnose fewer than 50 parasitic elements per 1 g. That was the reason why the result of quantification was done using the qualitative FEC method (Pittman *et al*., 2010). Qualitative FEC defines the obtained results as „positive" or „negative". In the method of conventional gravity flotation the number of parasite eggs is valued as „minus" (-) for negative findings, or „plus" (+) for positive findings (with three-level grading as „+, ++, +++"). The number of plus marks denotes the examiner's subjective opinion about the number of parasitic elements present beneath the two coverings of a microscopic specimen.

All the parasitological methods used in the study conform to the recommendations by the ISID (*International Society for Infectious Diseases*), OIE (*World Organisation for Animal Health*), WHO (*World Health Organization*) and WAAVP (World Association for the Advancement of Veterinary Parasitology) related to the diagnosis of parasitic diseases.

Statistics

The data was processed with the GraphPad Prism statistics software. The study results were presented in tables. The statistical analysis of the data involved the application of descriptive tests and analytical non-parametric tests (the Chi-square test). The descriptive statistics were performed to report the data analysis that was presented as mean and standard deviations. The categorical variables were shown as frequencies and percentages. The statistical significance cut-off was set at $p< 0.05$.

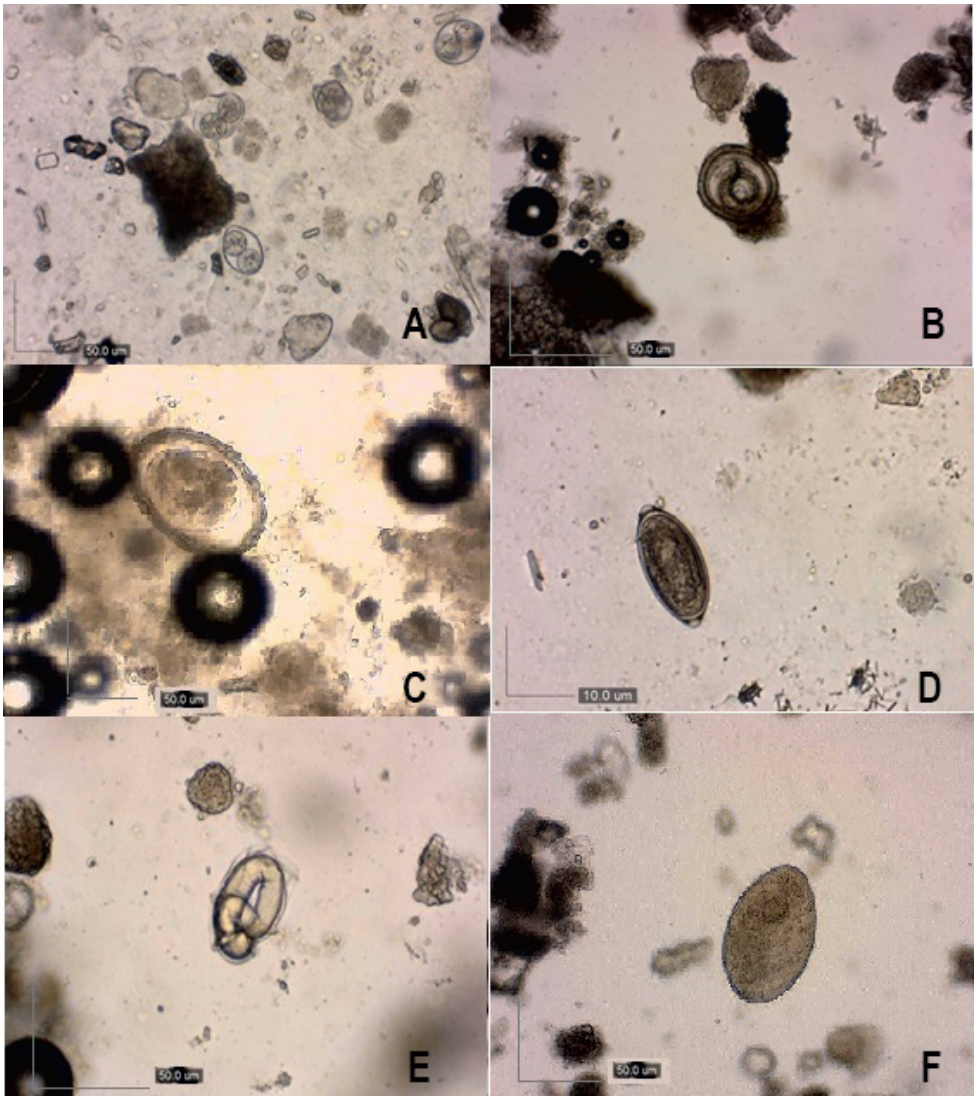

Figure 1. Parasitology diagnosis of soil samples. A) sporulated oocysts of *Cystoisospora* spp.; B) embryonated egg of *T. canis* (stage 1 larva); C) egg of *T. leonina*; D) egg of *T. vulpis*; E) embryonated egg of ancylostomatidae (stage 1 larva) (gravitation flotation method, $ZnSO_4$); F) egg of *A. alata*, sedimentation method), 100x

Table 1. Qualitative assessment of soil samples from the public parks in Niš – diagnosed endoparasites.

ENDOPARASITE	PARKS IN NIŠ (soil)								χ^2	P
	Park 1 N=50		Park 2 N=50		Park 3 N=50		Park 4 N=50			
	n	%	n	%	n	%	n	%		
Cystoisospora spp.	2	4	1	2	1	2	0	0	2.04	0.56
Toxocara canis	9	18	8	16	11	22	7	14	1.21	0.75
Toxascaris leonina	1	2	2	4	1	2	0	0	2.04	0.56
Ancylostoma caninum/ Uncinaria stenocephala	4	8	5	10	6	12	2	4	2.25	0.52
Trichuris vulpis	3	6	2	4	3	6	0	0	3.13	0.37
Alaria alata	0	0	1	2	1	2	1	2	1.02	0.80
Total positive	**19**	**38**	**19**	**38**	**23**	**46**	**10**	**20**		

n – number of positive samples; N – total number of samples

Ethical Approval and/or Informed Consent

Not applicable.

Results

In the examined soil and sand samples, seven endoparasites were identified: protozoa from the *Cystoisospora* genus (Fig. 1A), nematodes (*Toxocara canis* – Fig. 1B, *Toxascaris leonina* – Fig. 1C, *Trichuris vulpis* – Fig. 1D, ancylostomatidae – Fig. 1E) and a trematode *Alaria alata* (Fig. 1F).

Each of the 4 methods of parasitological diagnostics applied was used according to the degree of sensitivity of the selected method for the appropriate type of parasite elements, as expected in the samples of the test material.

Results of qualitative examination of soil samples

Developing forms of endoparasites were found in 38 % (19/50) soil samples from Park 1, 38 % (19/50) samples from Park 2, 46 % (23/50) samples from Park 3, and 20 % (10/50) samples from Park 4 (Table 1).

Table 2. Qualitative assessment of soil samples from the public parks in Niš – diagnosed coinfections.

MIXED ENDOPARASITIC CONTAMINATION	PARKS IN NIŠ (soil)								χ^2	P
	Park 1 N=50		Park 2 N=50		Park 3 N=50		Park 4 N=50			
	n	%	n	%	n	%	n	%		
Cystoisospora spp. *Toxocara canis*	1	2	0	0	1	2	0	0	2.02	0.57
Toxocara canis Toxascaris leonina	1	2	0	0	1	2	0	0	2.02	0.57
Toxocara canis Ancylostomatidae	1	2	3	6	2	4	1	2	1.63	0.65
Toxocara canis Trichuris vulpis	0	0	0	0	1	2	0	0	3.05	0.39
Toxascaris leonina Trichuris vulpis	0	0	1	2	0	0	0	0	3.05	0.39
Toxocara canis Alaria alata	0	0	0	0	0	0	1	2	3.05	0.39
Ancylostomatidae Trichuris vulpis	1	2	0	0	0	0	0	0	3.05	0.39
Cystoisospora spp. *Toxocara canis Trichuris vulpis*	0	0	1	2	0	0	0	0	3.05	0.39
Toxocara canis Ancylostomatidae Trichuris vulpis	1	2	0	0	0	0	0	0	3.05	0.39
Total positive	5	10	5	10	5	10	2	4		

n – number of positive samples; N – total number of samples

Table 3. Quantitative assessment of soil samples from the public parks in Niš.

Endoparasites	DEGREE OF CONTAMINATION (QUALITATIVE FEC method)																	
	Park 1						Park 2						Park 3					
	Low		Medium		High	Total positive	Low		Medium		High	Total positive	Low		Medium		High	Total positive
	n (%)	Mean ± SD	n (%)	Mean ± SD	n (%)		n (%)	Mean ± SD	n (%)	Mean ± SD	n (%)		n (%)	Mean ± SD	n (%)	Mean ± SD	n (%)	
Cystoisospora spp.	2 (100)	5.5±3.54	0	0	0	**2**	1 (100)	7	0	0	0	**1**	1 (100)	5	0	0	0	**1**
Toxocara canis	6 (66.66)	5.67±1.97	3 (33.33)	35±8.89	0	**9**	6 (75)	4.67±2.94	2 (25)	27±11.31	0	**8**	7 (63.63)	6.86±2.91	4 (36.36)	35.50±14.53	0	**11**
Toxascaris leonina	1 (100)	10	0	0	0	**1**	2 (100)	4±2.83	0	0	0	**2**	1 (100)	6	0	0	0	**1**
Ancylostomatidae	3 (75)	5.33±3.51	1 (25)	44	0	**4**	3 (60)	7.67±3.21	2 (40)	34±9.90	0	**5**	4 (66.66)	3.75±2.22	2 (33.33)	28.50±14.85	0	**6**
Trichuris vulpis	3 (100)	6.67±3.06	0	0	0	**3**	1 (50)	10	1 (50)	37	0	**2**	2 (66.66)	5.50±3.54	1 (33.33)	25	0	**3**
Alaria alata	0	0	0	0	0	**0**	0	0	1 (100)	43	0	**1**	1 (100)	9	0	0	0	**1**

Low: 1 – 10 eggs, oocysts; Medium: 11 – 49 eggs, oocysts; High: ≥ 50 eggs, oocysts (number of oocysts/eggs calculated per one covering – opc/epc)

Table 4. Qualitative assessment of samples of sand in Park 2 – free space.

ENDOPARASITES	Sand samples N=50	
	n	%
Cystoisospora spp.	3	6
Toxocara canis	13	26
Toxascaris leonina	1	2
Ancylostoma caninum/ Uncinaria stenocephala	4	8
Trichuris vulpis	2	4
Alaria alata	8	16
Total positive samples	**20**	**40**
MIXED ENDOPARASITIC CONTAMINATION	**n**	**%**
Cystoisospora spp. *Toxocara canis*	2	4
Toxocara canis Ancylostomatidae	2	4
Toxocara canis Alaria alata	3	6
Ancylostomatidae Alaria alata	2	4

n – number of positive samples; N – total number of samples

The most prevalent intestinal parasites were *T. canis* (18 % – 9/50) and ancylostomatids (8 % – 4/50) in Park 1, *T. canis* (16 % – 8/50) and ancylostomatids (10 % – 5/50) in Park 2, and *T. canis* (22 % – 11/50) and ancylostomatids (12 % – 6/50) in Park 3. The species from *Cystoisospora* genus were most prevalent in Park 1, with a prevalence rate of 4 % (2/50), *T. canis* nematode, with a prevalence rate of 22 % (11/50) was predominant in Park 3, *T. leonina,* with a prevalence rate of 4 % (2/50) in Park 2, *A. caninum*/*U. stenocephala* with a prevalence rate of 12 % (6/50) in Park 3, and *T. vulpis* with a prevalence rate of 6 % each (3/50) in Parks 1 and 3. The *A. alata* trematode was diagnosed in two soil samples, one from Park 2 (2 % – 1/50) and one from Park 3 (2 % – 1/50). In the examined soil samples we established the presence of eight mixed endoparasitic pathogens, and the most common was the double infection toxocariasis-ancylostomatidosis, with a prevalence of 6 % (3/50) in Park 2, and 4 % (2/50) in Park 3 (Tables 1 and 2). By analyzing

Table 5. Quantitative assessment of samples of sand in Park 2 – free space.

Endoparasites	DEGREE OF CONTAMINATION (QUALITATIVE FEC method)						
	Park 2						
	Low		Medium		High		Total positive
	n (%)	Mean ± SD	n (%)	Mean ± SD	n (%)	Mean ± SD	
Cystoisospora spp.	3 (100)	6±2	0	0	0	0	**3**
Toxocara canis	8 (61.54)	4.75±2.31	2 (15.38)	34±8.49	3 (23.07)	77±24.56	**13**
Toxascaris leonina	0	0	1 (100)	36	0	0	**1**
Ancylostomatidae	1 (25)	4	3 (75)	35.33±8.50	0	0	**4**
Trichuris vulpis	1 (50)	9	1 (50)	16	0	0	**2**
Alaria alata	5 (62.5)	4.60±3.05	3 (37.5)	20.67±10.97	0	0	**8**

Low: 1 – 10 eggs, oocysts; Medium: 11 – 49 eggs, oocysts; High: ≥ 50 eggs, oocysts (number of oocysts/eggs calculated per a covering – opc/epc)

Table 6. Comparison of the results obtained for soil and sand samples in the free space of Park 2.

ENDOPARASITES	PARK 2				P
	Soil N=50		Sand N=50		
	n	%	n	%	
Cystoisospora spp.	1	2	3	6	0.62
Toxocara canis	8	16	13	26	0.33
Toxascaris leonina	2	4	1	2	1
Ancylostoma caninum/ Uncinaria stenocephala	5	10	4	8	1
Trichuris vulpis	2	4	2	4	1
Alaria alata	1	2	8	16	0.04*
Total positive	**19**	**38**	**20**	**40**	

*p< 0.05; n – number of positive samples; N- total number of samples

the soil sampled from the "Pet Park", contamination with endoparasites was detected in 20 % (10/50) of samples. Three types of endoparasites were diagnosed: *T. canis* nematode 14 % (7/50), which was the most prevalent, ancylostomatids 4 % (2/50), and *A. alata* trematode 2 % (1/50) (Table 1).

Results of quantitative examination of soil samples

In the majority of soil samples, low levels of *T. canis* ascaridide contamination (66.66 %; 5.67±1.97 epc in P1, 75 %; 4.67±2.94 epc in P2 and 63.63 %; 6.86±2.91 epc in P3) and ancylostomatids (75 %; 5.33±3.51 epc in P1, 60 %; 7.67±3.21 epc in P2 and 66.66 %; 3.75±2.22 epc) were found in P3. Soil samples positive for the presence of *T. leonina* and *Cystoisospora* spp. showed low levels of contamination with this nematode in all three tested parks. A low degree of contamination of *T. vulpis* nematode (100 %; 6.67±3.06 epc in P1, 50 %; 10 epc in P2, 66.66 %; 5.50±3.54 epc in P3) was diagnosed in all the parks tested. In one sample from Park 2 (50 %; 37 epc) and Park 3 (33.33 %; 25 epc), a moderate degree of contamination with *T. vulpis* was determined. An average degree of contamination by trematode *A. alata* in the sample originating in Park 2 and a low degree of contamination in the sample from Park 3 were determined (Table 3).

Results of qualitative examination of sand samples

In the examined sand samples collected from the free space in Park 2, we diagnosed the protozoa from the *Cystoisospora* genus (6 % – 3/50), nematodes *T. canis* – 26 % (13/50), *T. leonina* – 2 % (1/50), ancylostomatids – 8 % (4/50), *T. vulpis* – 4 % (2/50) and a trematode *A. alata* – 16 % (8/50). The most common coinfection was toxocariasis-alariosis (6 % – 3/50), while other mixed infections (*Cystoisospora* spp. – *T. canis*; *T. canis* – ancylostomatids and ancylostomatids – *A. alata*) were present in 4 % (2/50) (Table 4).

Results of quantitative examination of sand samples

In most sand samples we detected a low degree of contamination with *T. canis* (61,54 %; 4.75±2.31 epc), while in 23,07 % (77±24.56 epc) of positive samples a high degree of contamination with this ascarid was seen. We also established a medium degree of contamination with the species *T. leonina* (100 %; 36 epc), *T. vulpis* (50 %; 16 epc), *A. alata* (37,5 %; 20.67±10.97 epc) and ancylostomatids (75 %; 35.33±8.50 epc) (Table 5).

The analysis of soil samples from public city parks in Niš and Park 4 did not reveal any statistically significant difference ($p>0.05$) (Tables 1 and 2). The analysis of soil and sand from the free space in Park 2, we found a significant difference ($p<0.05$) in the prevalence of individual endoparasitic infection with the trematode *A. alata*, with a higher prevalence in sand samples – 16 % (8/50) (Table 6).

Discussion

The study was performed in dry and humid periods in the spring season of 2019, investigating the prevalence of intestinal helminths eggs and protozoan oocysts in the samples of soil and sand from public parks and playgrounds for children in the city of Niš. The eggs of *T. canis* were diagnosed with the highest prevalence (16 – 22 % in the soil; 26 % in the sand), which agreed with other authors' findings, demonstrating the prevalence of this ascarid at 15.6 % in the playgrounds in Turkey (Aydenizoz, 2006) and 15.5 % – 23.3 % in the playgrounds in Croatia (Stojčević *et al*., 2010). The literature data suggest that depending on the climate and geographical location, the level of contamination varies among different countries and within individual countries, where it is determined by the action of local geoclimatic factors. Accordingly, the contamination of public parks in Italy with eggs of *T. canis* was 63.6 % (Giacometti

et al., 2000), in Slovakia 61.3 % (Rudohradská *et al*., 2011), in Serbia (Kruševac) 50.1 % (Raičević & Pavlović, 2019), while in Spain over 67 % of parks and 1.24 % of soil samples were contaminated (Ruiz *et al*., 2001).

The results of this study agree with the data about soil contamination with *T. canis* eggs obtained by the authors from the Czech Republic 5.0 to 20.4 % (Dubná *et al*., 2007), Croatia – 15.5 do 23.3 % (Stojčević *et al*., 2010), Spain – 16.4 % (Dado *et al*., 2012), Poland – 16.6 % (Bojar & Káapeü, 2012), Romania – 17.17 % (Tudor, 2015) and Greece – 17.2 % (Papavasilopoulos *et al*., 2018).

The degree of contamination of sand samples with the ascarid *T. canis*, as was found in the study, is following the authors' findings from Slovakia – 6.8 to 27.0 % (Ondriska *et al*., 2013), markedly higher than that from the Czech Republic – 11.9 % (Dubná *et al*., 2007), Slovakia – 11.8 % (Papajová *et al*., 2014) and slightly higher than the degree of contamination established in India – 17.64 % (Sudhakar *et al*., 2013).

The contamination with ancylostomatids eggs was diagnosed in 8 – 12 % of soil samples and 8 % of sand samples, while the presence of *T. vulpis* eggs was established in 4 – 6 % of soil samples and 4 % of sand samples. The studies from Croatia report a higher degree of contamination of soil and sand samples from the playgrounds in Pula with *T. vulpis* eggs – 10 – 17.7 % (Stojčević *et al*., 2010).

A statistically significant difference in the degree of contamination with *A. alata* eggs was found between sand (16 %) and soil samples (2 %). In the samples of soil from Park 1, the eggs of this trematode were not found. The reason might have been a dry period of sampling (May) since a higher degree of contamination and a greater number of identified species are always reported in humid periods of the year (Nurdian, 2004). An increased number of soil samples positive for the presence of ascarids and ancylostomatids, as well as sand samples for the presence of *A. alata*, can be explained by favorable climatic conditions, with moderate temperatures, appropriate soil humidity and adequate environmental conditions in general. During the spring months, the eggs accumulated and concentrated in the soil, and due to low precipitation, they were not washed down to deeper layers of soil. The analysis of soil sections revealed that most of the eggs were situated at the depth of 0 – 4 cm (Storey & Phillips, 1985). Since they are not situated on the surface, the eggs are probably protected from the action of direct sunlight and decay.

Such a long survival of ascarid eggs can be explained by the fact that these are most resilient helminth eggs, able to preserve their vitality for up to several years. On the other hand, protozoan cysts and oocysts have a reduced ability to survive in the natural environment (the cysts of *Giardia* spp. are less resilient than *Cryptosporidium* spp. oocysts). This fact may partly explain the absence of these protozoa in our study, in contrast to other authors' findings (Martínez-Moreno *et al*., 2007; Dado *et al*., 2012; Pavlović *et al*., 2015).

A difference was established in the number of positive soil (13) and sand samples (20). The sand was sampled only from the „free" part of Čair park (50 samples), since in other parks of interest for the study there were no sandpits.

Since the texture and humidity of samples have an impact on the length of survival of parasite eggs in the external environment, it should be emphasized that the samples of soil and sand in our study were almost identical as to their humidity. The reason is the vicinity of the Nišava river and its tributary Gabrovačka river, i.e. the fact that sand has an increased capacity to hold moisture. Nišava flows past the Parks 1 and 3 (from which exclusively soil was sampled), while the Park 2 is located further in the city core (the only park with free sand for sampling). In the Park 2, a higher degree of sand contamination (40 %) was found compared to soil contamination (38 %), which agreed with the results reported by Bojar and Kłapeć (2012), who detected the highest degree of contamination of sand (40 %) on the lake beaches in southeastern Poland. The authors stressed that in these places there was an increased chance for contamination of recreational areas with eggs of intestinal parasites from wild animals. The results are supported by the fact that in Poland and other European countries the phenomenon of synanthropization of wild animals, especially red foxes and feral pigs, has been observed, which opens new, additional opportunities for soil and sand pollution in public places in urban areas.

Most parasite eggs (especially embryonated ones) were found in the samples from shadowy places (under the trees or small shrubs). The reason is the ability of soil to hold humidity in such places and protection from direct sunlight, which significantly prolongs egg viability. The finding is also directly correlated with the behavior of dogs and their choice of spots for defecation (Rubel & Wisniveskyy, 2005).

In our study, 5 – 20g of soil and 5 – 20g of sand were examined for each of these methods. The amount of sample tested was dependent on the method used. According to literature data from Mandarino-Pereira *et al*. (2010) tested soil samples (25g) using a modified centrifugal-flotation technique with NaNO3 (Dunsmore *et al*. 1984) and an adaptation of Rugai's *et al*. method (Carvalho *et al*., 2005) is a spontaneous sedimentation method. This method checks 100g of soil and can be done the granulometric analyses of the samples were done and the soil classified into sand, silt, and clay according to their composition (Embrapa, 1997).

According to Kazacos (1983), the ability to diagnose parasitic elements in soil samples increases when a larger amount of soil (30g) is used for analysis. These authors claim that 30g of soil is the maximum amount of soil that can be effectively cultivated. In our study, the maximum amount of soil/sand sampled was 20g for technical reasons. Our experience shows that a larger amount of the sample leads to the formation of a dense suspension with the formation of air bubbles, which make it difficult to microscopically examine the preparations and to diagnose parasitic elements.

The experience of other authors shows that sedimentation techniques are more practical and economical than flotation for soil

and sand examination, but leave more impurity particles in the supernatant, which may interfere with the detection of the parasite by microscopy (Carvalho *et al*., 2005). Therefore, the flotation method with a saturated ZnSO4 solution is always recommended as an alternative. The flotation technique requires the use of a solution of appropriate specific gravity, which makes this technique more expensive than sedimentation (Dunsmore *et al*., 1984).

The diagnosed canine intestinal parasites from the soil and sand present an important potential serious human health hazard, especially for the children aged 3 – 5 years. Supporting this notion are the diagnosed cases of parasitic zoonoses in Serbia (Gvozdenović *et al*., 2012; Mijatović *et al*., 2015; Miladinović Tasić *et al*., 2017; Perić *et al*., 2017), and that is the reason why a synchronized cooperation of professionals in the fields of both veterinary and human medicine is mandatory.

In conclusion, in the areas of public parks in the city of Niš, we established contamination with endoparasites of 38 – 46 % of soil samples and 40 % of sand samples. In the samples of soil, the contamination with *T. canis* ascarid (a low and medium degree) and ancylostomatids (a low and medium degree) was predominant, while in the samples of sand different degrees of contamination were present with *T. canis* and *A. alata*. Such a finding suggests a serious risk for human health since the above geohelminths are the etiological agents of visceral, ocular and cutaneous *larva migrans* syndrome, as well as of human larval alariosis. The finding of *A. alata* trematode in the samples of soil (2 %) and sand (16 %) indicates a significant circulation of stray dogs in the areas of public parks in the city of Niš. For owned dogs and people, they represent a reservoir of zoonotic parasites found in the soil and sand in the studied public spaces.

The resolution of this significant public health problem is therefore necessary through the Suggestion of measures, involving: a) control of parasite transmission in the environment, b) guidelines/information for dog owners for the prevention, maintenance of health, and spread of zoonotic diseases, and c) education of medical and veterinary professionals, pet owners and the general public.

Conflict of Interest

The authors declare that they have no competing interests.

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The mouse bile duct tapeworm, *Hymenolepis microstoma* in free-living small mammals in Slovakia: occurrence and genetic analysis

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Summary

The mouse bile duct tapeworm *Hymenolepis microstoma*, is a potentially zoonotic species with a wide variety of reported definitive hosts of rodent genera. In the present study the occurrence of *H. microstoma* in free-living small mammals in selected areas of Slovakia and the retrospective analysis of epidemiological data published in Slovakia were performed. *Hymenolepis microstoma* was detected in two animal species, the common shrew (*Sorex araneus*) and the European hamster (*Cricetus cricetus*) of 186 small mammals examined from two ecosystems, urban and natural ecosystem of national park. No mention about the presence of this parasite in Slovakia in the past was found following a bibliographical search. Partial sequences of the nuclear paramyosin gene showed the shrew isolate placed in a subclade together with *H. microstoma* from Portugal, with high bootstrap value for its differentiation from the sister species *Hymenolepis nana*. Similarly, the analysis of the nuclear ribosomal ITS region placed the hamster isolate in the cluster composed of *H. microstoma* from Australia, Spain and Portugal. The Slovak isolate was the most distinctive sample among available *H. microstoma*, differing in 1.4 – 1.9% of nucleotides from the remaining isolates. The difference (seven of 17 nucleotide positions) was partially due to indel polymorphisms associated with two and five nucleotides. To our knowledge, these are the first reports of *H. microstoma* in Central Europe and also the first record of infection in the common shrew. A recently indicated zoonotic potential of *H. microstoma* along with a possibility of its direct transmission between animals and/or humans without the need of intermediate hosts pose a public health concern in contaminated areas of Slovakia. The use of molecular techniques may substantially facilitate more thorough understanding of the epidemiological situation of *H. microstoma* and related tapeworms in various ecosystems of the country.

Keywords: *Hymenolepis microstoma*; Small mammals; Occurrence; Genetic analyses; Slovakia

Introduction

Among tapeworms belonging to the genus *Hymenolepis*, three species are medically relevant and are commonly found worldwide in sites where murid rodents occur: *Hymenolepis nana* (Siebold, 1852), *Hymenolepis diminuta* (Rudolphi, 1819), and *Hymenolepis microstoma* (Dujardin, 1845) Blanchard, 1891 (Cunningham & Olson, 2010). *Hymenolepis* (= *Rodentolepis*) *microstoma*, the mouse bile duct tapeworm, has a wide variety of reported definitive hosts of rodent genera as mice (Hopkins *et al*., 1977), rats (Goodall, 1972), hamsters (Bogh *et al*., 1986), voles (Litchford, 1963), and gerbils (Schmidt, 1986). A zoonotic potential of this tapeworm

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was indicated by a single report in which mixed infections of *H. nana* and *H. microstoma* were identified in four humans from a remote region of Western Australia (Macnish *et al*., 2003). *H. microstoma* is recognized as requiring an appropriate tenebrionid grain beetle or other insect as intermediate host (originated mostly from *Tribolium* and *Tenebrio* genera), in which the eggs develop into cysticercoids after ingestion (Bogh *et al*., 1986; Smyth & Wakelin, 1994). Nevertheless, the feasibility of a direct life cycle in immunodeficient mice has also been demonstrated for the mouse bile duct tapeworm (Andreassen *et al.,* 2004).

H. microstoma has attracted considerable attention since it was first described by Dujardin in 1845. In 1891, Blanchard relocated the species to the genus *Hymenolepis* and provided a more detailed description of the species. Since 1950s, *H. microstoma* is being commonly used as an experimental model. Due to its easy maintenance in the laboratory in mice host and short life cycle, *H. microstoma* has been established as a preferable model to both *H. nana* and *H. diminuta,* the maintenance of which is more costly (Clark, 2013). Moreover, the mouse bile duct tapeworm is one of the four tapeworm species having its complete genome assembled and characterized (Cunningham & Olson, 2010; Tsai *et al*., 2013). Although adult *H. microstoma* specimens may be differentiated based on morphological characteristics and predilection site (Baer & Tenora, 1970; Czaplinski & Vaucher, 1994), in some cases these characteristics are difficult to use, especially when dead animals are sampled (e.g. in national parks) and the carcasses (along with their intestinal parasites) are in some stage of decay. Furthermore, a variety of other hymenolepidid species, morphologically similar to *H. microstoma*, can potentially infest free-living small mammals (Riebold *et al*., 2019). Thus, the use of molecular tools, especially in cases when identification via morphology is ambiguous, is essential for identifying and classifying species (Peniche *et al*., 2017). The study was designed to survey the occurrence of *H. microstoma* in free-living small mammals in selected areas of Slovakia and to conduct a retrospective analysis of epidemiological data published in Slovakia. In addition, the genetic typing at species level of collected isolates was carried out.

Materials and Methods

Collection of small mammals and isolation of tapeworms

Small mammals were obtained from two different ecosystems, specifically from the protected area of the Tatra National Park (TANAP) and the surroundings situated in north Slovakia and from the urban area of Košice city and Rozhanovce village, in southeast of Slovakia.

In Tatra National Park and its surroundings, small mammals were trapped during 2015 and 2016 within the zoological survey carried

Table 1. List of small mammal species collected from two localities in eastern and northern Slovakia.

Locality	Animal species	No. of tested individuals
Tatra National Park and surroundings	Brown rat (*Ratus norvegicus*)	1
	House mouse (*Mus musculus*	12
	Striped field mouse (*Apodemus agrarius)*	4
	Yellow-necked mouse (*Apodemus flavicollis*)	21
	Common shrew (*Sorex araneus*)	38
	Eurasian pygmy shrew (*Sorex minutus*)	14
	Alpine shrew (*Sorex alpinus*)	1
	Mediterranean water shrew (*Neomys anomalus*)	1
	Common vole (*Microtus arvalis)*	1
	Field vole (*Microtus agrestis*)	5
	Bank vole (*Clethrionomys glareolus*)	26
	European mole (*Talpa europaea*)	4
Košice city and Rozhanovce village and surroundings	Brown rat (*Ratus norvegicus*)	21
	House mouse (*Mus musculus*)	7
	European hamster (*Cricetus cricetus*)	2
	Eurasian harvest mouse (*Micromys minutus)*	1
	Hazel dormouse (*Muscardinus avellanarius)*	1
	Striped field mouse (*Apodemus agrarius)*	2
	Yellow-necked mouse (*Apodemus flavicollis)*	2
	Lesser shrew (*Crocidura suaveolens)*	5
	Eurasian pygmy shrew (*Sorex minutus)*	3
	Common shrew (*Sorex araneus)*	2
	Common vole (*Microtus arvalis)*	4
	European pine vole (*Microtus subterraneus)*	7
	European mole (*Talpa europea*)	1
	TOTAL	**186**

out after the wind storm in November 2004 or found dead by workers of Research Station and Museum of TANAP or by inhabitants and visitors of national park.

Wooden nutrient traps placed in a square grid (mesh size 15 x 15 m) on square dimensions of 75 x 75 m were used to capture small mammals in Stará Lesná, Tatranská Lomnica, Tatranské Matliare, Hrebienok, Smokovec pod Hrebienkom, Spišská Sobota or their close vicinity. For the collection of protected animal species, an "Exception for the approach outside of tourist trails, and for the collection, holding and relocation of dead protected animal species for museum processing and scientific research" was obtained from the Ministry of Environment of the Slovak Republic (MŽP SR 498/2018-6.3.).

In TANAP and its surroundings a total of 128 small mammals were sampled. In Košice city and Rozhanovce village and the vicinity, 58 synanthropic small mammals found dead (after road accidents, poisoning etc.) were collected (Table 1).

Small mammals were autopsied; intestinal tract was isolated, opened and searched for the presence of tapeworms. Whole isolated tapeworms or their parts were individually placed into the 1.5 mL microcentrifuge tubes and used for molecular analyses. Unfortunately, due to the poor *post-mortem* status of carcasses with the developing decay, it was impossible to characterize the tapeworms by morphological features.

Molecular analyses

Individual tapeworms (or their parts) were analysed by PCR-derived methods. For this purpose, genomic DNA was isolated by DNeasy tissue kit (Qiagen, Hilden, Germany**)** according to the manufacturer´s instructions. PCR reactions were performed using 5x FIREPol® Master Mix Ready to Load (SOLIS Biodyne, Estonia). The first internal transcribed spacer region (ITS1) with adjacent partial subunits (18S, 5.8S) of ribosomal DNA and the nuclear gene coding for paramyosin (*pmy*) gave satisfactory DNA profiles in single isolates allowing to derive their relationships with other hymenolepidids using GenBank® deposited sequences. Primers amplifying the ITS1 region (F3, R3) and running conditions were those previously described in Macnish *et al*. (2002). The final PCR product included 22 bp of the 18S, 587 bp of the ITS1 and 23 bp of the 5.8S (in total 632 bp). For the *pmy* amplification, a nested PCR approach was employed according to the protocol provided in the above study. Using the external set of primers (Ext-F, Ext-R) a 700 bp product was first amplified. The internal set of primers then amplified DNA template from the primary PCR reaction, resulting in a 617 bp product for *H. microstoma.* Compositions of primers and running conditions for primary and secondary nested PCRs were those outlined in Macnish *et al.* (2002), except that 40 cycles were carried out in both reactions.

Phylogenetic analyses

The consensus sequences were screened against the GenBank® database using the BLAST algorithm. For the ITS1 region, nucleotide sequences were aligned using the WebPrank global alignment tool (Löytynoja & Goldman, 2010) to better identify structural constraints of transcribed but not translated regions that include several indels. For the *pmy* gene, sequences were aligned using the Clustal Omega tool for multiple sequence alignment (Sievers *et al*., 2011). Phylogenetic trees were constructed by MEGA7 software (Kumar *et al*., 2016), using the neighbor-joining (NJ) method. Evolutionary distances were computed using the Tamura-3-parameter model of nucleotide substitutions, taking into account differences in transitional and transversional rates and G+C-content bias (Tamura, 1992).

Retrospective bibliographical search on Hymenolepis microstoma *occurrence*

To retrospectively verify records on the occurrence and distribution of *H. microstoma* in Slovakia over the last decades, a key inclusion criterion was a comprehensive ´Synopsis of Cestodes in Slovakia´, covering detailed data on hosts, localities and occurrences of tapeworms in small mammals and birds in Slovakia since 1955, and published during 1993 – 1999 as a series of articles, two of which provided records on the family Hymenolepididae (Hanzelová *et al*., 1995; Hanzelová & Ryšavý, 1996). In addition to this checklist, in particular for covering the synoptical period onwards, a bibliographical search of available literature in scientific databases (PubMed, ISI Web of Knowledge, SCOPUS, ScienceDirect and Google Scholar) was conducted to find studies reporting the occurrence of *H. microstoma* in small mammals in Slovakia. The following key terms and their combinations were used for the database searches: *Hymenolepis microstoma, Rodentolepis microstoma*, *Vampirolepis microstoma*, Slovakia, Slovak Republic and Czechoslovakia. Besides, the books, journals and annual reports deposited in the Library of the Institute of Parasitology of Slovak Academy of Sciences were searched.

Ethical Approval and/or Informed Consent

The research related to animals has been complied with all the relevant national regulations and institutional policies for the care and use of animals.

Results

Occurrence of Hymenolepis microstoma *in small mammals in Slovakia*

From a total of 186 examined animals collected from the two localities in eastern and northern Slovakia, in 32 small mammals hymenolepidid tapeworms were detected. All individual tapeworms or their parts were examined molecularly, in scope of which the ITS1 and paramyosin markers were amplified. Ten isolates gave positive PCR signal with either DNA region and were subsequently sequenced. As a result, two isolates from two animal species coming from different ecosystems were identified as *H. microstoma*.

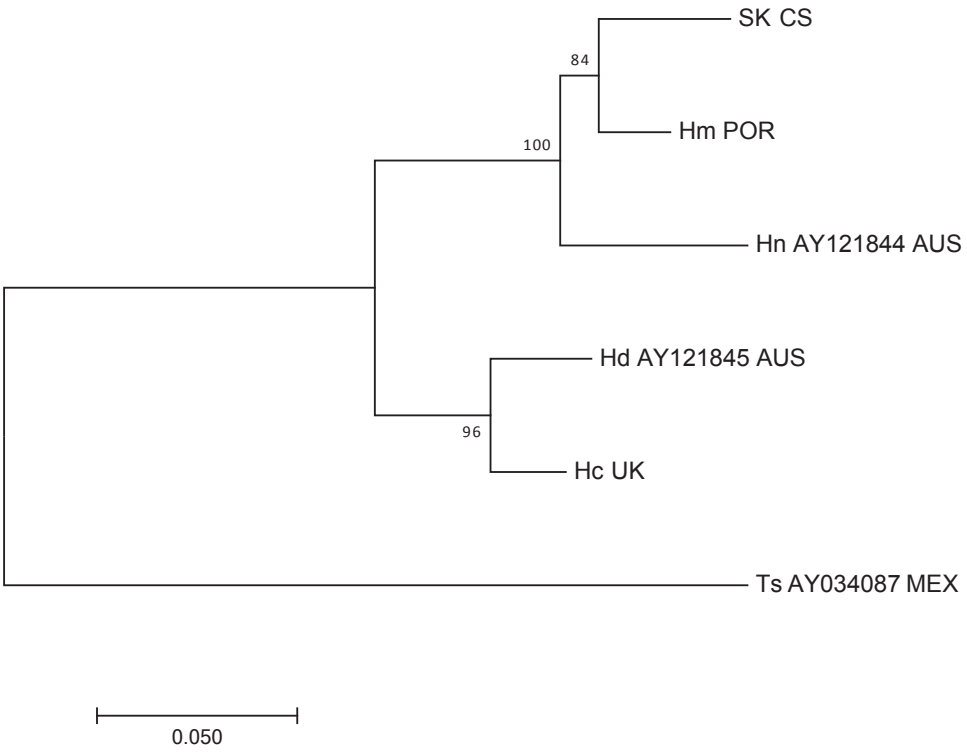

Fig. 1 Neighbor-joining (NJ) phylogram generated from the sequences of the paramyosine (*pmy,* 617 bp) partial gene showing the relationships among the SK-CS isolate from common shrew in Slovakia and GenBank-retrieved hymenolepididae sequences. Reference sequence for *Hymenolepis microstoma* (Hm) and *H. citelli* (Hc) were taken from the PhD thesis of Macnish (2001). *Taenia solium* cestode was used as the outgroup. Numbers next to the branches indicate the bootstrap value calculated from 1,000 replicates. Only bootstrap values >50% are shown at the branching points.
Species involved in the phylogram: *H. microstoma* (Hm), *H. nana* (Hn), *H. diminuta* (Hd), *H. citelli* (Hc), *Taenia solium* (Ts).
Geographical origins of the isolates: POR – Portugal, AUS – Australia, UK – United Kingdom, MEX – Mexico.

The first isolate (assigned as SK-CS) originated from the common shrew (*Sorex araneus*) caught in surroundings of Tatranské Matliare village (TANAP, north Slovakia). The second isolate (assigned as SK-EH) was obtained from the European hamster (*Cricetus cricetus*), which was found dead in an urban ecosystem of Košice city in south-east of Slovakia.
Gene sequences obtained in the study were deposited in GenBank® under accession numbers MN617852 for a SK-CS isolate (common shrew, Tatranské Matliare) and MN620506 for a SK-EH isolate (European hamster, Košice).

Genetic analyses of Hymenolepis microstoma *isolates*
A consensus sequence of 617 bp was obtained from the amplification of the partial *pmy* gene in the SK-CS isolate. A two-base-deletion at positions 506 and 507 was identified in SK-CS compared to other *Hymenolepis* spp. with available sequences, *H. nana*, *H. microstoma*, *H. diminuta* and *H. citelli*. In the N-J phylogram, the SK-CS sample clustered together with *H. microstoma* and *H. nana* (100 % bootstrap support), in the subclade composed of SK-CS and *H. microstoma* that also received a high bootstrap value (84 %) for its differentiation from *H. nana* (Fig. 1). The percentage nucleotide difference between the *H. microstoma* sequence derived from a Portuguese mouse isolate (the sequence provided by Macnish, 2001) and the SK-CS isolate was 4.86 %.
The analysis of the ITS region and the adjacent rRNA partial genes (18S, 5.8S) of the total size of 632 bp showed that the SK-EH isolate was grouped with seven *H. microstoma* specimens (Australia, Spain, Portugal) in the highly bootstrapped cluster (100 %), and clearly differentiated from the adjacent *H. nana* cluster (phylogram shown in Fig. 2). Interestingly, SK-EH constituted the most distinct sample within the *H. microstoma* assemblage, differing in 1.4 – 1.9 % of nucleotides from the remaining samples. Ten nucleotide substitutions, five-base deletion (-CGTTG) at nucleotide positions 271 – 275 and two-base insertion (+CA) at positions 107 – 108, accounted for this difference. In the rest of *H. microstoma* isolates only single-, two-, and three-nucleotide polymorphisms were detected although the samples originated from distinct geographical locations (Australia and southern Europe). Furthermore, indels were recorded only in SK-EH among the *H. microstoma* representatives. The observed deletion (-CGTTG) is responsible for the absence of arginine in the corresponding protein sequence.

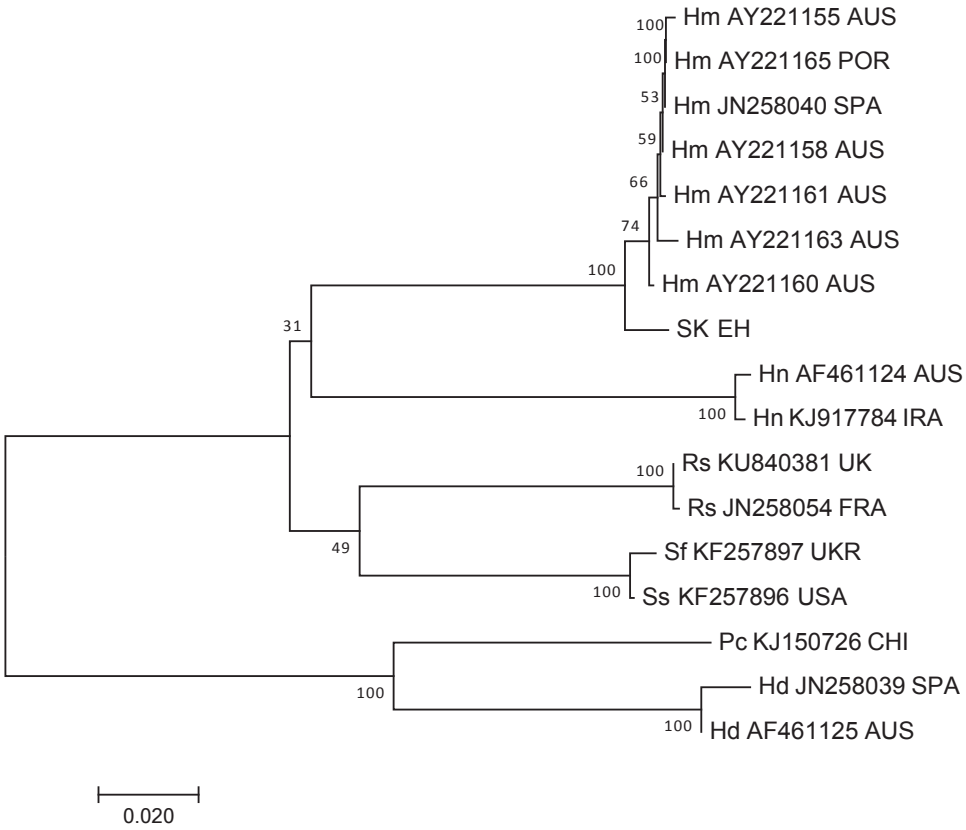

Fig. 2 Neighbor-joining (NJ) phylogram generated from the sequences of the ribosomal internal transcribed spacer (ITS1, 632 bp) showing the relationships among SK-EH isolate from European hamster in Slovakia and GenBank-retrieved hymenolepididae sequences. Numbers next to the branches indicate the bootstrap value calculated from 1,000 replicates. Only bootstrap values >50% are shown at the branching points.
Species involved in the phylogram: *Hymenolepis microstoma* (Hm), *H. nana* (Hn), *H. diminuta* (Hd), *Rodentolepis straminea* (Rs), *Pseudoanoplocephala crawfordi* (Pc), *Staphylocystis furcata* (Sf), *Staphylocystis schilleri* (Ss). Geographical origins of the isolates: AUS – Australia, POR – Portugal, SPA – Spain, IRA – Iran, UK - United Kindom, UKR – Ukraine, FRA – France, USA – United States of America, CHI – China.

Retrospective analysis of epidemiological data published in Slovakia

The search of scientific databases has not shown any publication reporting the presence of *Hymenolepis* (*Rodentolepis*) *microstoma* in the territory of Slovakia in the past. Likewise, no record on this tapeworm was included in the ´Synopsis of Cestodes in Slovakia´, confined to the period from 1955 to 1996 in small mammals, in the two articles involving findings of 114 taxa belonging to the family Hymenolepididae in Slovakia (Hanzelová *et al*., 1995; Hanzelová & Ryšavý, 1996).

Likewise, no mention about the presence of *H. microstoma* was found within the retrospective search of annual reports of the Institute of Parasitology SAS, reflecting the occurrence of helminths in different species of rodents, soricimorphs and small mammals from a variety of areas in Slovakia. Specifically, *H. microstoma* was not recorded in brown rats (*Rattus norvegicus*) from 52 districts of Slovakia in the period of 1956 – 1957 (Mituch, 1958), in 936 soricimorphs from 147 localities of Slovakia in 1958 – 1960 (Mituch, 1961), in 2,302 rodents and 1,040 soricimorphs from the territory of TANAP in 1966 – 1970 (Mituch, 1970), in 425 rodents and 98 soricimorphs from the Slovak territory of the Carpathian arc in 1986 – 1987 (Mituch *et al*., 1987), and in 55 soricimorphs and 245 rodents from the surroundings of Košice city in 1985 – 1988 (Gajdoš, 1990).

Discussion

Although *H. microstoma* was not recorded to date in any study of intestinal parasites of small mammals conducted in the territory of Slovakia, the present research documented its occurrence in the country. The parasite was recorded in two animals, in the common shrew (*Sorex araneus*) and in the European hamster (*Cricetus cricetus*), in two geographically distinct sites and ecosystems of Slovakia.

To our knowledge, these are also the first documented reports *H. microstoma* in Central Europe, considering that no data about its occurrence are available in the literature including records deposited in the Host-Parasite database of the Natural History Museum (Gibson *et al.,* 2005). As the parasite was not included in the checklist ´Synopsis of Cestodes in Slovakia´, encompassing

records from a number of local and broader surveys in Slovakia during 1955 – 1996 (Hanzelová & Ryšavý, 1996), it is likely that the mouse bile duct tapeworm was introduced into the Slovak territory over the last two decades.

The parasite was found close to Tatranské Matliare village in the vicinity of the protected area of the Tatra National Park in a common shrew (*Sorex araneus*). As far as we are aware, the present study also provides the first record of *H. microstoma* infection in *S. araneus*, which is one of the most common and abundant woodland micromammals in different types of forests (Mitchell-Jones *et al*., 1999; Rychlik, 2000), whose presence has also been confirmed in 90.3 % of the area of Slovakia (Krištofík *et al.,* 2012).

Two *H. microstoma*-specific DNA profiles (assigned as SK-CS and SK-EH) were identified among 10 hymenolepidid isolates examined with *pmy* and ITS-1 markers. In the partial *pmy* gene, the shrew isolate (SK-CS) has formed the subgroup with *H. microstoma*, thus differentiating this couple from the close *H. nana* with whom one of two main clades in the NJ tree inferred from *Hymenolepis*-deposited sequences was formed. The percentage nucleotide difference between the SK-CS isolate and the *H. microstoma* sequence obtained from a Portuguese mouse isolate (Macnish, 2001) was 4.86 %, which is a relatively high value, comparable to the interspecific nucleotide difference (4.21 %) between *H. diminuta* and *H. citelli* in the screened *pmy* region. Nevertheless, given that we have found no record of the *H. microstoma* grouping with any other species when *H. nana* was present as the closest relative in phylograms inferred from nuclear and/or mitochondrial data, and the strong genetic cohesiveness between the *H. microstoma* reference and our sample (their subclade received the bootstrap value of 84 %), the classification of the studied isolate as belonging to *H. microstoma* is highly plausible.

After analysis of the nuclear ribosomal ITS region, the hamster isolate (SK-EH) originated from an urban ecosystem in south-east Slovakia clustered with *H. microstoma* specimens from the two continents (Europe, Australia). Interestingly, SK-EH appeared to be the most distinctive sample among *H. microstoma* examined so far in ITS-1, differing in 1.4 – 1.9 % of nucleotide bases from the remaining isolates that markedly exceeded the value of the average intercontinental base difference measured between isolates from southern Europe and Australia (0.14 %). The difference was partially due to the two indel polymorphisms associated with two and five nucleotides, respectively, the latter of which accounted for the absence of arginine in the protein composition. It has been found that especially during starvation, flatworm parasites including *H. diminuta* develop enzymes to synthesize arginine and urea, offering an advantage for osmoregulation and serving as effective nutrients in the fasting state (Campbell, 1963; Mohamed *et al*., 2005). Nevertheless, some studies have suggested that arginine is not necessarily an essential amino acid for a variety of invertebrates including flatworms if ornithine can be synthesized (Campbell & Speeg, 1968; Tielens & van Hellemond, 2005).

With regard to the three most important *Hymenolepis* spp., phylogenetic analysis of nuclear targets herein employed corroborated that representatives of *H. nana* and *H. microstoma* form a strongly supported clade with respect to *H. diminuta*, which may have diverged from the common ancestral line earlier. Similar results with respect to the systematic position of these species were also obtained by other mitochondrial (*cox1*) and nuclear (ITS2, 18S) evidences (e.g. Okamoto *et al*., 1997; Macnish *et al*., 2002; Olson *et al*., 2003; Sharma *et al*., 2016). Besides, studies on immunobiological characteristics and cysticercoid development from eggs suggested that *H. microstoma* is more similar to *H. nana* than to *H. diminuta* (Voge, 1964; Ito *et al*., 1988).

In previous surveys, *H. microstoma* was genetically identified in two consecutive studies by Macnish *et al*. (2002, 2003) in mice (*Mus musculus*) from Portugal, Australia and the United Kingdom, and in humans from Australia. In the Canary Islands (Spain), Foronda *et al*. (2011) detected *H. microstoma* in 10.8 % of the examined house mice (*Mus musculus*) and in 0.3 % of black rats (*Rattus rattus*), but none of 13 brown rats (*Rattus norvegicus*) was positive. In twelve cestodes collected from house mice (*Mus musculus*) in Lima (Peru), morphologically and genetically (*cox1* mt gene) categorized as *H. microstoma*, lower genetic variability between specimens from Peru and Spain compared to Japan indicated that the parasite was introduced into Peru from a European country (Gomez-Puerta *et al*., 2018). A close phylogenetic match to *H. microstoma* was also determined in the tapeworm derived from captive dormouse (*Muscardinus avellanarius*) in the United Kingdom (Peniche *et al*., 2017).

The dwarf tapeworm *H. nana* is the only cestode recognized as not requiring an intermediate host to develop into maturity under natural conditions (Dovč *et al*., 2017). The potential capability of the direct life cycle for *H. microstoma* (as another common feature with *H. nana*) has been discussed in the literature in the past (Skrjabin & Kalantarian, 1942; Macnish *et al*., 2003), and later evidenced in immunocompromised mice by Andreassen *et al*. (2004) who showed that oncospheres penetrated the intestinal tissues and developed into cysticercoids and subsequently into the adult stage in the bile duct and duodenum when infected with parasite eggs. It is therefore presumed that the development of patent infections of *H. microstoma* in humans could more likely occur in immunocompromised individuals. Malnourishment, type II diabetes, alcoholism, cancer, HIV-infection, immunosuppressive therapy, etc. (e.g. Kontogiorgi *et al*., 2013; Kumari *et al*., 2014) are factors contributing to depression of the immune system and may enhance the development of infections with parasites such as *H. microstoma* in humans.

To date, the majority of published surveys in free-living and domestic small mammals reported the occurrence of hymenolepidid tapeworms based on coprological examination (Jarošová *et al*., 2020). Due to striking morphological similarity of the eggs of *H. microstoma* and *H. nana* that are difficult to distinguish by morphology alone (Fitzgerald *et al*., 1970; Macnish, 2001), it is possible that some infections of *H. microstoma* in animals and humans

may have been previously misdiagnosed as *H. nana* (Whary *et al*., 2015; Riebold *et al*., 2019). In addition, infection in humans may have been underdiagnosed due to sporadic or reduced egg shedding that was documented in *H. nana*, *H. diminuta* and *H. citelli* in response to factors such as the quality and quantity of the host diet (Kennedy, 1983; Schantz, 1996) and can occur also in *H. microstoma*.

A recently indicated zoonotic potential of *H. microstoma* (Macnish *et al*., 2003) along with a possibility of its direct transmission between animals and/or humans without the need for intermediate hosts pose a public health concern in contaminated areas of Slovakia. In the present study, the use of molecular techniques has enabled identification of the mouse bile duct tapeworm circulating in the two animal populations of Slovakia and will greatly facilitate a more thorough understanding of the epidemiological situation of *H. microstoma* and related tapeworms in various ecosystems of the country in the future.

Conflict of Interest

Authors state no conflict of interest

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The effect of soil type and ecosystems on the soil nematode and microbial communities

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Article info

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Summary

Integrated studies are required to better understand the relationships between groups of soil microfauna under the influence of various biotic and abiotic factors that drive and characterise ecosystems. We analysed soil nematode communities and microbial diversity and the properties of three soil types to assess the effect of these environmental variables on biological diversity in natural (forest), semi-natural (meadow), and managed (agriculture) habitats of the Slovak Republic. The type of ecosystem and soil and the interaction of both factors had considerable effects on most monitored abiotic and biotic soil properties. The forest with a Chernozem soil had the most nematode species, highest nematode diversity, highest abundance of nematode within functional guilds, best values of ecological and functional indices, highest microbial biomass, highest microbial richness and diversity, and the highest values of various soil properties, followed by meadows with a Cambisol soil. The agricultural ecosystem with a Stagnosol soil had the lowest biological diversity and values of the soil properties. Several nematode species were new for Slovak nematode fauna. Sampling date and the interaction of all three factors (ecosystem × soil × date) had minor or no effect on most of the parameters, except soil moisture content, microbial richness, nematode channel ratio, nematode maturity index, and plant parasitic index. Both the biological indicators and basic soil properties indicated that the natural forest with a Chernozem soil was the best habitat from an ecological point of view. This ecosystem is thus the most appropriate for ecological studies.

Keywords: soil trophic web; indicators; soil properties; nematodes; microbes; multivariate analysis

Introduction

Soil is an extremely heterogeneous environment in all aspects: biological, physical, chemical, and structural. Biological diversity is substantially higher in soil than above it, numbers are much larger for populations of soil organisms than aboveground communities (Young & Ritz, 1998). Microbes (fungi, bacteria, and algae), microfauna (protozoa), and mesofauna (arthropods and nematodes) belong to the most diverse soil organisms (Neher, 2001), affected mainly by vegetation and edaphic factors (Nielsen *et al.,* 2014). Nematodes inhabit nearly every environment and as biotic indicators are one of the most studied groups of soil organisms (Bhusal *et al.,* 2014). Since nematodes have diverse feeding behaviour and life strategies and play a key role in soil food web, they function as important indicator for ecosystems processes (Ferris 2010). As nematodes show different degrees of sensitivity to the environmental stimuli, alterations or disturbances because they have different long life cycles and reproduction capacity (Bongers, 1990),

* – corresponding author

species diversity and structure of community what is important indicator of soil health and conditions (Neher, 2001). In addition, nematode indices allow the evaluation of ecosystem nutrient status (enriched vs. depleted), structure of soil food web (complexity vs. simplicity) and the prevailing decomposition of organic matter (slower fungal vs. faster bacterial) (Ferris *et al.*, 2001).

In contrast to nematode community's structure, however, potential microbial community structure for use as indicators of soil quality and functioning are hampered by a lack of standardised assays of microbial ecological diversity (Schutter *et al.,* 2001). Culturing techniques have been used to identify the number of specific taxonomic or functional groups, but only a small fraction of a microbial community (1 – 10 %) can be identified with these methods (Olembo & Hawksworth, 1991; Nannipieri *et al.,* 2003). Analyses of microbial DNA (Martin-Laurent *et al.,* 2001, Zhang *et al.*, 2014) can identify taxonomic groups at different levels, but developing specific primers, for example, is problematic. PLFA analyses cannot identify organisms to the species level but can be used to estimate gross changes in community structure (Kaur *et al.,* 2005). BIOLOG® EcoPlates are now commonly used for measuring microbial functional diversity based on the use of sources of available carbon (C) (Garland & Mills, 1991), and the method proposed by Degens and Harris (1997) for measuring the catabolic potential of microbial communities is often used in measurements of the impacts of soil management due to the easy use of both methods. Nevertheless, several microbial and biochemical attributes such as respiration, N mineralisation, or enzymatic activities can be reliably measured and are also frequently used as indicators of soil quality (Blagodatskii *et al.,* 2008; Gömöryová *et al.,* 2013, Bobuľská *et al.,* 2015, Zhang *et al.,* 2014).

Worldwide, several particular studies have revealed that environmental conditions determines the degree of species diversity of soil nematodes or nematode abundance e.g. ecosystem type and its properties (Neher *et al.,* 2005; Nielsen *et al.,* 2014), soil type and its properties (Lišková *et al.,* 2008, Hu *et al.,* 2018; Lima da Silva *et al.,* 2019); vegetation and its species diversity (Cesarz *et al.,* 2013; Renčo & Baležentienė, 2015). Similar, microbial activity and biomass have been evaluated in arable soils due to crop production as affected by tillage (Mangalassery *et al,* 2015); fertilizers (Kautz *et al.,* 2004; Zakarauskaitė, *et al.,* 2008) or management system (Bloem *et al.,* 1992); in forest soil as affected by forest type (Fang *et al.,* 2016) or in grasslands affected by plant diversity (Lange *et al.,* 2015). The structure of soil nematode communities and microbial diversity, however, have not been investigated or compared amongst various land use (ecosystems) and main soil types in the territory of Slovak Republic in collaborative study. We studied the soil properties and nematode-microbial assemblages in three soil types and three ecosystems to evaluate 1) nematode and microbial diversity in ecosystems with different soil types, 2) the fundamental variability in soil properties amongst and within the ecosystems and soil types, and 3) the effects of soil properties and sampling date on the nematodes and microbes in the ecosystems and soil types. We hypothesised that biological diversity would be lower in agroecosystems, that soil trophic webs would be more coherent in natural habitats, but that the differences between ecosystems would vary with the physicochemical properties of the soil type.

Materials and Methods

Site selection

We examined the physical and chemical properties, nematode communities, and microbial attributes in soil samples collected from a Stagnosol (SS), a Cambisol (CS), and a Chernozem (CM) in each of a forest (FOR), a meadow (MEA), and an agricultural field (AGR) ecosystems. The soil types, ecosystems, locations, and vegetation characteristics are shown in Table 1.

Soil samples and properties

Soil samples from each soil type and ecosystem were collected from five randomly established 1 × 1 m quadrats in selected plots of 20 × 20 m in May (M), July (J), and September (S) 2016. Five randomised subsamples were collected from the quadrats, one from each corner and one from the centre of the plots, for analysing the soil nematode communities, microbial activities, and physicochemical properties. The subsamples were bulked to produce a representative sample for the plot (1 kg). Samples were collected from a depth of 10 cm, excluding the surface humus layer. A total of 135 representative samples were collected; 5 from each ecosystem (FOR, MEA, and AGR, 5x3=15), from three soil type (SS, CS, and CM; 15x3=45), in three sampling date (M, J, and S; 45x3=135). The samples were transferred to the laboratory in sealed plastic bags and stored at 5 °C until processing for the nematode analysis or at -20 °C for the microbial analysis.

Total soil C and nitrogen (N) contents, soil moisture (SM) contents, and pH were measured in all samples. The organic C and total N contents were determined using a Vario MACRO Elemental Analyzer (CNS Version; Elementar, Hanau, Germany). Organic C content was determined based on the difference between total C and C bound in carbonates. SM content was estimated gravimetrically by oven-drying fresh soil at 105 °C overnight, and pH was measured potentiometrically in 1M KCl suspension by a digital pH meter separately for each representative sample.

Analysis of nematode communities

Each sample was homogenised by gentle hand mixing, and stones were manually removed. The nematodes were extracted from 100 g of fresh soil by a combination of Cobb sieving and decanting (Cobb 1918) and a modified Baermann technique (van Benzoijen, 2006). One hundred grams of soil from each representative sample were soaked in l L of tap water for 60 min to disrupt soil aggregates and promote nematode movement. The soaked sample was carefully passed through a 1-mm sieve (16 mesh) to remove plant parts and debris, and this suspension was passed

Table 1. Soil type, location, ecosystem type, and vegetation characteristics of the study plots.

Soil type	Location/characteristics	Ecosystem	Vegetation
Stagnosol	Hanušovce nad Topľou Altitude 258 – 308 m a.s.l., slope 3 – 7° Soil with strong mottling of the soil profile due to redox processes caused by stagnating surface water, The topsoil can also be completely bleached (albic horizon). A common name in many national classification systems for most Stagnosols is pseudogley.	Forest 49°00.339'N, 21°31.248'E	*Carpinus betulus* (90 %), *Pinus sylvestris* (5 %), sporadically *Prunus avium*, *Fagus sylvatica*, and *Betula pendula.* Understory vegetation dominated by grasses *Carex pilosa*, *Festuca drymej*a, and *Poa memoralis* and herbs *Dentaria bulbifera* and *Fragaria vesca*
		Meadow 49°00.658'N, 21°30.058'E	*Carex* sp., *Lolium perenne, Fragaria vesca, Trifolium pratense, Plantago* sp. *Leucanthemum* sp.
		Agricultural field 49°00.727'N, 21°30.344'E	*Zea mays* monoculture
Cambisol	Tŕnie Altitude 550 – 554 m a.s.l., slope 3 – 7° Soil with a beginning of soil formation. The horizon differentiation is weak. This is evident from weak, mostly brownish discolouration and/ or structure formation in the soil profile. Cambisols are developed in medium and fine-textured materials derived from a wide range of rocks, mostly in alluvial, colluvial and aeolian deposits.	Forest 48°36.712'N, 19°01.462'E	*Carpinus betulus* (75 %), *Quercus robur* (10 %), *Tilia cordata* (10 %), and sporadically *Prunus avium* Understory herbaceous vegetation dominated by *Viola reichenbachiana*, *Geranium robertianum*, *Asarum europaeum*, *Luzula sylvatica*, *Galium odoratum*, and *Hedera helix.*
		Meadow 48°36.683'N, 19°01.494'E	*Trifolium pratense*, *Agrimonia eupatoria*, and grasses such as *Carex* sp., *Poa* sp., *Dactylis glomerata, Trifolium pratense, Rumex acetosa*
		Agricultural field N 48°36.660'N, E 19°01.503'E	*Zea mays* monoculture
Chernozem	Močenok Altitude 135 – 180 m a.s.l., slope 0 – 3° Black-colored soil containing a high percentage of humus (4 % to 16 %) and high percentages of phosphoric cids, phosphorus, and ammonia. Chernozem is very fertile and can produce high agricultural yields with its high moisture storage capacity. Chernozems are also a reference soil group of the World reference base for soil resources	Forest 48°12.960'N, 17°57.854'E	*Fraxinus excelsior* (80 %), *Quercus petraea* (20 %), and sporadically *Robinia pseudoacacia.* Understory vegetation dominated by grasses *Poa nemoralis*, *Brachypodium sylvaticum*, *Melica uniflora*, and *Dactylis polygama*
		Meadow 49°00.339'N, 21°30.344'E	*Carex* sp*., Phleum pratense, Arrhenatherum elatius, Trifolium pratense, Vicia* sp., *Rumex acetosa, Achillea millefolium*
		Agricultural field 49°00.339'N, 21°30.344'E	*Zea mays* monoculture

through a 50-µm sieve (300 mesh) 2 min later to remove water and very fine soil particles. The nematodes were then extracted from the soil/water suspension by a set of two cotton-propylene filters in the Baermann funnels. Two filter trays were used per sample to limit material thickness to <0.5 cm. Suspensions containing the nematodes were collected after extraction for 24 h at room temperature. The nematodes were killed and fixed in a hot 99:1 solution of 4 % formaldehyde and pure glycerol (Seinhorst, 1962). The all nematodes were microscopically (100, 200, 400, 600, and 1000× magnification) identified to the species level (juveniles to the genus level) from temporary slides using an Eclipse 90*i* light microscope (Nikon Instruments Europe BV, Netherlands). Nematode abundance was expressed as the number of individuals per 100 g of dry soil.

The nematodes were assigned to fifteen functional guilds integrating nematode feeding strategies (trophic groups) and the nematode coloniser-persister (c-p) scale (Bongers & Bongers, 1998). The five nematode trophic groups were: bacterivores (Ba), fungivores (Fu), carnivores (Ca), omnivores (Om), and plant parasites (Pp) (Wasilewska, 1997). The Pp group included both obligatory plant parasites and facultative plant parasites that may attack plants or fungi. Colonisers-persisters characterising nematode life strategies are classified on a scale of 1 to 5 (Bongers, 1990). C-p1 represents "r-strategists" (colonisers) with short life cycles, small eggs, high fecundity, high colonisation ability, and high tolerance to disturbance, eutrophication, and anoxybiosis. Colonisers generally live in ephemeral habitats. At the other end of the scale, c-p5 nematodes represent "k-strategists" (persisters) with the longest generation times, largest bodies, lowest fecundities, and the highest sensitivity to disturbance. Persisters are never dominant in a sample and generally live in stable habitats where they become very abundant (Bongers, 1990). C-p scaling allows the calculation of the basal maturity index (MI) for non-parasitic nematodes, the plant parasitic index (PPI) for plant parasites only (Bongers, 1990), and the summ maturity index (ΣMI) (Yeates, 1994) for all nematode taxa. Functional guilds allow the calculation of the enrichment index (EI), the structure index (SI), and the channel index (CI) proposed by Ferris *et al.,* (2001). The species-diversity index (H´) defined by Shannon and Weaver (1949), the nematode channel ratio (NCR) defined by Yeates (2003), and trophic diversity (TD) defined by Heip *et al.,* (1998) were also calculated.

Nematode species were characterised as dominant at D >5 % (the species represents more than 5 % of the total nematode abundance in the ecosystem or soil type) and subdominant at D >2 % (the species represents more than 2 % of the total nematode abundance in the ecosystem or soil type) (Losos *et al.,* 1984).

Microbial biomass

Microbial biomass C (Cmic) content was determined following the procedure described by Islam and Weil (1998). Ten grams of oven-dried equivalent (ODE) of field moist soil adjusted to 80 % water-filled porosity was irradiated twice by microwaves (MW) at 400 J g^{-1} ODE soil to kill the microorganisms. The cooled samples were extracted with 0.5 M K_2SO_4, and the C content of the extract was quantified by oxidation with $K_2Cr_2O_7/H_2SO_4$. The same procedure was performed with a non-irradiated sample. Cmic content was determined as (Cirradiated content - Cnon-irradiated content)/KME, where KME represents the extraction efficiency (0.213) recommended by Islam and Weil (1998).

Functional diversity of microbial communities

The functional diversity of the soil microbiota was determined using the methods described by Insam (1997). Each well in a BIOLOG EcoPlate received 150 µl of an extract prepared by resuspending of fresh soil in 0.85 % NaCl and diluted 1:10000. The plates with the extracts were then incubated at 27 °C for 6 d, and absorbance at 590 nm was recorded every 24 h using a Sunrise Microplate reader (Tecan, Salzburg, Austria). The data were corrected against the initial readings at time zero and were expressed as optical densities of individual wells. The richness of the soil microbial community (Richn) was determined as the number of substrates used by the microbial community, i.e. the number of wells with a positive response after background correction. Hill's diversity index (Diver) (Hill, 1973) based on Eq. 1 was calculated for estimating the diversities of the microbial functional groups:

$$\text{Diver} = 1/\sum p_i^2 \quad (1)$$

in which p_i is the ratio of the activity on a substrate to the sum of activities on all substrates.

Data analysis

Data were log-transformed before analysis to improve normality. Soil and ecosystem types were included as fixed factors. The effects of soil type (SS, CS, and CM), type of ecosystem (FOR, MEA, and AGR), and sampling date (M, J, and S) on nematode trophic-web descriptors and functional guilds, soil properties, and microbial biomass, diversity, and richness were analysed by factorial analyses of variance (ANOVAs). Nonparametric Spearman's correlation coefficient (rs) was calculated to test the relationships between nematode functional guilds, microbial parameters, and soil parameters for each sample using STATISTICA v9.0. Correlations obtained at $P<0.05$ were considered significant.

We then used multivariate analyses to evaluate the effects of soil and ecosystem types on nematode-community composition and the microbial characteristics. The composition of the nematode functional guilds and the microbial parameters were thus used as response variables, and the soil and ecosystem types were used as explanatory variables in a multivariate framework of a redundancy analysis (RDA). The soil physicochemical parameters were used as supplementary variables. Canoco 5 for Windows was used for the multivariate analyses (vers. 5.04; Ter Braak & Šmilauer, 2012).

Ethical Approval and/or Informed Consent

This article does not contain any studies with human participants or animals by any of the authors, so formal consent is not required. Authors have no potential conflict of interest pertaining to this submission to Helminthologia.

Results

Soil properties

The factorial ANOVA found that ecosystem type (FOR, MEA, and AGR) and soil type (SS, CS, and CM) significantly affected all soil properties (except SM content vs. soil type) and that sampling date (M, J, and S) affected only SM content (P<0.01, Table 2). The bi-factorial interaction ecosystem × soil significantly affected all soil properties, ecosystem × date affected half of the properties, and soil × date and the interaction of all three factors (ecosystem × soil × date) had minor or no effects on the soil properties. The values of the soil properties were generally higher in the FOR soils (except pH) than the MEA and AGR soils and higher in CM (including pH) than CS and SS. pH and the C/N ratio were correlated negatively in FOR but positively in AGR and MEA (Fig. 1).

Nematode and microbial trophic webs

The three ecosystems and soil types contained 133 nematode species (32 bacterivores, 26 fungivores, 9 carnivores, 24 om-

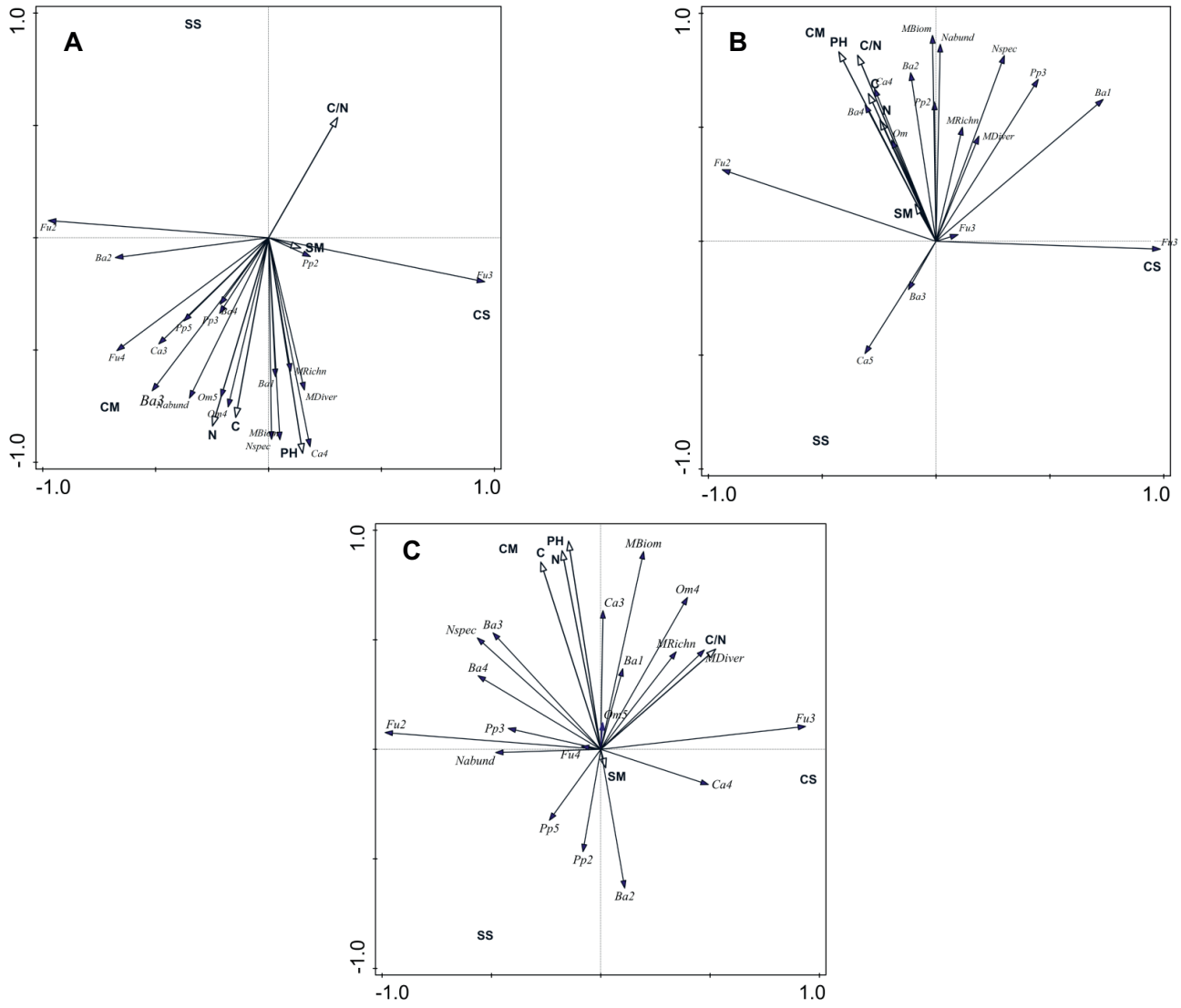

Fig. 1. RDA triplots of the relationships of abundance of nematode functional guilds, microbial parameters, and soil properties in the forest (A), agricultural field (B), and meadow (C) ecosystems and the Stagnosol (SS), Cambisol (CC), and Chernozem (CM) soil types.

Table 2. Effects of ecosystem type (E), type of soil (S), sampling date (D), and their interactions on basic soil physical and chemical characteristics.

	Ecosystem		Soil		Date		E × S		E × D		S × D		Ecosystem			Soil			Date			E × S × D	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	Meadow	Agricultural field	Forest	Stagnosol	Cambisol	Chernozem	May	July	Sept	*F*	*P*
pH/H_2O	28.91	< 0.01	598.43	< 0.01	2.95	ns	80.12	< 0.01	2.63	< 0.05	2.05	ns	5.88±0.78	5.79±0.75	5.54±1.08	4.82±0.61	5.71±0.41	6.68±0.32	5.68±0.88	5.71±0.87	5.81±0.92	1.08	ns
SM	60.14	< 0.01	0.99	ns	131.43	< 0.01	2.72	< 0.05	5.39	< 0.01	33.88	ns	26.33±7.28	22.14±5.14	30.78±8.64	26.15±8.96	26.93±8.85	26.15±5.75	26.68±8.04	20.52±5.47	32.10±5.3	2.53	< 0.05
N	156.52	< 0.01	122.56	< 0.01	0.15	ns	11.22	< 0.01	0.29	ns	2.60	ns	0.377±0.10	0.205±0.07	0.395±0.13	0.244±0.06	0.300±0.09	0.434±0.15	0.326±0.12	0.321±0.12	0.331±0.16	1.36	ns
C	275.46	< 0.01	140.58	< 0.01	0.08	ns	6.33	< 0.01	0.41	ns	1.89	ns	3.588±1.05	1.955±0.85	4.395±1.28	2.429±0.84	3.127±1.321	4.382±1.48	3.298±1.38	3.271±1.40	3.369±1.64	1.87	ns
S	32.62	< 0.01	51.84	< 0.01	2.11	ns	10.39	< 0.01	4.79	< 0.01	3.75	ns	0.071±0.02	0.048±0.01	0.070±0.02	0.049±0.02	0.060±0.01	0.082±0.02	0.067±0.03	0.060±0.18	0.063±0.02	2.71	< 0.01
C/N	171.64	< 0.01	7.51	< 0.01	1.81	ns	49.09	< 0.01	1.86	ns	1.21	ns	9.49±0.54	9.35±0.98	11.28±1.06	9.86±1.70	10.08±1.21	10.18±0.56	9.95±1.32	10.04±1.24	10.14±1.16	1.80	ns

E: meadow, agriculture and forest; S: Stagnosol, Cambisol and Chernozem; D: May, July, and September

pH/H_2O, acidity; SM, soil moisture content; N, total nitrogen content; C, organic carbon content; S, total sulphur content; C/N, carbon to nitrogen ratio. The means ±SEs of nematode abundance for the three ecosystems, soil types, and sampling dates are shown.

Table 3. Effects of ecosystem type (E), type of soil (S), sampling date (D), and their interactions on nematode abundance, functional guilds and microbial communities.

	Ecosystem		Soil		Date		E × S		E × D		S × D		Ecosystem			Soil			Date			E × S × D	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	F	*P*	*F*	*P*	Meadow	Agricultural field	Forest	Stagnosol	Cambisol	Chernozem	May	July	Sept	*F*	*P*
Nabund	2.36	ns	92.49	< 0.01	15.04	< 0.01	37.97	< 0.01	5.57	< 0.01	1.63	ns	479.8±119.8	540.1±322.5	451.8±169.5	351.6±150.1	435.8±104.9	671.3±260.3	549.2±276.3	418.1±165.9	504.1±201.8	1.59	ns
Nspec	5.69	< 0.01	4.87	< 0.01	0.88	ns	12.45	< 0.01	1.98	ns	2.98	ns	33.2±5.7	25.2±6.2	37.1±4.1	24.1±5.8	32.5±6.3	38.5±7.5	33.0±7.7	29.0±11	31.7±8.4	1.12	ns
Ba1	6.19	< 0.01	85.42	< 0.01	1.24	ns	21.63	< 0.01	1.78	ns	1.76	ns	10.9±11.1	38.6±43.9	22.18±21.8	4.2±5.8	41.9±41.6	25.6±20.7	22.6±30.4	21.1±25.6	28.1±36.3	0.97	ns
Ba2	5.49	< 0.01	12.36	< 0.01	11.48	< 0.01	35.72	< 0.01	6.11	< 0.01	2.44	ns	159.4±80.3	181.8±118.6	190.8±84.5	145.8±87.3	150.3±52.0	214.6±122.0	207.8±117.8	147.2±75.4	165.8±81.2	1.70	ns
Ba3	39.96	< 0.01	117.06	< 0.01	2.9	ns	29.93	< 0.01	1.24	ns	3.98	ns	2.3±5.0	0.0±0.0	3.1±5.5	0.0±0.0	0.2±0.7	5.11±6.5	1.0±2.6	2.9±6.4	1.4±3.3	2.12	ns
Ba4	5.82	< 0.01	28.8	< 0.01	0.07	ns	1.9	ns	0.69	ns	0.77	ns	3.7±7.2	1.5±3.6	2.9±4.0	0.9±1.6	0.6±1.3	6.6±7.5	2.9±6.3	2.5±4.8	2.7±4.7	0.83	ns
Ca3	15.89	< 0.01	24.98	< 0.01	0.67	ns	7.47	< 0.01	0.47	ns	0.27	ns	1.0±1.7	0.0±0.0	0.6±1.2	0.0 ±0.0	0.3±0.9	1.3±1.8	0.4±1.2	0.6±1.4	0.5±1.3	0.22	ns
Ca4	45.67	< 0.01	63.63	< 0.01	0.08	ns	31.3	< 0.01	0.23	ns	0.14	ns	3.9±3.9	1.8±3.5	10.4±9.7	1.0±2.2	7.0±7.6	8.1±8.4	5.1±6.8	5.8±8.3	5.2±6.9	0.80	ns
Ca5	11.63	< 0.01	11.63	< 0.01	1.22	ns	11.63	< 0.01	1.22	ns	1.22	ns	0.0±0.0	0.6±1.6	0.0±0.0	0.6±1.6	0.0±0.0	0.0±0.0	0.2±0.9	0.3±1.3	0.1±0.4	1.22	ns
Fu2	7.03	< 0.01	50.64	< 0.01	3.42	< 0.05	30.43	< 0.01	6.69	< 0.01	1.42	ns	97.8±34.7	155.6±156.7	87.5±52.6	68.1±38.5	90.1±40.4	182.7±143.5	122.4±127.5	92.2±126.2	126.2±104.5	1.42	ns
Fu3	4.06	< 0.05	4.06	< 0.05	0.79	ns	7.88	< 0.01	0.57	ns	0.57	ns	1.4±4.5	0.0±0.0	0.3±1.2	0.3±1.2	0.0±0.0	1.4±4.5	0.5±0.3	0.5±1.2	0.8±2.7	0.68	ns
Fu4	29.26	< 0.01	18.65	< 0.01	0.18	ns	14.74	< 0.01	1.20	ns	0.90	ns	3.7±4.9	0.0±0.0	5.3±9.3	1.9±4.4	0.9±2.2	6.2±9.2	3.6±8.3	2.8±5.4	2.5±5.3	0.89	ns
Om4	4.24	< 0.05	43.16	< 0.01	2.41	ns	10.80	< 0.01	0.62	ns	1.13	ns	31.2±29.8	21.7±19.0	41.0±37.0	16.0±16.8	35.8±22.2	52.1±35.1	41.5±37.2	33.2±25.2	29.3±24.0	0.55	ns
Om5	5.32	< 0.05	22.74	< 0.01	0.94	ns	4.84	< 0.01	0.65	ns	2.36	ns	19.8±7.4	8.7±12.1	15.4±13.2	8.2±9.1	8.6±5.3	20.1±13.7	12.8±10.3	12.5±14.2	11.7±9.1	1.87	ns
Pp2	19.66	< 0.01	1.02	ns	6.47	< 0.01	9.28	< 0.01	1.03	ns	2.55	ns	62.4±42.9	54.8±47.0	25.3±20.1	46.7±46.9	43.3±29.1	52.4±46.3	49.4±33.9	33.0±27.5	59.9±54.4	2.55	ns
Pp3	27.71	< 0.01	24.21	< 0.01	0.11	ns	14.19	< 0.01	0.75	ns	5.04	< 0.01	74.9±43.3	57.8±39.2	28.1±24.5	36.6±36.2	49.2±30.5	74.7±46.3	58.4±46.8	49.1±34.6	52.9±41.5	2.38	ns
Pp5	3.46	< 0.05	3.78	< 0.05	0.59	ns	7.78	< 0.01	0.37	ns	0.37	ns	0.27±0.95	0.0±0.0	0.5±1.3	0.3±0.9	0.0±0.0	0.5±1.3	0.4±1.2	0.2±0.8	0.2±0.8	0.48	ns
Cmic	175.55	< 0.01	295.91	< 0.01	2.84	ns	5.02	< 0.01	0.69	ns	2.64	< 0.05	335.2±118.3	207.9±91.5	400.1±145.5	183.0±65.9	325.9±119.4	434.4±109.2	316.0±129.1	300.4±143.4	326.8±157.8	19.0	ns
Richn	3.60	< 0.05	50.90	< 0.01	11.28	< 0.01	1.88	ns	0.68	ns	14.01	< 0.01	26.2±2.39	25.5±1.86	26.5±2.93	24.2±2.3	26.8±1.96	27.2±1.9	25.8±1.8	26.9±1.9	25.5±3.2	3.19	< 0.01
Diver	3.15	< 0.05	53.52	< 0.01	0.69	ns	1.96	ns	2.24	ns	4.69	< 0.01	14.51±2.6	13.85±2.4	14.9±3.0	12.1±2.9	15.6±2.3	15.6±2.4	14.2±2.1	14.7±2.7	14.5±3.2	34.2	< 0.01

E: meadow, agriculture and forest; S: Stagnosol, Cambisol and Chernozem; D: May, July, and September

$Ba_{1,2,3,4}$, bacterivores; $Fu_{2,3,4}$, fungivores; $Ca_{3,4,5}$, carnivores; $Om_{4,5}$, omnivores; $Pp_{2,3,5}$, plant parasites; Nabund, mean nematode abundance/100 g dry soil, Nspec, mean nematode species number/100 g dry soil; Cmic, microbial biomass carbon content; Richn, richness of microbial functional groups; Diver, diversity of microbial functional groups. The means ±SEs of nematode abundance for the three ecosystems, soil types, and sampling dates are shown.

Table 4. Effects of ecosystem type (E), type of soil (S), sampling date (D), and their interactions on nematode trophic-web descriptors.

	Ecosystem		Soil		Date		E × S		E × D		S × D		Ecosystem			Soil			Date			E × S × D	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	Meadow	Agricultural field	Forest	Stagnosol	Cambisol	Chernozem	May	July	Sept	*F*	*P*
MI	18.86	< 0.01	21.28	< 0.01	5.56	< 0.01	44.11	< 0.01	4.22	< 0.01	2.04	< 0.05	2.32±0.19	2.21±0.23	2.37±0.23	2.25±0.20	2.24±0.23	2.41±0.23	2.33±0.25	2.32±0.24	2.24±0.19	1.83	< 0.05
ΣMI	11.34	< 0.01	25.74	< 0.01	3.89	< 0.05	28.66	< 0.01	2.27	ns	0.95	ns	2.38±0.17	2.28±0.16	2.39±0.21	2.28±0.15	2.30±0.15	2.45±0.20	2.35±0.22	2.38±0.18	2.31±0.16	1.46	ns
PPI	0.70	ns	16.05	< 0.01	7.03	< 0.01	8.30	< 0.01	2.24	ns	6.25	< 0.01	2.5±0.20	2.53±0.22	2.51±0.26	2.43±0.24	2.53±0.20	2.62±0.20	2.59±0.23	2.60±0.21	2.38±0.22	6.41	< 0.01
H´	125.08	< 0.01	87.76	< 0.01	0.73	ns	22.99	< 0.01	0.99	ns	2.50	< 0.05	2.91±0.22	2.60±0.15	3.02±0.29	2.64±0.18	2.88±0.25	3.01±0.30	2.82±0.26	2.85±0.28	2.86±0.33	2.67	< 0.05
EI	4.52	< 0.05	9.05	< 0.01	1.46	ns	14.80	< 0.01	0.76	ns	2.32	ns	41.3±9.9	46.7±12.4	46.2±11.3	38.9±5.6	47.8±14.8	47.1±10.5	42.3±9.4	46.3±13.2	45.3±11.8	4.70	< 0.01
SI	4.53	< 0.05	61.88	< 0.01	1.78	ns	25.55	< 0.01	6.20	< 0.01	3.60	< 0.01	44.3±18.4	41.0±13.3	49.2±18.4	30.7±12.1	50.5±10.8	53.2±18.2	44.5±19.4	47.1±16.5	42.9±15.7	2.06	< 0.05
CI	53.82	< 0.01	61.48	< 0.01	0.11	ns	182.05	< 0.01	13.74	< 0.01	3.14	< 0.05	68.5±16.8	57.9±30.1	52.7±16.3	64.4±18.9	52.7±28.8	61.9±18.4	60.1±23.8	61.6±24.9	57.5±20.3	15.57	< 0.01
NCR	7.77	< 0.01	34.25	< 0.01	5.17	< 0.01	42.19	< 0.01	6.01	< 0.01	1.88	ns	0.65±0.12	0.63±0.16	0.69±0.13	0.69±0.14	0.70±0.12	0.57±0.12	0.69±0.12	0.65±0.13	0.64±0.16	4.02	< 0.01
TD	19.17	< 0.01	15.09	< 0.01	0.009	ns	16.81	< 0.01	1.75	ns	1.15	ns	12.2±2.4	10.5±4.2	14.7±2.1	10.5±3.2	13.2±1.9	13.7±2.4	12.3±0.8	12.5±0.7	12.9±0.7	1.34	ns

E: meadow, agriculture and forest; S: Stagnosol, Cambisol and Chernozem; D: May, July, and September

MI, maturity index; ΣMI, sigma maturity index; PPI, plant parasitic index; H´, Shannon diversity index; EI, enrichment index; SI, structure index; CI, channel index; NCR, nematode channel ratio; TD, trophic diversity. The means ±SEs of the nematode-community descriptors for the three ecosystems, soil types, and sampling dates are shown

Table 5. Spearman´s rank correlation coefficients between nematode abundance, species number, functional guild, microbial parameters and soil properties.

	pH/H_2O	SM	N	C	S	C/N
Nabund	0.35**	0.68***	0.42***	0.29**	ns	ns
Nspec	0.22*	ns	0.31*	0.37***	ns	ns
Ba_1	0.44**	ns	0.25***	0.22*	0.31***	ns
Ba_2	ns	-0.23***	ns	ns	ns	ns
Ba_3	0.54***	0.26*	0.44***	0.41***	0.42***	0.21*
Ba_4	0.43***	ns	0.30***	0.29***	0.30***	ns
Ca_3	0.44***	0.46***	0.39***	0.40***	0.39***	0.33***
Ca_4	0.41***	ns	0.21*	0.22*	0.22**	0.21**
Ca_5	ns	0.41***	0.35*	ns	0.36*	0.26*
Fu_2	0.55***	ns	0.35***	0.29**	0.37***	ns
Fu_3	ns	ns	ns	ns	ns	ns
Fu_4	0.39***	0.37***	0.29***	0.26**	0.27**	ns
Om_4	0.42***	ns	ns	ns	0.33**	ns
Om_5	0.38***	ns	0.21*	ns	0.23**	ns
Pp_2	ns	ns	ns	ns	ns	-0.27***
Pp_3	0.47***	0.24***	ns	ns	ns	ns
Pp_5	0.39***	0.37***	0.28***	0.28***	0.29**	0.38***
Cmic	0.70***	0.39***	0.90***	0.91***	0.83***	0.51***
Richn	0.46***	ns	0.47***	0.45***	0.53***	ns
Diver	0.47***	ns	0.40***	0.38***	0.50***	ns

*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ns, not significant

$Ba_{1,2,3,4}$, bacteriovores; $Fu_{2,3,4}$, fungivores; $Ca_{3,4,5}$, carnivores; $Om_{4,5}$, omnivores; $Pp_{2,3,5}$, plant parasites; Cmic, microbial biomass carbon content; Richn, richness of microbial functional groups; Diver, diversity of microbial functional groups; pH/H_2O, acidity; SM, soil moisture content; N, total nitrogen content; C, organic carbon content; S, total sulphur content; C/N, carbon to nitrogen ratio

nivores, and 42 plant parasites) (Table S1). *Heterocephalobus eurystoma*, *Stegetellina leopolitensis*, *Ditylenchus parvus*, *Ditylenchus tenuides*, *Paraphelenchus obscurus*, *Boleodorus volutus*, *Cephalenchus intermedius*, and *Ecphyadophora tenusissima* were new to the list of Slovak nematode fauna, increasing the total number of soil nematode species to 732. The number of species (99) and diversity were highest in the FOR soils, followed by the MEA (90) and AGR (53) soils. Nematode species number and diversity were higher in CM than CS and SS (102, 81, and 60, respectively) (Tables S1, 4). The most abundant nematode species by trophic group were *Acrobeloides nanus* and *Chiloplacus propinquus* (bacterivores), *Aphelenchus avenae* and *Filenchus vulgaris* (fungivores), *Clarkus papillatus* and *Mylonchulus brachyuris* (carnivores), *Eudorylaimus carteri* (omnivores), and *Aglenchus agricola*, *Boleodorus thylactus*, and *Bitylenchus dubius* (facultative and obligate plant parasites) (Table S1).

Soil type and sampling date had significant effects on overall nematode abundance ($P<0.01$), but ecosystem type did not (Table 3). Ecosystem and soil types significantly influenced the abundances of all nematode functional guilds (except Om_5 and Pp_2, respectively), but the nematode-community compositions were similar.

The mean abundance of Ba_2 nematodes was significantly higher in FOR than MEA and AGR ($P<0.01$) and in CM than SS and CS ($P<0.01$). The amount of microbial biomass and microbial richness and diversity had tendencies similar to those of the Ba_2 nematodes; all were higher in FOR and CM (Table 3). Ba_4, Fu_3, and $Pp_{2,3}$ nematodes were most abundant in MEA, Ba_1 and Fu_2 were most abundant in AGR, and Ba_3, $Ca_{3,4}$, Fu_4, $Om_{4,5}$, and Pp_5 were most abundant in FOR. The majority of the nematode functional guilds were more abundant in CM than SS and CS. Only Ba_1 was significantly more abundant in CS ($P<0.01$). Sampling date only significantly affected the abundance of c-p2 nematode (Ba, Fu, and Pp) trophic groups, with higher values in M and S than J. Microbial richness was also affected by sampling date and was highest in J ($P<0.01$).

Nematode abundance, species number, and functional guilds and the microbial parameters were positively correlated with all soil properties. Only the Ba_2 nematode parameters were negatively correlated with SM content, and the Pp_2 nematode parameters were negatively correlated with the C/N ratio (Table 5). The RDA analysis, however, indicated that the abundance of most of the nematode guilds, total nematode abundance, nematode species

number, and the microbial parameters tended to be higher in environments with higher pHs and that N and C contents tended to be higher in FOR and CM soil (Figure 1A), except for Fu_2 and Fu_3 nematodes. The presence and distribution of nematodes within functional guilds, number of species, nematode abundance, and the microbial parameters in MEA were more affected by soil type than soil properties.

Nematode trophic-web descriptors

The ANOVA found that ecosystem and soil types significantly affected all descriptors (except PP vs. ecosystem type). Sampling date had a significant effect on MI, ΣMI, PPI, and NCR (P<0.01, 0.05, Table 4). The interaction ecosystem × soil significantly affected all descriptors (P<0.01), ecosystem × date and soil × date significantly affected half of the descriptors, and the interaction of all three factors (ecosystem × soil × date) affected the majority of the descriptors. MI, ΣMI, PPI, H´, SI, and TD were generally higher in FOR than MEA and AGR soils and in CM than CS and SS. EI was highest in AGR and CS, CI was highest in MEA and SS, and NCR was highest in FOR and CS.

Discussion

Nematode and microbial communities have been evaluated for their ability to detect changes in response to environmental impacts (e.g. wildfire, windstorms, and plant invasion) or human activities (e.g. pollution, land management, and ecosystem conversions) in many studies (Schutter *et al.,* 2001; Gömöryová *et al.,* 2011; Jangid *et al.,* 2011; Whitford *et al.,* 2014; Čerevková *et al.,* 2013; Renčo *et al.,* 2015; Renčo & Baležentienė, 2015; Sánchez-Moreno *et al.,* 2018). In present comprehensive study we evaluated their differences amongst ecosystems (natural, semi-natural, and managed) and soil types (CM, CS, and SS) measured by various community parameters. Such works where nematodes and microbes are surveyed together are rare (Ekschmitt *et al.,* 2001; Briar *et al.,* 2007). We also analysed the basal soil physicochemical properties and interactions with both nematode and microbial communities.

Relationships of ecosystem type with soil properties and nematode and microbial communities

Ecosystem type was an important factor shaping soil nematode and microbial communities and affecting soil properties. The abiotic and biotic soil properties and interactions amongst them were best for the FOR ecosystem. FOR had the highest SM, C, and N contents and C/N ratio but the lowest pH. C and N contents were twice as high in FOR than AGR but were similar to those in MEA. The supposed benefits of management of agricultural land (e.g. tillage, fertilisation, and crop rotation) include increased soil C and N contents, fertility, water retention, and overall provision of ecosystem services (Garbach *et al.,* 2017; Sánchez-Moreno *et al.,* 2018). The low C and N contents in our AGR soils, however, suggested differences in the quantity and quality of inputs to the soil, nutrient inputs and losses, low plant diversity and stimulation of decomposition by soil disturbance compared to the semi-natural (MEA) and natural (FOR) ecosystems. These results are in agreement with many studies of differences in soil C and N contents and changes following conversion of forest to managed agricultural land, well summarised in a review by Murty *et al.,* (2002). This review revealed that large amounts of C and N could be lost when forest is converted into cultivated land but that no changes in soil C and N contents were recorded when forests were converted to uncultivated pasture (similar to our meadow). In contrast, the abandonment and reforestation of agricultural land can substantially increase C and N storage (Compton and Boone 2000), due to increase in plant diversity (Lange *et al.,* 2015). Additionally, cultivated soils usually have lower C/N ratios than forest soils (Murty *et al.,* 2002), consistent with our and other results (Fernandes *et al.,* 1997; Smil, 1999; Compton & Boone, 2000). Our C/N ratio was negatively correlated with pH in FOR, consistent with the results reported by Högberg *et al.,* (2007).

Food, water, and temperature are the three primary factors that determine the habitats occupied by nematodes and microbes, the degree of species diversity, and the composition and structure of their communities. The availability of food, water, and temperature, however, are determined by ecosystem type, soil characteristics (e.g. structure, pH, and chemistry), plant composition, and microclimatic (Neher, 2010) or seasonal (Gaugler & Bilgrami, 2004) variations. More diverse nematode and microbial assemblages contribute to more resilient ecosystem services (Yeates, 2007; Fuhrman, 2009; Háněl, 2017). Forest soils, for example, contain more species than agricultural soils (Domsch *et al.,* 1983; Neher *et al.,* 2005), some with >400 nematode species (Yeates, 2007). This finding is consistent with our results; nematode species numbers and diversity (H´) were highest for FOR, even though FOR had the lowest overall nematode abundance, suggesting that established forests represent relatively stable environments providing suitable conditions for maintaining balanced and rich nematode trophic webs (Yeates, 2007). This was supported also by values of ecological and functional indices (Bongers, 1990; Ferris *et al.,* 2001). All maturity indices (MI, ΣMI, PPI) as well as Structure index and Trophic diversity were generally higher in forests than in grasslands and/or cultivated soils. These results partially agree with those by Neher *et al.,* (2005), who reported that MI, PPI and SI were higher in forests than in wetlands and agricultural soils. Ecosystem type has significant effect on values of CI, which was the highest in meadow soils in our study, indicates a higher proportion of fungal decomposition (fungal decomposition channels) and low abundance of c-p1 bacterial feeders (e.g. Rhabditidae and Panagrolaimidae) (Ferris *et al.,* 2001). In contrast, Neher *et al.*, (2005) revealed the highest CI value in forest soils.

We found several nematode species exclusively in one ecosystem e.g. *Paraphelenchus obscurus* in AGR, *Paratylenchus microdorus* in MEA, and *Filenchus polyhypnus* in FOR. Extreme disturbanc-

Table S1. Mean abundance of nematode species (100 g of dry soil) in the three ecosystems (forest (FOR), meadow (MEA), and agricultural field (AGR)) and types of soil (Stagnosol (SS), Cambisol (CS), and Chernozem (CM)) (n=45). Bold figures indicate dominance >2 but <5%, and bold and underlined figures indicate dominance >5%.

		Ecosystem			Soil		
Taxon	TG/FG	FOR	MEA	AGR	SS	CS	CM
Mesorhabditis spp. juvs	Ba1	**13.5**	0.6	**13.4**		**17.4**	10.1
Panagrolaimus rigidus	Ba1	2.0	6.1	7.2	1.3	5.8	8.2
Rhabditis spp. juvs	Ba1	6.7	4.3	**18.1**	2.9	**18.7**	7.4
Acrobeles ciliatus	Ba2	**20.3**	2.8	0.4			**23.4**
Acrobeloides buetschlii	Ba2		0.4		0.4		
Acrobeloides nanus	Ba2	**43.0**	**40.9**	**30.1**	**30.2**	**45.1**	**38.2**
Acrobelophis minimus	Ba2	0.6				0.6	
Acrolobus emarginatus	Ba2			0.1	0.1		
Anaplectus granulosus	Ba2	2.5	5.1	2.7	2.4	1.9	7.2
Cervidellus cervus	Ba2	0.1				0.1	
Cervidellus vexiliger	Ba2	5.8	2.7			0.6	8.4
Cephalobus persegnis	Ba2	**17.7**	**14.3**	**22.7**	**24.4**	**27.6**	11.5
Ereptonema arcticum	Ba2	1.9					1.9
Eucephalobus mucronatus	Ba2		2.9		1.3		1.6
Eucephalobus oxyuroides	Ba2	12.6	**13.5**	**17.5**	**22.1**	7.7	13.9
Eucephalobus striatus	Ba2	7.2	3.3	**44.8**	13.5	5.1	**39.4**
Eumonhystera dispar	Ba2	1.0					1.0
Eumonystera filiformis	Ba2	1.7				2.4	
Geomonhystera villosa	Ba2	1.0			1.0		
Heterocephalobus elongatus	Ba2	8.6	4.0	9.7	**10.2**	**9.9**	2.2
Heterocephalobus eurystoma (N)	Ba2		0.7				0.7
Chiloplacus demani	Ba2	7.4			5.8		1.6
Chiloplacus propinquus	Ba2	**18.2**	**22.6**	**23.8**	**21.6**	**12.2**	**30.8**
Chiloplacus symmetricus	Ba2		1.0			1.0	
Plectus acuminatus	Ba2	1.1		8.1	0.9	5.6	2.6
Plectus cirratus	Ba2	7.3	3.1		3.9	4.3	2.1
Plectus communis	Ba2	3.1			3.1		
Plectus longicaudatus	Ba2	3.8				2.0	1.8
Plectus parietinus	Ba2	6.3	**9.9**	7.6	7.0	4.4	12.4
Plectus parvus	Ba2	**10.9**	**15.0**		**15.5**	4.6	5.8
Plectus rhizophilus	Ba2	0.9				0.9	
Plectus silvaticus	Ba2	1.3					1.3
Seleborca complexa	Ba2		0.7				0.7
Stegelletina leopolitensis (N)	Ba2		0.1	3.2	0.9		2.4
Wilsonema schuurmansstekhoveni	Ba2	6.5	1.7		1.8	3.6	2.9
Aulolaimus oxycephalus	Ba3		1.1				1.1
Bastiania gracilis	Ba3		0.2				
Prismatolaimus intermedius	Ba3	2.3	1.2				3.5
Teratocephalus lirellus	Ba3	0.1					0.1
Teratocephalus terrestris	Ba3	0.7				0.2	0.5
Alaimus parvus	Ba4	0.6			0.6		
Alaimus primitivus	Ba4	2.2	3.7	1.3	0.4	0.5	6.3
Amphidelus coronatus	Ba4			0.2			0.2
Amphidelus elegans	Ba4	0.2	0.1			0.2	0.1
Tripyla affinis	Ca3	0.6	0.8				1.4
Trischistoma monohystera	Ca3		0.3			0.3	
Clarkus papillatus	Ca4	2.6	1.8	0.8		2.3	3.0
Coomansus parvus	Ca4	1.3		0.1		1.3	0.1
Coomanus zschokkei	Ca4	0.7				0.7	
Ironus macramphis	Ca4	0.4				0.4	

Mylonchulus brachyuris	Ca4	3.2	1.7	0.9	1.0	2.4	2.5
Prionchulus muscorum	Ca4	2.5					2.5
Paravulus hartingii	Ca5			0.6	0.6		
Aphelenchoides bicaudatus	Fu2	0.3	0.1			0.1	0.3
Aphelenchoides composticola	Fu2	**9.7**	**11.0**	0.9	**7.4**	5.3	8.9
Aphelenchoides limberi	Fu2			1.4	0.2		1.2
Aphelenchoides parietinus	Fu2	5.5	7.4	**14.8**	3.2	3.0	**21.6**
Aphelenchoides saprophilus	Fu2	1.1	1.3		1.0	0.1	1.3
Aphelenchus avenae	Fu2	**22.8**	**39.5**	**82.4**	**21.2**	**35.6**	**88.0**
Ditylenchus dipsaci	Fu2	0.6		0.5			1.1
Ditylenchus intermedius	Fu2	3.6		**15.7***	**8.3**	4.7	6.3
Ditylenchus longicauda	Fu2		1.1			0.7	0.4
Ditylenchus longimetricalis	Fu2	2.7				1.0	1.7
Ditylenchus myceliophagus	Fu2	1.7	0.7		0.6		1.7
Ditylenchus parvus (N)	Fu2		1.2		1.2		
Ditylenchus tenuidens (N)	Fu2	0.8			0.8		
Ditylenchus sp.	Fu2		1.7		1.7		
Filenchus discrepans	Fu2	2.6				2.6	
Filenchus misellus	Fu2	1.0				1.0	
Filenchus polyhypnus	Fu2	7.2					7.2
Filenchus thornei	Fu2	3.3	2.1		2.1	3.3	
Filenchus vulgaris	Fu2	8.1	**19.1**	**32.2**	**17.6**	**13.7**	**35.2**
Hexatylus viviparus	Fu2	0.5				0.5	
Nothotylenchus acris	Fu2			0.7			0.7
Paraphelenchus obscurus (N)	Fu2			4.6			4.6
Paraphelenchus pseudoparietinus	Fu2	1.5	1.8	0.8		2.1	2.1
Diphtherophora communis	Fu3	0.3	1.4		0.3		1.4
Tylencholaimus mirablis	Fu4	0.7	0.9				1.6
Tylencholaimus stecki	Fu4	4.3	2.8		1.9	0.9	
Tylencholaimus teres	Fu4	0.3					0.3
Aporcelaimus superbus	Om4	0.4					0.4
Campydora demonstrans	Om4		0.2				0.2
Crassolabium ettersbergense	Om4	2.4	1.0			1.4	2.0
Dorydorella bryophila	Om4	3.7	6.7				10.4
Dorylaimoides micoletzkyi	Om4	1.4	2.4		1.7	1.3	0.8
Ecumenicus monohystera	Om4	0.2	0.4	2.4		0.2	2.8
Eudorylaimus carteri	Om4	3.9	**19.5**	**9.2**	**10.8**	**10.5**	11.3
Eudorylaimus leuckarti	Om4	**9.9**	2.3	2.5	1.5	2.7	10.5
Eudorylaimus iners	Om4	3.4				3.4	
Eudorylaimus opistohystera	Om4	2.9	2.1	1.4		2.3	4.1
Eudorylaimus spp. juvs	Om4	7.5	3.1	3.3	2.1	6.5	5.3
Mesodorylaimus meyli	Om4	2.0				2.0	
Microdorylaimus parvus	Om4	2.8	0.2	2.4		5.6	0.2
Pungentus silvestris	Om4	0.7	3.5				4.2
Aporcelaimellus obtusicaudatus	Om5	7.8	2.2	6.8	2.3	4.7	11.6
Axonchium propinquum	Om5		0.1		0.1		
Discolaimus major	Om5	0.9					0.9
Discolaimus texanus	Om5			0.5			0.5
Epidorylaimus agilis	Om5	0.4				0.4	
Mesodorylaimus bastiani	Om5	2.6	2.9	2.1	4.8	0.6	2.1
Metaxonchium coronatum	Om5		0.6				0.6
Nygolaimus brachyuris	Om5		1.1			0.3	0.8
Oxydirus oxycephalus	Om5	2.5	2.1	1.7	0.6	2.0	2.6
Paraxonchium laetificans	Om5	0.6	0.1	0.5			1.2
Prodorylaimus acris	Om5	0.6	0.4		0.4	0.6	

Aglenchus agricola	Pp2	1.9	6.4	**32.9**	**10.3**	4.8	**28.7**
Basiria gracilis	Pp2	0.7				0.7	
Basiria similis	Pp2		0.6		0.3		0.3
Basiria tumida	Pp2	2.0			1.8		0.2
Boleodorus thylactus	Pp2	0.5	**24.8**	6.8	**21.3**	6.6	4.0
Boleodorus volutus (N)	Pp2	0.4	0.4				0.8
Cephalenchus intermedius (N)	Pp2		0.2		0.2		
Coslenchus andrássyi (N)	Pp2		0.7			0.7	
Coslenchus costatus	Pp2	1.8	1.9				3.7
Ecphyadophora tenuissima (N)	Pp2	0.4					0.4
Malenchus acarayensis	Pp2	**9.1**			**7.6**	4.4	
Malenchus bryophilus	Pp2	12.4	2.2			**14.6**	
Malenchus exiguus	Pp2	2.6	**10.1**	2.7		1.0	6.8
Malenchus gratiosus	Pp2	0.9					0.9
Neopsilenchus magnidens	Pp2		0.5		0.5		
Tylenchus davainei	Pp2	4.6	5.4	1.6	2.7	2.9	5.9
Paratylenchus bukowinensis	Pp2			**15.8**		**11.5**	4.3
Paratylenchus microdorus	Pp2		**10.9**		3.5	6.4	1.0
Paratylenchus projectus	Pp2	3.4				3.4	
Psilenchus hilarulus	Pp2		2.0	0.9		2.6	0.2
Amplimerlinius macrurus	Pp3	0.7	**14.8**		**14.3**		0.7
Bitylenchus dubius	Pp3		**10.8**	**12.8**	4.1	**19.5**	
Bitylenchus maximus	Pp3		2.2			1.7	0.5
Geocenamus brevidens	Pp3	7.1	5.2		6.5		1.7
Geocenamus microdorus	Pp3		**21.0**				**21.0**
Geocenamus nanus	Po3			8.8		**8.8**	5.0
Helicotylenchus canadensis	Pp3	3.8	1.3			5.1	
Helicotylenhus digonicus	Pp3	**11.0**	0.6			3.3	8.4
Helicotylencus dihystera	Pp3			10.2			10.2
Heterodera mani juvs	Pp3		1.2		1.2		
Heterodera avenae juvs	Pp3		0.3	0.6		0.9	
Meloidogyne hapla	Pp3		1.1			1.1	
Nagelus obscurus	Pp3		1.1				1.1
Pratylenchoides crenicauda	Pp3	0.8	0.5				1.3
Pratylenchus crenatus	Pp3			1.3		0.7	0.6
Pratylenchus penetrans	Pp3	4.6		9.7		7.5	9.7
Pratylenchus pratensis	Pp3		**9.1**	7.4	6.3	0.6	6.8
Tylenchorhynchus bicaudatus	Pp3	0.1					0.1
Tylenchorhynchus cylindricus	Pp3			2.3			2.3
Longidorus elongatus	Pp5		0.3		0.3		
Longidorus intermedius	Pp5	0.5					0.5
Total number of species		**99**	**90**	**53**	**60**	**81**	**102**

juvs, juveniles; (N), species new for Slovak fauna

es, such as bulldozing, slash-and-burn management, windstorms, and wildfires in forests, however, can substantially reduce nematode diversity (Yeates, 2007; Čerevková *et al.,* 2013). The species richness of the nematode fauna in FOR in our study was higher than in the soils of a protected forest in the Slovak Tatra National Park nine years after a windstorm and wildfire, likely due to the persistent influence of changes in the plant community and basal soil physicochemical properties (Renčo & Čerevková, 2015; Renčo *et al.,* 2015).

The FOR soils also had the highest microbial biomass, richness, and diversity, what positively correlated with C and N contents, and was consistent with the observations of Yergeau *et al.,* (2006)). Microbial biomass is involved in the control of the synthesis and decomposition of soil organic matter and acts as an accessible storage system for nutrients in ecosystems. Sites with high microbial biomass can therefore stock and recycle more nutrients for plant nutrition and thus improve the sustainability of an ecosystem (Kaschuk *et al*., 2010). In contrast, the number and diversity

of nematode species and diversity of microbial functional groups in our study were lowest in AGR. Additionally, AGR had half the amount of microbial biomass than FOR and MEA, and microbial biomass was negatively correlated with C and N contents. These findings support our hypothesis that biological diversity would be lowest in the agricultural soils due to periodic perturbation, land management, and crop monoculturing, consistent with the results by Neher *et al.,* (2005); even though AGR had the highest overall nematode abundance, likely due the periodic organic manure inputs (Hu *et al.,* 2018).

Bacterivorous nematodes are often the most dominant feeding group in forest (Neher *et al.,* 2005; Yeates, 2007; Renčo & Čerevková, 2017) and agricultural (Neher *et al.,* 2005, Renčo *et al.,* 2010) soils. The preponderance of Ba_2 bacterivores (*A. nanus*, *C. persegnis*, and *C. propinquus*) in all ecosystems in our study was likely due to the high microbial biomasses in FOR and MEA and to the management (tillage and fertilisation) in the corn monoculture in AGR. Microbial biomass was nevertheless significantly lower in AGR than FOR and MEA. Microbial diversity is often lower after a natural habitat has been cultivated (Buckley & Schmidt, 2001). These results are in agreement with Wasilewska (1997), who stated that a higher abundance of microflora would support larger numbers of bacterivorous nematodes. An increase in the abundance of this group is indicative of enhanced microbiological activity e.g. after the addition of cow and chicken manure or slurry (Wasilewska, 1997; Neher & Olson, 1999). Our study thus demonstrated the synchronisation between bacterivorous nematodes and their food resources, which has not been frequently reported (Wardle *et al.,* 2001, Papatheodorou *et al.,* 2004). Fungivorous nematodes (Fu_2) were the second most abundant trophic group in all ecosystems. AGR had the highest abundance of fungivores, mainly *A. avenae*, *F. vulgaris*, *Ditylenchus intermedius*, and *Aphelenchoides parietinus*, likely due to the high density of fungal hyphae and spores under *Z. mays* monoculture from the association of corn with arbuscular mycorrhizal fungi (Bai *et al.,* 2008).

Plants and their root systems serve as food for plant parasitic nematodes (Flis *et al.,* 2018; Le *et al.,* 2019) before they serve as a food source for microbivorous nematodes during decomposition. Root systems are more diverse in natural ecosystems with rich communities of plant species than for monocultured crops. Root growth is also more extensive and less ephemeral in perennial plants than annual crops and supports a soil community with many species of plant parasites, omnivores, and predators (Neher, 2010). Plant parasites are common in natural grasslands (Popovici & Ciobanu, 2000; Čerevková, 2006). The abundance of plant parasites, such as *Boleodorus thylactus*, *Malenchus exiguus*, and *Paratylenchus microdorus* (Pp_2) or *Amplimerlinius macrurus*, *Geocenamus microdorus*, and *Bitylenchus dubius* (Pp_3) was highest in MEA.

The importance and high population densities of plant parasitic nematodes in agriculture are mainly associated with specific crop pests, e.g. root-knot and cyst-forming endoparasites (e.g. *Meloidogyne*, *Heterodera*, and *Globodera*). The high overall abundance of Pp nematodes in AGR (*Aglenchus agricola* Pp_2, *Paratylenchus bukowinensis* Pp_2, *Bitylenchus dubius* Pp_3, and *Helicotylenchus dihystera* Pp_3 are all ectoparasites) suggests their close relationship with cultured crops. Omnivores and carnivores were significantly more abundant in MEA and FOR than AGR, consistent with previous findings by Neher *et al.,* (2005) and Renčo *et al.,* (2010).

Relationships of soil type with soil properties and nematode and microbial communities

Soil type was also an important factor affecting the nematode and microbial communities and soil properties. Soil properties were best in CM, with a neutral pH and the highest C and N contents and C/N ratio, followed by CS and SS. C and N contents were twice as high in CM than SS, in agreement with the general soil classification (www.vupop.sk).

Soil type was more important than ecosystem type for both the nematode and microbial communities. For example, nematode abundance, number of nematode species, and microbial biomass or diversity positively correlated in the CM soil type in two out of three ecosystems studied. Significant effects of soil type on the composition of nematode communities have been documented by Alphei (1998) and Lišková *et al.,* (2008) in forests, by Popovici and Ciobanu (2000) in grasslands, and by Neher *et al.,* (2005) in agricultural land. The populations of bacterivores (mainly *A. nanus*, *Eucephalobus striatus*, and *C. propinquus*) and fungivores (*A. avenae* and *F. vulgaris*) and microbial biomass in our study were highest in CM with aerobic conditions, a neutral pH, and a high humus content beneficial to microbial activities and associated nematodes (Wasilewska, 1997). In contrast, the abundances of bacterivores and fungivores were low in SS because of its oxygen deficiency and acidic conditions. These results partially agreed with those by Lišková *et al.,* (2008), who reported that Cephalobidae bacterivores (*Acrobeloides*, *Acrobeles* and *Cervidellus*) were more abundant in a light sandy Regosol with a high pH, but disagreed with those by Wasilewska (1997) and Lišková *et al.,* (2008), who reported that fungivores were more abundant in an acidic Cambisol.

The abundance of facultative plant parasites (Pp_2) did not differ amongst the soil types. *A. agricola* in CM, *Malenchus bryophilus* in CS, and *B. thylactus* in SS were nevertheless the most abundant, supporting the preference of various species of Pp_2 nematodes for different soil types, also reported by Lišková *et al.,* (2008). In contrast, obligate plant parasites (Pp_3) were most abundance in CM, followed by CS and SS, probably due to the different levels and distributions of food sources between these soil types, as also suggested by Popovici and Ciobanu (2000) and Lišková *et al.,* (2008). Natural ecosystems are characterised by high proportions of omnivores and predators (Wasilewska, 1997; Ferris *et al.,* 2001). Omnivores and predators were most abundant in CM, but only in FOR and MEA.

In our study soil type was also as important factor affecting values

of all ecological and functional indices, contradicting findings of Lišková *at al.,* (2008), who reported that only fungal to bacteria (F/B) ratio and channel index (CI) was significantly different among Cambisol, Regosol, Fluvisol and Rendzina soil types. Ruess (2003) studied CI and F/B at various sites and stated that soil and climate affect CI more strongly than does ecosystem type. In our study CI was significantly affected by both, ecosystem and soil type as well as their interactions, and sampling date has no impact on CI values.

In general, season (sampling date) in our study had relatively minor effects on both the abiotic and biotic characteristics. Only SM content fluctuated with the season (lowest in summer) what significantly affecting microbial biomass, confirming results of Buchanan and King (1992). Similar overall nematode abundance influences sampling date, which can partly be explained by changes in SM, in agreement with observation of Sohlenius and Boström (2001) from Swedish Scot pine forest soils. Out of functional guilds, Ba_2, Fu_2, and Pp_2 nematodes were influenced by sampling date, however only Ba_2 were negatively correlated with SM content.

Conclusion

The differences in soil properties, nematode communities, and microbial biomasses amongst the soil and ecosystem types suggest an obvious impact of environmental variables on biotic and abiotic soil characteristics. The differences were larger amongst the soil types than the ecosystems. CM had the best soil properties, with a neutral pH and the highest C and N contents and C/N ratio and thus the highest number of species and diversity of nematode communities, as well as the MI, ΣMI, PPI, SI, and TD nematode ecological indices, and microbial biomass, richness, and diversity. The majority of the abiotic and biotic characteristics varied the most between CM and SS. The abiotic and biotic soil properties and their interactions were best in FOR, where the number of species and diversity of nematode communities, as well as the MI, ΣMI, PPI, SI, and TD ecological indices, and microbial biomass, richness, and diversity were highest. SM, C, and N contents and the C/N ratio were also highest in FOR, but the pH was lowest. C and N contents were twice as high in FOR than AGR but were similar to those in MEA, suggesting that established forests and natural meadows represent relatively stable environments, providing suitable conditions for soil microbial and nematode communities. C/N ratios and biological diversity were lower in the cultivated soils than in the natural ecosystems soils, likely due to periodic perturbation. This resulted in a lower abundance and diversity of nematode communities and microbial diversity. FOR and AGR generally differed the most. The soil properties, nematode communities, and microbial biomass were more similar in FOR and MEA. A multivariate analysis indicated that the abundance of most of the nematode guilds, total nematode abundance, number of nematode species, and microbial characteristics tended to be higher in the environment with a higher pH, the N and C contents. Sampling dates had a minor or no effect on most of the parameters, except the SM content, abundance of c-p2 nematodes, microbial richness, and several of the nematode ecological indices.

Conflict of Interest

Authors have no potential conflict of interest pertaining to this submission to Helminthologia.

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Short-term effects of cadmium and mercury on soil nematode communities in a pot experiment

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Summary

Analysis of soil nematode feeding groups and functional guilds were used as a valuable tool to detect heavy metal pollution. Effects of cadmium (Cd) at 5 mg/kg, mercury (Hg) at 20mg/kg, combined Cd and Hg at 5+20mg/kg on the nematode communities were studied after three months application. Nematodes were collected from soil in rhizosphere of Morning glories (*Pharhiris nil*) which were applied as heavy metal accumulators and were grown in the experimental pots. Both single and combined heavy metals had marked effects on the nematode abundance, life-history strategies and feeding type composition. Bacteriovores and c-p 2 group were found to be the most abundant trophic group and functional guild, respectively. *Acrobeloides* and *Pratylenchus* were the most two abundant genera, decreasing number of them was responsible for the significant difference between control and polluted treatments. Cd-5 and Cd-Hg 5+20 presented lower values of nematode diversity index (H') and evenness index (J') than Hg-20. The combination of Cd and Hg showed lower nematode trophic diversity (TD), in comparison with single Cd or Hg. Conversely, heavy metals addition exhibited no pronounced effect on Maturity index (MI), structural index (SI) and enrichment index (EI). Our results demonstrate that genera composition is a better indicator to short-term heavy metal effects than some common indicator indices and emphasize that deeper assemblage analyses are needed for a correct interpretation of short-term disturbance on soil nematodes.
Keywords: Morning glory; heavy metals; nematodes; trophic group; c-p group

Introduction

Anthropic activities such as mining, manufacturing, transporting and fertilizing have caused heavy metal contamination in urban and agricultural soils. The heavy metal contamination has resulted in severe threats to human and environment health (Chen *et al*., 2005; Xia *et al*., 2004). Cadmium (Cd) and mercury (Hg) are the two most predominant metals in sewage irrigation region of Liaoning province (Li & Tong 2008). How to deal with the toxic pollutants efficiently and safely is becoming one of the most popular environmental-related themes. Phytoremediation has been developed in recent years (Yoon 2007), and has become a new economical method which can reduce some toxic pollutants in an environmentally friendly way (Pilon-Smits, 2005). Morning glory (*Pharhiris nil*) is a remediation plant which absorbs pollutants from soil and accumulates them in stalk, leaf, and root, in this way, contaminant could be reduced through harvesting the plant body. Xu *et al*. (2012) reported that petroleum degrading rate was increased by 13 % after 2 months of planting Morning glory. With respect to metal bioaccumulation, plants accumulated higher amounts of

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metal in root than stem in previous studies (Ali *et al*., 2004), so the toxicity created by heavy metals impacts roots first, which means soil quality and plant-microbe interactions around the rhizosphere can influence the efficiency of metal phytoremediation (Cherian & Oliveira 2005). However, most investigations about accumulating plants were focused on the aboveground parts, the structure and assemblage of soil fauna around rhizosphere are not well known. Nematodes are one of the most abundant groups of soil invertebrates (Fu *et al*., 2000) and occupy a central position in the soil food web. Soil nematode communities can provide unique insights into soil processes and supply useful information about soil environment. Soil nematode as an indicator for heavy metal pollution has been widely studied in recent years. (Zhang *et al*., 2011; Šalamún, 2011; Martinez *et al*., 2018). Most of previous studies took place in an open environment such as mining area, however, under laboratory condition, the characteristics of rhizosphere nematode communities affected by specific concentrations of single and mixed heavy metals are not well studied. The present study investigated the changes of Cd, Hg and Cd+Hg concentrations after 90 days remediation and characterized the nematode community structure and assemblage around rhizosphere of Morning glory. The objectives of this study were: 1) to evaluate soil quality after phytoremediation experiment; 2) to represent the responses of nematodes to different heavy metal treatments; 3) to provide basic data for using soil nematodes to assess the level of phytoremediation. We hypothesize that only small part of heavy metals could be absorbed by plants during short-term remediation, hence the remaining part still has adverse effects on nematode communities, furthermore, the degree of influence may depend on feeding groups and life-history strategies.

Material and Methods

The seeds of Morning glory were planted in non-contaminated garden soil for 7 days before experiment, then, five seedlings were subsequently transplanted into one pot. Each pot (15cm – diam×12cm – -depth) was loaded by 600g soil collected from Dalian Xishan National Forest Park, soil was loamy with 13.1 % clay, 50.4 % silt and 36.5 % sand and was completely mixed. Based on the concentrations of heavy metals obtained from the wastewater irrigation area (max concentration of Cd = 5 mg/kg) (Liao, 1993) and mercury mining area (Hg = 20.4 mg/kg) (Yu *et al*., 2017), the concentrations of heavy metal were selected at Cd-5mg/kg, Hg-20mg/kg and Cd-Hg5+20mg/kg. Heave metal solutions were applied as $Cd(NO_3)_2 \cdot 4H_2O$ and $HgCl_2$, three replicates at each level were established, concentrations of heavy metals before and after treatment were shown in Table 1. The seedlings were grown for three months in a climate chamber at 20±1 °C. Intensity of light was 5000LX with 16 hour of light alternating with 8 hour of darkness. Each pot was watered with 250 ml deionized water every 2 days.

Nematodes were extracted from 100g (fresh weight) soil per pot using elutriate-sieving-flotation and centrifugation method (Barker *et al*., 1985). Extracted nematodes were heat killed at 60 °C, counted and preserved in 4 % formalin aqueous solution (Steinberger & Sarig, 1993). One hundred nematodes randomly selected specimens per sample were identified to genus level using Olympus inverted compound microscope based on stoma and esophageal morphology (Liang *et al*., 2001, 2003). The genus was identified according to Bongers (1988) and Li *et al*. (2017).

Soil moisture of each sample was gravimetric determined by weight loss at 105 °C for 8 hours and expressed as percent dry weight. Another 100g fresh soil per pot was air-dried for 14 days at room temperature to text soil organic matter and heavy metals. Electronic pH meter (model SevenGo™ pH-SG2), the potassium dichromate external heating method, graphite furnace atomic absorption spectrometry and microwave dissolution/atomic fluorescence spectrometry were used to determined soil pH, organic matter, Cd and Hg, respectively (Ru, 1999; China National Environmental Monitoring Centre,1997; Ministry of Ecology and Environment of the People's Republic of China,2013).

The assemblage and characteristics of nematode community were investigated by following approaches: (1) feeding groups (bacterivores-BF, fungivores-FF, plant parasites-PP, omnivores-predators-

Table 1. Soil chemical properties and heavy metal concentrations.

Heavy metals addition (mg/kg)		Heavy metals contents after remediation (mg/kg)		Average root weight (g/ DW plant)	pH	Soil organic matter (g/kg)
Cd	Hg	Cd	Hg			
0	0	0.21 ± 0.01	0.14 ± 0.01	0.023	6.86 ± 0.27a	40.76 ± 1.95a
5	0	4.12 ± 0.32	0.13 ± 0.01	0.032	6.33 ± 0.21b	42.88 ± 1.54a
0	20	0.21 ± 0.01	18.17 ± 0.67	0.024	6.68 ± 0.30ab	41.31 ± 2.86a
5	20	4.19 ± 0.45	16.38 ± 0.58	0.036	6.42 ± 0.33ab	40.69 ± 3.07a

Heavy metal, root weight and pH data were supplied by Hou (2012); Mean values in a line with different letters are significantly different from each other at $P < 0.05$; values are expressed as mean ± standard error (n = 3).

OP) (Yeates *et al*., 1993; Pen-Mouratov *et al*., 2004); (2) life-history groups (c–p values range from 1 to 5 represent colonizers to persistors (Bongers, 1990, 1999). (3) ratio of f/b (fungivores/ bacterivores); (4) diversity index H'=–∑ni (lnni) where ni is the proportion of individuals in the i-th taxon (Shannon & Weaver, 1949) , (5) trophic diversity index TD = TD = 1/∑pi2, where pi is the proportion of each trophic group; (6) evenness J'= H'/ln(S), where S is the number of taxa; (7) maturity indices MI=∑vifi, where vi is the c–p value of the i-th taxa and fi is the frequency of the i-th taxa in the sample (excluding PP) (Bongers, 1990); (8) enrichment index (EI) and structure index (SI), EI = 100(e/e + b), SI = 100(s/s + b), where e =∑kene, s =∑ksns, and b =∑kbnb (Ferris *et al*., 2001). All nematode data were 1n (x +1) transformed prior to statistical analysis. The significance of the effects of heavy metals on nematode communities was tested by one-way analysis of variance (ANOVA), SPSS 18.0 statistical software, and means compared by LSD's Test (Least Significant Difference). Differences with P < 0.05 were considered significant. Principal component analysis

Table 2. Relative abundance (%) of nematode genera in control (CK) and polluted pots.

Genus	Guild	CK	Cd-5	Hg-20	Cd5 + Hg20
Panagrolaimus	BF1	0.62 ± 0.61b	0.00 ± 0.00b	5.51 ± 1.14a	5.15 ± 0.67a
Rhabditis	BF1	11.04 ± 0.86a	3.33 ± 3.33b	9.74 ± 1.12ab	7.72 ± 1.92ab
Mesorhabditis	BF1	3.46 ± 1.03a	1.75 ± 1.75a	4.23 ± 0.38a	2.29 ± 1.17a
Acrobeles	BF2	1.68 ± 0.92a	0.00 ± 0.00a	1.28 ± 1.28a	0.00 ± 0.00a
Acrobeloides	BF2	32.30 ± 1.52ab	29.70 ± 2.68ab	27.95 ± 4.56b	42.18 ± 6.71a
Heterocephalobus	BF2	2.89 ± 0.56a	1.75 ± 1.75a	1.67 ± 1.67a	1.01 ± 1.01a
Eucephalobus	BF2	2.27 ± 0.46a	0.00 ± 0.00a	2.56 ± 1.23a	3.57 ± 2.23a
Cervidellus	BF2	0.61 ± 0.61a	0.00 ± 0.00a	1.28 ± 1.28a	1.01 ± 1.01a
Plectus	BF2	0.60 ± 0.60a	0.00 ± 0.00a	1.28 ± 1.28a	1.28 ± 1.28a
Chrohogaster	BF2	0.53 ± 0.53a	0.00 ± 0.00a	0.00 ± 0.00a	1.01 ± 1.01a
Prismatolaimus	BF3	1.19 ± 1.03a	5.46 ± 3.21a	0.00 ± 0.00a	2.29 ± 1.17a
Alaimus	BF4	2.20 ± 1.39b	0.00 ± 0.00b	0.00 ± 0.00b	5.43 ± 1.35a
Paraphelenchus	FF2	0.53 ± 0.53b	0.00 ± 0.00b	0.00 ± 0.00b	5.43 ± 1.35a
Pseudhalenchus	FF2	0.61 ± 0.61a	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a
Aphelenchoides	FF2	8.19 ± 1.78a	6.94 ± 1.53ab	2.95 ± 1.51b	1.01 ± 1.01b
Bursaphelenchus	FF2	0.00 ± 0.00a	0.00 ± 0.00a	2.56 ± 1.28a	1.28 ± 1.28a
Filenchus	FF2	5.79 ± 0.71b	5.27 ± 0.16b	27.56 ± 1.70a	1.01 ± 1.01c
Diphtherophora	FF3	0.53 ± 0.53ab	3.42 ± 1.71a	0.00 ± 0.00b	1.01 ± 1.01ab
Paratylenchus	PP2	17.62 ± 0.52b	33.58 ± 4.33a	8.19 ± 2.05c	10.14 ± 3.30bc
Helicotylenchus	PP3	0.00 ± 0.00b	3.61 ± 1.81a	0.00 ± 0.00b	1.01 ± 1.01ab
Heterodera	PP3	0.53 ± 0.53a	1.67 ± 1.67a	1.28 ± 1.28a	1.01 ± 1.01a
Eudorylaimus	OP4	1.20 ± 0.60a	3.52 ± 1.77a	1.28 ± 1.28a	2.29 ± 1.17a
Aporcelaimellus	OP5	0.61 ± 0.61a	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a
Aporcelaimium	OP5	1.65 ± 0.92ab	0.00 ± 0.00a	2.95 ± 1.51b	2.86 ± 1.61a
Prodorylaimus	OP5	0.61 ± 0.61a	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a
Discolaimus	OP5	1.14 ± 0.57a	0.00 ± 0.00b	0.00 ± 0.00b	0.00 ± 0.00b

BF: bacterivores, FF: fungivores, PP: plant parasites, OP: omnivores-predators, numbers following the letters in Guild indicate the c-p value of each taxon
Mean values in a line with different letters are significantly different from each other at $P < 0.05$; values are expressed as mean ± standard error ($n = 3$).

Table 3. Ecological indices for nematode community structure in different treatments.

	CK	Cd5	Hg20	Cd+Hg 5+20
H'	2.31 ± 0.21a	1.44 ± 0.14b	1.91 ± 0.20ab	1.68 ± 0.50b
TD	2.34 ± 0.22b	2.85 ± 0.20a	2.42 ± 0.06b	1.78 ± 0.22c
J'	0.85 ± 0.02a	0.70 ± 0.05b	0.84 ± 0.03a	0.70 ± 0.07b
f/b	0.25 ± 0.07b	0.29 ± 0.04b	0.55 ± 0.02a	0.10 ± 0.02c
MI	1.71 ± 0.15a	2.13 ± 0.23a	1.91 ± 0.04a	2.16 ± 0.11a
SI	47.61 ± 7.76a	35.12 ± 17.40a	25.76 ± 6.71a	43.58 ± 0.13a
EI	64.19 ± 4.25a	39.15 ± 15.42a	61.98 ± 1.05a	49.04 ± 15.73a

Mean values in a line with different letters are significantly different from each other at $P < 0.05$; values are expressed as mean ± standard error ($n = 3$).

(PCA) was applied to represent the composition of the soil nematode community using CANOCO software.

Results

Soil chemical properties and heavy metal concentrations

Soil pH dropped dramatically at Cd-5, while soil organic matter increased slightly at Cd-5 and Hg-20. The absorption rates of heavy metals were 17.6 %, 9.15 % and 16.2 %+18.1 % for Cd-5, Hg-20 and Cd+Hg 5+20, respectively (Table1).

Nematode abundance and ecological indices

The average nematode abundance ranged from 150 to 812 individuals per 100g dry soil. Significant difference in the total nematode abundance was found between the control and other treatments ($P<0.05$) (Table 4). Twenty six nematode genera were identified; of them, 12 genera belonged to the bacteriovores, 4 to fungivores, 4 to plant parasites and 6 to omnivores and predators. *Acrobeloides* was the most abundant genus in all treatments with the highest dominance (42.18 %) at Cd-Hg5+20; *Pratylenchus* dominated the control, Cd-5 and Cd-Hg5+20; *Rhabditis* dominated the control (Table 2). All samples with heavy metal contamination appeared to be dissimilar from the control. The distinction between Cd-5 and CK was most pronounced, whereas the distinction between Hg-20 and Cd-Hg5+20 was not obvious. The abundance of *Panagrolaimus* and *Bursaphelenchus* were positively correlated with Hg and Cd-Hg. The abundance of *Helicotylenchus* was strongly related to Cd (Fig. 1).

Both single and joint toxicity of Cd and Hg to the nematode ecological indices were significant. Cd-5 and Cd-Hg 5+20 reduced the values of diversity index (H') and evenness index (J') ($P< 0.05$); single Cd heighten the trophic diversity (TD), while the combination of Cd and Hg diminish TD ($P< 0.05$); Hg increased f/b value, in contrast Cd-Hg reduced the f/b ($P< 0.05$); heavy metal treatments moderately affected values of MI, SI and EI which fell in relative narrow limits throughout the experiment ($P> 0.05$) (Table 3).

Nematode assemblage

Bacteriovores were found to be the most abundant group in all treatments, they concentrated at Cd-Hg5+20. Plant parasites were the second most common trophic group with highest proportion at Cd-5. Fungivores were the third most frequent group, their highest relative abundance was found at Hg-20 and the lowest was discovered at Cd-Hg 5+20 (Table 4).

Nematodes classed as c-p 1 and c-p 2 accounted for 87.67 % of all nematodes identified (Table 4). Although the abundance of nematode dropped strongly in polluted soil, the proportion with c-p value 1 and 2 at Hg-20 was much higher than that in the control. Both the highest proportion of c-p3 group and the lowest proportion of c-p 1 group occurred at Cd-5. The relative importance of nematodes with a c-p value of 4 and 5 varied slightly among all treatments.

Discussion

Building on previous researches, population structure that was measured by abundance and genus number (Bakonyi *et al*., 2003), function indexes (MI, SI and EI) (Bongers, 1990; Korthals *et al*., 1996; Wang & McSorley, 2005) and functional variables such as feeding groups (Yeates *et al*., 1993) and c-p groups (Bongers, 1990, 1999) were always applied to analyze the responses of nematode communities to disturbance.

The population of nematodes may increase or decrease with heavy metal concentration (Sánchez-Moreno & Navas, 2007). In the present case the pronounced drop of nematode abundance already sent an alert on the heavy metal pollution (Table 4). Our result is different from previous findings by Bakonyi *et al*. (2003) who reported that Cd had a moderate influence on nematodes. This difference might be contributed by the short-term and high level of pollution. Cadmium could negatively impact on soil enzymatic activities and microbial community structure (Wang *et al*., 2019). We expect that the abundance of nematode was inhibited by the degradation of their feeding sources in contamination soil. In addition, Hg exposure can lead to multitoxicity, and most of these

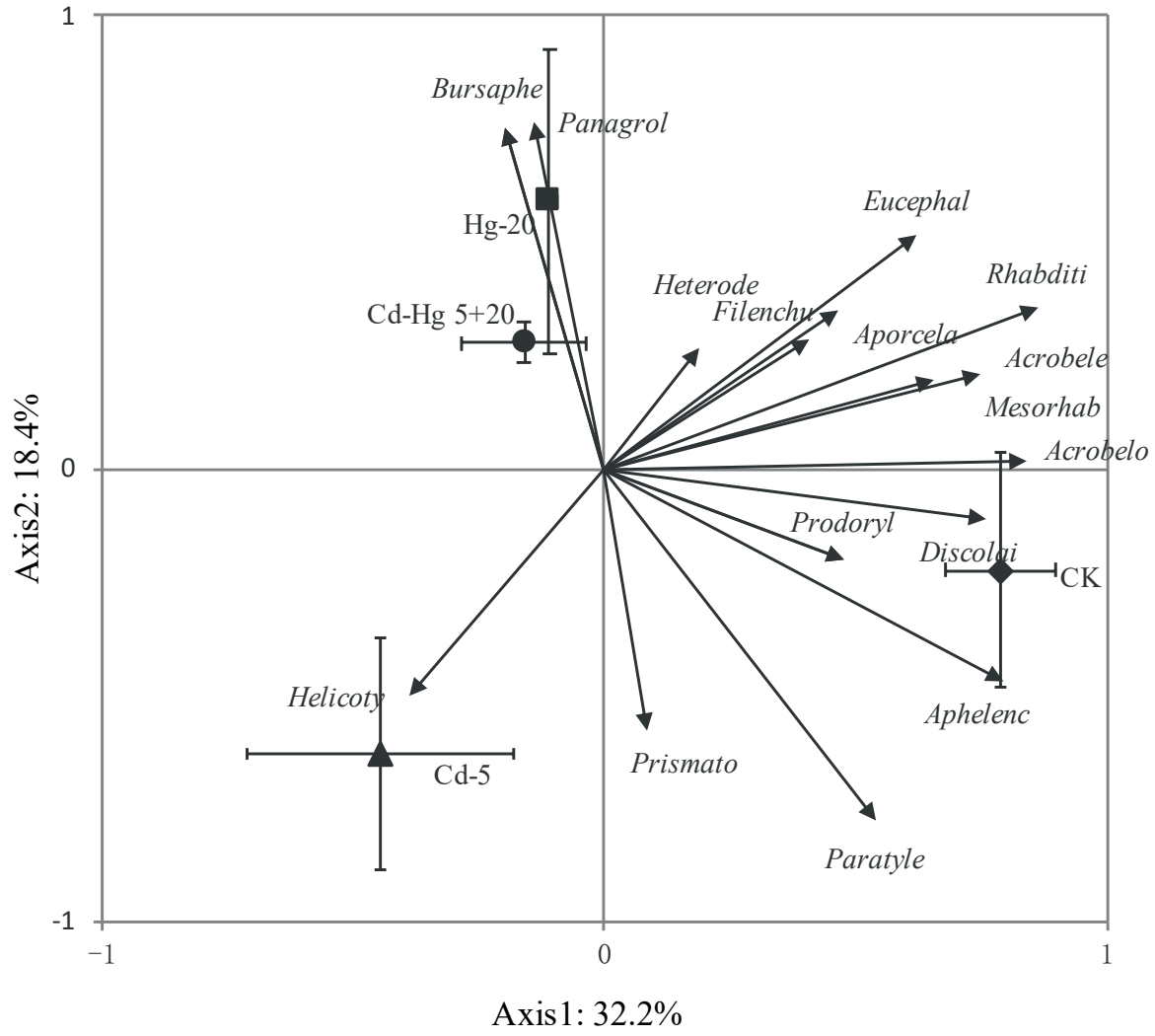

Fig. 1. Principal components analysis (PCA) of nematode abundance in different treatments. Species fit range more than 20% were presented.

harmful effects on nematode can be transferred to progeny (Wu *et al*., 2010). Nematode diversity (H') and evenness (J') were lower at Cd-5 and Cd-Hg 5+20 than the control and Hg-20 (Table 3), which suggested single Cd and mixing of Cd and Hg create worse effects on nematode communities than single Hg. Martinez *et al*. (2019) proposed that nematode-based environmental evaluations should be interpreted in a context-dependent way. Acidic soils can inhibit the diffusion of cadmium (Lu *et al*., 2005) and enhance its bioavailability (Kim *et al*., 2009), but the bioavailability of Hg tends to be lower in acidic soils (Mahbub *et al*., 2016). So the lower values of pH at Cd-5 and Cd-Hg 5+20 seem to be the reason for the lower values of H' and J'. Xie *et al*. (2011) found that the mixing of Cd and Hg had stronger toxic effects on soil microbial community than the single Cd or Hg. Similarly, nematode trophic diversity index (TD) was lowest at Cd-Hg 5+20, which indicated the combination of Cd and Hg exert more adverse impacts on nematode trophic groups than individual Cd or Hg in this study.

Nematode assemblage and function level structure presented by MI, SI and EI was found to be skewed away from the theory of these indices which predicts a reduction of the MI and SI as a consequence of heavy metal pollution. The values of MI, SI and EI were relatively uniform throughout all treatments. Our result was in line with Martinez *et al*. (2018) who discovered a short range for maturity index from different levels of disturbance. The reason for this phenomenon is the rarity and stabilization of relative abundance of high c-p value groups among all treatments. Therefore, we propose that changes in nematode communities could be represented better from abundance and diversity point of view. Since MI, SI and EI are expanded indices derive from proportions of feeding groups and life-history strategies, current findings highlight that more detailed analysis about trophic and c-p groups are needed for a correct interpretation of short-term high level pollution effects on soil nematodes.

Table 4. Absolute abundance (individuals per 100 g dry soil) and relative abundance (%) of nematode guilds in control and polluted pots.

Absolute abundance				
	CK	Cd-5	Hg-20	Cd-Hg 5+20
BF1	120.51 ± 11.68a	8.40 ± 4.84c	30.88 ± 1.56b	23.6 ± 4.43b
BF2	327.48 ± 26.67a	50.04 ± 4.38b	57.36 ± 6.00b	74.68 ± 8.11b
BF3	14.16 ± 8.31a	8.28 ± 4.22ab	0.00 ± 0.00b	3.88 ± 1.49ab
BF4	17.61 ± 11.09a	0.00 ± 0.00b	0.00 ± 0.00b	9.61 ± 2.39a
FF2	121.58 ± 23.30a	19.52 ± 2.79b	52.92 ± 7.46b	14.93 ± 2.96b
FF3	0.00 ± 0.00a	5.52 ± 1.67a	0.00 ± 0.00a	1.84 ± 0.46a
PP2	152.25 ± 5.31a	52.6 ± 4.23b	8.80 ± 2.55c	13.92 ± 2.25c
PP3	4.50 ± 4.50a	4.08 ± 0.10a	2.20 ± 2. 20a	3.72 ± 3.72a
OP4	9.45 ± 4.73a	2.42 ± 1.21b	2.16 ±2.16b	3.68 ± 1.49b
OP5	32.01 ± 8.81a	0.00 ± 0.00b	4.45 ± 2.25b	3.87 ±1.94b
BF	492.05 ± 48.55a	67.61 ± 16.46c	88.21 ± 14.12bc	115.67 ± 43.74b
FF	121.58 ± 23.30a	25.14 ± 7.15c	52.92 ± 7.46b	16.74 ± 6.72c
PP	156.91 ± 9.83a	56.28 ± 4.89b	11.45 ± 3.97c	17.98 ± 6.55c
OP(c-p 4-5)	42.26 ± 13.04a	2.42 ± 1.21b	6.61 ± 2.66b	7.84 ± 3.72b
c-p 1-2	727.47 ± 49.04a	130.48 ± 14.24b	150.12 ± 21.96b	125.92 ± 24.92b
c-p 3-5	85.71 ± 30.07a	20.32 ± 6.31b	8.84 ± 6.68b	27.06 ± 7.40b
Relative abundance (%)				
	CK	Cd-5	Hg-20	Cd-Hg5+20
BF	60.69 ± 4.31b	42.00 ± 3.06c	55.52 ± 0.50b	73.43 ± 5.71a
FF	15.30 ± 3.23b	12.21 ± 2.43b	30.54 ± 1.27a	7.66 ± 1.63c
PP	18.17 ± 1.69b	38.85 ± 7.75a	9.70 ± 1.57b	12.59 ± 4.84b
OP(c-p 4-5)	5.84 ± 1.99a	6.94 ± 2.65a	4.23 ± 0.68a	6.32 ± 2.57a
c-p 1-2	89.53 ± 2.31ab	86.33 ± 3.96b	94.58 ±2.09a	82.10 ± 5.07b
c-p 3-5	11.62 ± 3.33ab	14.67 ± 3.96a	5.42 ± 2.09b	17.90 ± 5.07a

Mean values in a line with different letters are significantly different from each other at $P < 0.05$; values are expressed as mean ± standard error ($n = 3$).
BF: bacterivores, FF: fungivores, PP: plant parasites, OP: omnivores-predators, numbers following the letters in Guild indicate the c-p value of each taxon

Nematode trophic structure

Distribution of soil nematodes within four trophic groups reflects their food-web relations and helps to investigate the trophic structure inside nematode community. Different trophic groups of nematode demonstrated their varied ability to adapt to the environment in present study. Bacteriovores are considered as species insensitive or resistant to various disturbances of environment (Nagy *et al*., 2004). Cd-Hg 5+20 which could produce greater side effects presented highest proportion of bacteriovores (Table 4). Among all the bacteriovores, *Acrobeloides*, *Rhabditis* were the dominant genera, which are partially comparable with results by Zhang *et al*. (2011). Martinez *et al.* (2012) demonstrated that the series of Cd concentrations did not significantly affect *Acrobeloides*. In contrast, the abundance of *Acrobeloides* in contaminated soil was extremely lower than in unpolluted soil. The steep decrease of *Acrobeloides* may result from the fact that heavy metal which likely concentrated around rhizosphere lead to the irregular ultrastructural changes in esophageal and intestinal cells of nematode, which cause the conflict of nutrient absorption and digestion (Harada, 2006). Consequently, Cd can decrease body growth (Álvarez *et al*., 2006) and reproductive capacity (Harada, 2006), which contributed to the lowest values for both absolute and relative abundance of bacteriovores.

Plant parasites may be affected more by vegetation than contents of heavy metals (Šalamún *et al*., 2011). Plant parasites were the second abundant trophic group (Table 4), and their proportion peaked at Cd-5. Jin and Wang (2019) found Cd (<30 mg/kg) promoted the growth of plants, moreover Cd could combine with car-

boxyl functional groups, cellulose, proteins, lignin or hemicellulose on the cell wall to form precipitation, which helps plant parasites easily pierce the skin of roots and enhances their growth and reproduction. *Helicotylenchus* feeds mainly on the outside of the plant root (Sasser, 1989) only appeared at Cd-5 and Cd+Hg 5+20, our result was partially in accordance with findings by Šalamún *et al.* (2011) who reported *Helicotylenchus* was dominate genus in heavy metal polluted soil. Plant feeding nematodes *Paratylenchus* was negatively correlated with the heavy metals (Zhang *et al.*, 2007). Our data showed that *Paratylenchus* was the second abundant genus among all genera, its sub-adult stage made it have more advantage in adaptability. Ekschmitt *et al*. (2006) suggested *Paratylenchus* was a good candidate for substance-specific bioindication of Cr.

High ratio of fungivores compared with bacterivores may be a mark of heavy metal contamination (Bongers & Bongers, 1998). The highest proportion of fungivores at Hg-20 resulted in the highest value of f/b. Our finding was partially in line with Pen-Mouratov *et al*. (2008) who reported that fungivores and plant parasites were the most two abundant groups near the source of heavy metal pollution. Since the functional diversity and genetic structure of microbial communities could be influenced by dramatic increase of Hg (20 mg/kg) (Harris-Hellal *et al*., 2008), and fungi are more tolerant than bacteria to heavy metal pollution (Rieder *et al*., 2013), the relationship between fungivores and their food source fungi in polluted soil should be further investigated .

C-p group structure

Investigation of the c-p group structure of the nematode assemblage is a useful method to detect heavy metal pollution (de Goede *et al*., 1993; Bongers & Ferris, 1999). It is worthy to notice that bacteriovores with c-p1 nematodes were fewest at Cd-5, lower pH might result in the lack of c-p1 nematodes. Similar result can be found in the study of Sánchez-moreno and Navas (2007) who obtained that BF1 and BF2 nematodes were more abundant in the control than the polluted area. Omnivores-predators are always classified as high c-p value groups, as k-strategists they are sensitive to environmental changes, so omnivores-predator was the least abundance trophic group in this investigation. However, the proportion of c-p4-5 didn't show any regular change, this can be explained by the significant decline of the total abundance of nematodes in polluted soil. Šalamún *et al*. (2011) also discussed that the proportion of omnivores-predator was surprisingly relatively constant, fluctuating around 15 % at all sites. It is well known that c-p 3 group is more sensitive than c-p 1 and 2 group. Interestingly, nematodes classified as c-p 3 from different feeding groups all showed a tolerant response to disturbance rather than c-p 1-2 group at Cd-5. This finding is partially in accordance with Korthals *et al*. (1996) who reported that some c-p groups with lower values were as sensitive as groups with higher values. Another interesting phenomenon was that even the two genera have the same feeding type and c-p value, could have distinct responses to heavy metal pollution such as *Acrobeles* and *Acrobeloides*. Martinez *et al*. (2012) suggested that different bacterivorous species have different pollutant tolerances which influence their ecological interactions, then lead to a higher population fitness of one species under intensive pollution. The above analyses indicated that more deep investigations should be conducted to evaluate the relationship between the c-p groups and the sensitivities to disturbance especially in a short-term experiment.

Conclusions

After 3 months application, morning glories absorbed small part of heavy metals. Heavy metals had a deleterious effect on soil nematode assemblage, decreased nematode abundance, changed the structure of feeding groups and c-p groups. Single Hg seemed to have smaller impacts than Cd, and single Cd and Hg had fewer side effects than Cd-Hg on nematode communities. Direct analysis of nematode abundance, diversity, trophic and c-p groups could be more useful tools than some indices to assess the degree of soil disturbance in the short-term high level pollution experiment.

Conflict of interest statement

The authors declared that they have no conflicts of interest to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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Case Report

The fatal case of an autochthonous heartworm disease in a dog from a non-endemic region of south-eastern Slovakia

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Summary

During the past few years, several localities with increasing *Dirofilaria immitis* occurrences have been identified in Slovakia; particularly in areas regarded as endemic for *Dirofilaria repens* up until now. In terms of that, dogs with clinically manifested heartworm disease have been referred to the veterinary ambulances more frequently. We report in this study, two autochthonous cases of *D. immitis*-infections diagnosed in two seven-year-old siblings of Tibetan Mastiff dogs from the Košice region of south-eastern Slovakia. The course of the disease in both dogs were very different. The female dog did not manifest any unusual findings, however the male dog exhibited severe clinical signs of heartworm disease that lead to his death. The subsequent autopsy revealed adult *D. immitis* worms in the right heart ventricle and pulmonary arteries.
Keywords: *Dirofilaria immitis*; heartworm disease; fatal outcome; dog; Slovakia

Introduction

The causative agent of canine heartworm disease is the mosquito-borne *Dirofilaria immitis* (Spirurida: Onchocercidae). The disease within Europe is wide-spread mainly in the temperate Mediterranean regions of Italy, Spain, Portugal and Greece (Simón *et al*., 2012). In other parts of Europe a related species (*Dirofilaria repens*) is responsible for the less serious subcutaneous and ocular dirofilariosis which occurs more frequently (Capelli *et al*., 2018). In Slovakia, the first cases of canine dirofilariosis were diagnosed fifteen years ago (in 2005) in the south-western part of the country. During the first epidemiological monitoring, encompassing an investigations of more than 4,000 dogs, *D. repens* was detected with a mean prevalence ranging between 2 % and 25 % depending upon the region. On the other hand, *D. immitis* was confirmed very rarely; between 2005 and 2015 only ten dogs were reported to have been infected (Miterpáková *et al*., 2016). This situation has been altered during the last five years and *D. immitis* has increased its prevalence. Only recently, have heartworm cases been detected in a breeding establishment in south-western Slovakia (Miterpáková *et al*., 2018) and new autochthonous infections have been recorded regularly during routine diagnostic procedures at the Institute of Parasitology SAS (IP SAS).

The clinical presentation of heartworm disease is very heterogeneous; from no symptoms (for several months, even years) up to a serious "caval syndrome" leading to the displacement of worms from the pulmonary arteries into the right cardiac chambers. Liver congestion and jugular pulsation may be observed as a result of the tricuspid valve obstruction by the parasites. This condition is often associated with anaemia, haemoglobinaemia and haemoglobinuria. Changes in haematological and biochemical profile often become evident only during the last stages of the infection (ESDA, 2017).

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Here, we report for the first time a fatal case of heartworm disease in a dog from a previously non-endemic area in south-eastern Slovakia.

Cases presentation

The patients described here were two seven-year-old canine Tibetan Mastiff siblings; an intact male (Dog 1) and a female (Dog 2) who were kept together by the same owner. The dogs had never been treated with heartworm preventatives, no anti-ectoparasites prophylaxis had been used, and even routine vaccinations had been omitted in the last few years.

Dog 1

In December 2019, a 7-year-old 50 kg male Tibetan Mastiff dog was referred to the veterinary hospital at the University of Veterinary Medicine and Pharmacy (UVMP) in Košice because of acute weakness and apathy. He suffered from anorexia and vomiting. The clinical examination revealed: an elevated body temperature (39.8 °C), anaemic mucous membranes and pain in the abdomen. Epistaxis from both nostrils was also observed. The breathing frequency and pulse were normal. The lymph nodes were not enlarged, auscultation of the heart was normal, without any murmurs, and the respiratory sounds were normal. No jugular vein pulsations were observed. During the hospitalization, the patient was found to be oliguric.

A blood examination and ultrasonography (USG) of the abdomen were performed. The haematological examination revealed a leucocytosis and neutrophilia; biochemistry showed an elevated alkaline phosphatase. Other parameters were within the normal reference ranges. However, the creatinine and urea levels started to rise sharply during following days. The USG exam revealed an increased liver hyperechogenicity, mild hepatomegaly with highlighted portal veins and the presence of the sediment in the gallbladder. The stomach contractions and intestine peristalsis were slowed down, with fluid stagnation in the stomach. The caecum was filled with fluid as well as some segments of the small intestine. The urinary bladder was distended with significant sediment inside. The prostate and both of the kidneys were of normal size and echogenicity.

Canine pancreatic lipase test (SNAP® cPL™ Test IDEXX Laboratories, Inc., Westbrook, ME, USA) was performed with positive results. The SNAP® Lepto test (IDEXX Laboratories, Inc., Westbrook, ME, USA) for the detection of *Leptospira*-antibodies was negative.

The specific gravity of the urine was measured with a value of 1018 kg/m^3; the pH of the urine was 6.0. Leukocytes, erythrocytes, squamous and tubular cells were detected in the urinary sediment. The dog was also tested using a rapid test system registered for the detection *of D. immitis* antigen and specific antibodies to synthetic peptides of *Anaplasma phagocytophilum*/*Anaplasma platys, Borrelia burgdorferi s.l.*, *Ehrlichia canis* and *Ehrlichia ewingii*

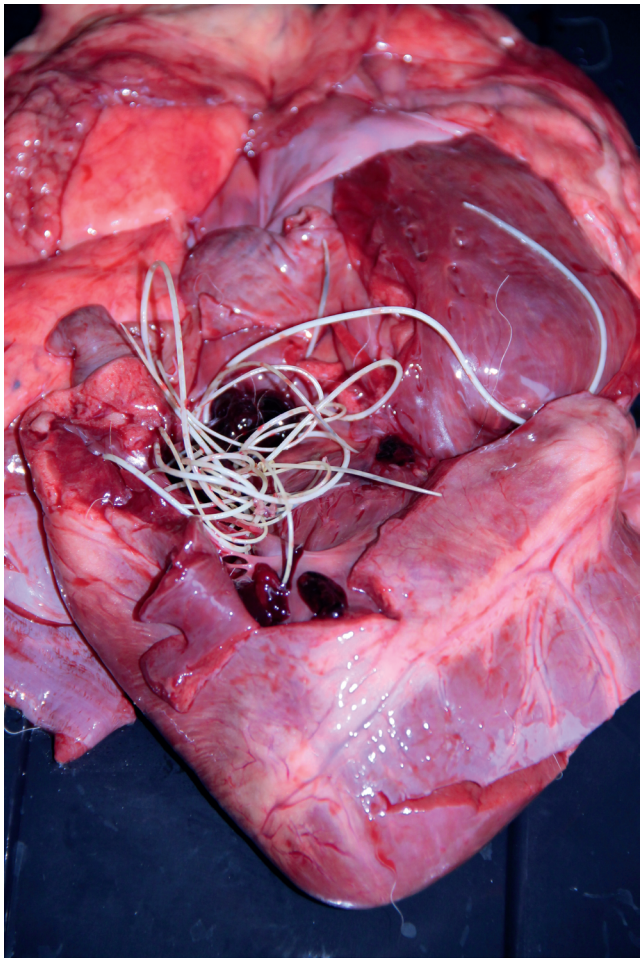

Fig. 1. Adult *Dirofilaria immitis* worms in the right heart ventricle of Tibetan Mastiff male dog.

(SNAP® 4Dx® Plus, IDEXX Laboratories, Inc., Westbrook, ME, USA) with positive results for *D. immitis*. Subsequently, the diagnosis was confirmed at IP SAS, Košice, using the PCR approach. Briefly, DNA was extracted from the blood using DNeasy Blood and Tissue Kit (Qiagen, Germany) and subsequently fragments of cytochrome *c* oxidase were amplified with *D. immitis* and *D. repens* specific primers using conventional PCR (Rishniw *et al.,* 2006).

The cardiologic examination was planned, however, the condition deteriorated and dyspnoea appeared. The X-ray revealed an alveolar pattern in the cranial lung lobes and the heart silhouette was not visible due to changes in the lungs. The dog died after 5 days and no other examinations could be performed. The dog was submitted for post-mortem examination and adult *D. immitis* worms were found in the right heart ventricle, and several specimens were identified in the pulmonary arteries (Fig. 1). In addition, pulmonary oedema was discovered and the right ventricle was dilated. The necropsy findings included: chronic interstitial nephri-

tis and chronic cystitis, acute catarrhal jejunitis, sediment in the gallbladder and icterus. Cardiovascular and respiratory failure in connection with kidney damage was assigned as an immediate cause of the death.

Dog 2

Another dog from the same owner was referred to our clinic several days later. This 7-year-old female Tibetan Mastiff, was born in the same litter as dog 1 and they were kept together in the same household. This dog did not manifest any signs of the heartworm disease, but numerous microfilariae were detected in a blood smear. Additionally, a SNAP® 4Dx® Plus Test was performed for the detection of circulating *D. immitis* female antigen and was positive. The DNA analysis unambiguously confirmed the heartworm infection. The results of further haematological and biochemical examinations yielded values within the normal reference ranges. The value of renal biomarker SDMA (symmetric dimethylarginine) was 8 µg/dL (reference interval for adult dogs: 0 – 14 µg/dL) and the echocardiography revealed no abnormalities.

An alternative therapeutic protocol with macrocyclic lactones and doxycycline was proposed in the female patient due to the unavailability of the melarsomin dihydrochloride (Savadelis *et al*., 2017; ESDA, 2017) regimen. Eventually, the treatment selected involved monthly applications of imidacloprid/moxidectin (10 %/2.5 %) for 9 months and doxycycline administration at a dose of 10 mg/kg twice daily for the first 30 days. Additionally, prednisone was administered at a dose of 0.5 mg/kg twice daily for the first week, then it was reduced to 0.5 mg/kg once daily for another week and subsequently the dose of 0.5 mg/kg every 48 hours was given during the third and fourth weeks.

Currently (in February 2020), the treatment is still in progress and the patient is monitored at the University Hospital of UVMP in Košice.

Discussion and Conclusions

Both dogs came from the village of Nižná Myšľa situated 15 km from Košice which is the largest city of eastern Slovakia (48°37'25"N; 21°21'55"E). In this region, canine dirofilariosis, caused by *D. repens*, was first diagnosed in 2007. Since then, a total of 948 blood samples of dogs living in the districts of Košice and Košice-Okolie (eng. Košice-Surrounding) have been examined for microfilariae at IP SAS until the end of 2019. Of them, 89 were tested positive for *D. repens* (9.4 %). On the contrary, *D. immitis* was confirmed only in five dogs; in 2011 (a dog from Čaňa village in Košice-Okolie district), in 2017 (a dog from Košice city) and finally, in 2019 in three dogs from Nižná Myšľa, Košice-Okolie, including the two cases presented in this report. It was confirmed that the autochthonous heartworm infections occurred in that village as no travelling history was mentioned in the anamnesis. Anyway, in recent years, localities with increased occurrences of *D. immitis* were identified in Slovakia. Interestingly, these areas have been regarded as endemic for *D. repens* until now (Miterpáková *et al*., 2018). In this regard, the competent authorities, including veterinary practitioners in affected regions should take note. The significance of preventive measures should not be underestimated and dogs should be periodically monitored for microfilariae and for circulating antigens, especially in localities where dirofilariae were previously detected, because microfilaremic dogs serve as an important reservoir of the parasite for both humans and other animals (Stoyanova *et al*., 2019; Velev *et al*., 2019). Also, taking into account the recent global trends in traveling and trading, the risk of accidental imported heartworm infections is constantly increasing (Sabūnas *et al*., 2019).

Ethical Approval and Informed Consent

No animals were killed for any purpose in this study.

Conflict of interest

The authors declare that they have no conflict of interest.

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Case Report

First documented cases of *Pearsonema plica* (syn. *Capillaria plica*) infections in dogs from Western Slovakia

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Summary

Three clinical cases of dogs with *Pearsonema plica* infection were detected in the western part of Slovakia. All cases were detected within five months. Infections were confirmed after positive findings of capillarid eggs in the urine sediment in following breeds. The eight years old Jack Russell Terrier, one year old Italian Greyhound, and eleven years old Yorkshire terrier were examined and treated. In one case, the infection was found accidentally in clinically healthy dog. Two other patients had nonspecific clinical signs such as apathy, inappetence, vomiting, polydipsia and frequent urination. This paper describes three individual cases, including the case history, clinical signs, examinations, and therapies. All data were obtained by attending veterinarian as well as by dog owners.
Keywords: Urinary capillariasis; urine bladder; bladder worms; dogs

Introduction

Urinary capillariasis caused by *Pearsonema plica* nematode of family Capillariidae is often detected in wild canids. Especially in red foxes (*Vulpes vulpes*), where the *P. plica* infection was confirmed in many European countries. To be exact in Hungary (Sréter *et al*., 2003), Denmark (Saeed *et al*., 2006; Petersen *et al*., 2018), Germany (Bork-Mimm & Rinder, 2011), Italy (Magi *et al*., 2014), Norway (Davidson *et al*., 2006), Estonia (Laurimaa *et al*., 2016), Lithuania (Bruzinskaite-Schmidhalter *et al*., 2011), Bosnia and Herzegovina (Alic *et al*., 2015), and Belarus (Shimalov & Shimalov, 2003). In all these studies, the prevalence ranged from the lowest 21.3 % in Belarus and the highest in 93.3 % in Lithuania. Similarly Franssen *et al*. (2014) recorded *P. plica* in four out of four red foxes from Netherlands. Foxes as the hosts are considered to be the wild reservoirs of *P. plica* in the natural environment (Mariacher *et al*., 2016) and their role in epidemiology of bladder worms seems to be very important (Petersen *et al*., 2018). The prevalence in domestic dog population is unknown. The occurrence of *P. plica* in domestic dogs was observed and described in quite a few case reports from Poland (Studzinska *et al*., 2015), Italy (Callegari *et al*., 2010; Mariacher *et al*., 2016), and Switzerland (Basso *et al*., 2014).

The life cycle of *P. plica* comprises of obligate intermediate hosts. The earthworms of the Lumbricidae family, such as *Lumbricus terrestris, L. rubellus,* and *Dendrodrilus rubidus.* A dog or fox will become infected after ingestion of earthworms containing infective first-stage larvae of *P. plica* (Moravec *et al*., 1987). Petersen *et al*. (2018) considers also another way of transmission, which is typical by feeding on paratenic hosts, such as rodents and birds.

Case I: Jack Russell Terrier

Urinary capillariasis was diagnosed for the first time in eight years old Jack Russell Terrier female primarily presented with pronounced apathy. The animal owner also described the other

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Fig. 1. *Pearsonema plica* capillarid egg.

symptoms, such as inappetence, vomiting, and polydipsia. Clinical examination confirmed slight dehydration, pink sticky mucous membranes, and prolonged capillary refill time. The body temperature was 38 °C. Lymph nodes were not enlarged or changed and abdominal pain was not present. The urine obtained by cystocenthesis was odorless, with pH 7, and of a yellowish color. The urine sample was centrifuged (2000 RPM/5 min). Microscopic examination of the urine sediment confirmed the presence of *Pearsonema plica* capillarid eggs (51 eggs in 0.1 mL) (Fig. 1) and bacterial infection associated with cystitis was also present. Ultrasonography revealed thickness of the urinary bladder wall and confirmed the presence of few very small uroliths. No history of a urinary tract disease was detected until this examination. Hematological examination confirmed eosinophilia, leukocytosis, and thrombocytosis. Biochemical examination indicated on increased level of urea concentration (15.8 mmol/L; normal range 2.5 – 9.6).

The dog has lived outdoors in the owner's garden in Bratislava and never went to the other places, except the veterinary clinic visits. Dog was not constantly under supervision. The owner did not exclude the possibility that the dog may have eaten intermediate hosts. Therapy applied therapy was Fenbendazole at the dose of 50 mg/kg for 4 days. The dog's condition improved within few days after the therapy initiation. The symptoms observed by dog owner have disappeared and dog became lively and active. Control examination was performed on day 13 and at that time the animal's condition was very good. However, the eggs of *P. plica* were still present in the urine sediment (39 eggs in 0.1 ml). The Fenbendazole therapy had to be repeated for another four days. Although a follow-up veterinary check-up examination was recommended the animal owner did not bring the patient for another examination. Therefore, the efficiency of the treatment could not be verified.

Case II: Italian Greyhound

Five months later, another case of urinary capillariasis was confirmed. The infection of *P. plica* was found in the one year old Italian Greyhound male from Šamorín (Western Slovakia) living indoors in its owner's flat. The dog had no clinical signs and capillarid eggs were found accidentally during the pre-operative assessment prior stomatological treatment. Beside the eggs, many struvite crystals were present in the urine sediment as well. Within two months five different biochemical blood examinations were performed. Each measurement confirmed an increased concentration of urea (ranging from 12.9 to 19.7; norm < 9.6). The other renal function parameters (CREAT, PHOS, and SDMA) were normal and the average number of capillarid eggs per 0.1 mL in urine sediment was 10. Four day Fenbendazole therapy was applied to treat parasitic infection. The treatment had to be repeated after 12 days due to reappearance of *P. plica* eggs (8t eggs in 0.1 ml of urine sediment). Following second treatment the egg production stopped and the urea level returned to normal. Two months later, control examinations confirmed the efficiency of the therapy. The dog was finally cured.

Case III: Yorkshire Terrier

During the treatment of previous case another case of *P. plica* infection emerged. The eleven years old Yorkshire Terrier female from Bratislava was examined due to the loss of appetite and frequent urination. The dog lived indoors and owner excluded any contact with intermediate hosts. Biochemical examination revealed slightly increased urea level in blood (9.35; norm <8.05). Together with capillarid eggs in the urine sediment (15 eggs in

0.1 ml), the transitional epithelial cells and erythrocytes were present and proteinuria was confirmed. The results of the X-ray examination and ultrasonography were normal. The dog was treated with Fenbendazole (50 mg/kg for 4 days). Unfortunately, 3 days after the therapy initiation she died due to the obstruction of esophagus with a chicken bone. The owner refused the autopsy. Therefore, the post-mortem examination of the urine bladder could not be performed.

Discussion

Since October 2018, three different clinical cases of *P. plica* infections were observed in the western part of Slovakia. Historically, only a little attention was paid to this helminth species and no such findings in our country have ever been published. In this paper, one veterinarian identified three naturally infected dogs within a relatively short time period (5 months). The urine sediment of all examined dogs contained typical thick wall and bipolar plugs capillarid eggs. The clinical signs in individual dogs ranged from none to quite serious. Inappetence was observed in two of three dogs. The bladder worm infection in Yorkshire Terrier was associated with pollakiuria, proteinuria, and the presence of erythrocytes as well as epithelial cells in the urine sediment. This was very similar to the clinical case in Switzerland where the same symptoms were observed (Basso *et al*., 2014). Erythrocytes, leucocytes, and transitional epithelial cells were found in the urine sediment of an infected dog in Italy (Callegari *et al*., 2010). This was similar to another case of canine urinary capillariasis in Poland in which the erythrocytes and leucocytes were detected along with bacteria and struvite crystals (Studzinska *et al*., 2015). Large number of erythrocytes and leucocytes were also seen in urine of infected dog from Netherlands (van Veen, 2002). The cystitis confirmed in Jack Russel Terrier is one of the most common clinical signs associated with *P. plica* infection generally detected in dogs (Mariacher *et al*., 2016), foxes (Alic *et al*., 2015; Fernández-Aguilar *et al*., 2010), wolves (Mariacher *et al*., 2015), and cats (Rossi *et al*., 2011). However, the pathologic effects of bladder worms in canids include also: reddish and thickened bladder mucosa (Alic *et al*., 2015; Callegari *et al*., 2010), inflammatory reactions and edema of submucosa of the bladder and ureter (Senior *et al*., 1980), chronic inflammation of the urine bladder and renal pelvis, nephritis (Callegari *et al*., 2010), and glomerular amyloidosis (Callegari *et al*., 2010; Mariacher *et al*., 2016). Additionally the renal failure and urethral obstruction was observed in cats (Rossi *et al*., 2011).

Regarding diagnostic methods the urine sedimentation technique usually detects the presence of *P. plica* eggs. Maurelli *et al*. (2014) used FLOTAC and Mini-FLOTAC quantitative techniques for diagnosis of capillarid eggs in dog urine. Both above mentioned methods are considered more sensitive than standard sedimentation technique.

In our study, biochemical blood examinations revealed elevated urea in all three dogs. This might be associated with the *P. plica* infection. In an Italian Greyhound case we had the opportunity to compare urea values during and after the Fenbendazole treatment. When the parasitic infection was completely cured the urea level returned back to normal. Callegari *et al*. (2010) also measured the level of urea in a dog with the *P. plica* infection and found the same elevated urea concentration. The two other dogs from our study could not be examined repeatedly. As a consequence we do not know if the urea decreased after the Fenbendazole therapy. The thickness of the urinary bladder wall confirmed in Jack Russell Terrier might also be related to the urinary capillariasis. The same finding was described by Basso *et al*. (2014).

The data regarding the treatment of bladder worms are inconsistent and the experiences with particular drugs vary. In some cases Fenbendazole seemed to be efficient (van Veen, 2002; Mariacher *et al*., 2016), and according to the other authors this therapy has failed. Instead of Fenbendazole, Kirkpatrick & Nelson (1987) and Studzinska *et al*. (2015) used Ivermectin to treat dog successfully. Del Angel Caraza *et al*. (2018) eliminated the infection with Fenbendzole in a dog and Ivermectin in a cat. On contrary, Basso *et al*. (2014) considered Ivermectin, Fenbendazole and Moxidectin-imidacloprid as inefficient. Based on their experience and due to high excretion of metabolites via urine Levamisole is the most appropriate drug for the treatment of the urinary bladder capillariasis. In our study, the Italian Greyhound was successfully treated with Fenbendazole. At the beginning of therapy the dose 50 mg/kg for 4 days was used for each dog. The veterinarian counted number of eggs in 0.1 ml of urine sediment at each examination. Although the urine sedimentation technique is considered as qualitative method with low sensitivity (Maurelli *et al*., 2014) the vet found out that the number of eggs decreased as the patient´s health condition was improving. The information whether the infection in Jack Russel Terrier has been definitely eliminated is missing, but the clinical signs observed at the first examination such as apathy, inappetence, vomiting, and polydipsia were not present during second vet checkup. The Yorkshire Terrier died shortly after initiation of treatment. Therefore it was impossible to monitor treatment efficacy. In general, Fenbendazole was efficient, or partially efficient, in all these cases.

The transmission of *P. plica* by earthworms is well known where intermediate hosts are essential for the development of this parasite. Many authors suggest that the ingestion of earthworms by dogs is the main source for the bladder worm infection (Fernández-Aguilar *et al*., 2010; Bork-Mimm & Rinder, 2011; Mariacher *et al*., 2016; Petersen *et al*., 2018). The importance of paratenic hosts is also under consideration, but there is a lack of relevant information. No direct life cycle has been experimentally demonstrated (Senior *et al*., 1980).

With regard to our study, it is not clear how dogs acquired the infection. Two dogs lived indoors and one outdoors and all of them in urban areas in the Western Slovakia. The owner of the Yorkshire Terrier excluded the consumption of earthworms. A contact between the Jack Russel Terrier and the Italian Greyhound and an

intermediate host could not be absolutely ruled out. According to Petersen *et al*. (2019), foxes can acquire the infection by feeding on rodents and birds as a paratenic hosts. This way of transmission might be possible in dogs and cats as well. The consumption of earthworms by cats is not unusual while rodents and birds are common preys of felids. For that reason, it is necessary we should to consider also another ways of transmission.

This study shows that dogs living exclusively in urban areas are also at the risk of worm bladder infection. It means that infected intermediate hosts could be present not only in rural areas, but also in big cities, such as Bratislava. Similar results were found in urban areas in Mexico where domestic animals (dogs and cats) were infected, but no wild animal as a definitive host of *P. plica* did occurred in the area (Del Angel Caraza *et al*., 2018).

Based on our findings, more attention should be paid to the parasitological examination of urine sediments in dogs and cats. This may reveal more other cases of *P. plica* infection and also provide more information about urinary capillariasis. Additional data will help to clarify how domestic dogs and cats acquire this infection.

Ethical Approval and Informed Consent

No animals were killed for the purpose of this study.

Conflict of interest

Authors state no conflict of interest.

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Research Note

A controlled study on efficacy and egg reappearance period of Ivermectin in donkeys naturally infected with small strongyles

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Summary

The aim of the present study was to investigate the efficacy and the egg reappearance period (ERP) of ivermectin (IVM) in donkeys during a 13-week period. The study involved a total of 14 adult Amiata breed donkeys, 7 – 13 years of age, and naturally infected with small strongyles. A group of 10 donkeys was treated with IVM oral paste at a dose rate of 200 mcg/kg BW. Another group of 4 donkeys was kept as untreated control group. Faecal samples were collected and examined for strongyle eggs on day 0 before treatment. IVM efficacy was based on the faecal egg count reduction test (FECRT) on day 14 post-treatment. Then individual faecal samples were collected and examined by FECRT at weekly intervals. A FECRT of 100 % was found after treatment with IVM and its ERP, defined as the week when the mean FECRT decreased until to become lower than 90 % efficacy, was estimated to be 11 weeks without signs of developing anthelmintic resistance. No adverse reactions were observed during the study period. Our findings may be useful to veterinary practitioners and breeders as they show that IVM, at the recommended dose rate, can be still considered a highly effective and safe pharmacological tool for the treatment of small strongyles in donkeys. Therefore, it is strongly recommended that all possible strategies are undertaken to avoid the risk of emergence of anthelmintic resistance to IVM in donkeys.
Keywords: donkeys; small strongyles; ivermectin; efficacy; egg reappearance period

Introduction

Donkeys (*Equus asinus*) are very important working animals as they provide vital support to human beings in many developing countries. Moreover, they are increasingly used for recreational activities (agritourism, trekking), onotherapy (Borioni *et al.,* 2012), meat production (Polidori *et al., 2008*), and mostly milk production, since jenny's milk can be used in children with allergy to cow's milk (Polidori *et al.,* 2013). In addition, the potent anti-proliferative activity of whey protein of donkey milk against human lung cancer cells has been reported (Mao *et al.,* 2009). Therefore, improving the donkey health status is important to owners, farmers, and consumers.

Small strongyles, also commonly called cyathostomins, are among the most important intestinal parasites of donkeys due to the high prevalence values commonly reported in several countries around

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the world (Matthee *et al.,* 2000; Bu *et al*., 2009; Getachew *et al.*, 2010; Matthews *et al*., 2013; Ismail *et al.,* 2016; Jajere *et al.,* 2016; Dibaba *et al.,* 2017). They include more than 40 species and several genera, they belong to the subfamily Cyathostominae and to the family Strongylidae, and they are found in the cecum and colon of domestic equids. It is known that small strongyles can be a threat to donkeys' health and welfare (Matthee *et al.,* 2002; Yoseph *et al.,* 2005; Orian *et al.*, 2015; Waqas *et al.,* 2015).

The control of endoparasites, including small strongyles, in donkeys depends mainly on the use of anthelmintics at doses determined for horses (Grosenbaugh *et al.,* 2011). These include macrocyclic lactones (i.e. Ivermectin and Moxidectin), benzimidazoles (i.e. Fenbendazole), and tetrahydropyrimidines (i.e. Pyrantel embonate) (Grosenbaugh *et al.,* 2011; Matthews *et al.,* 2013). However, the first report of resistance to moxidectin in cyathostomins was observed in donkeys (Trawford *et al.,* 2005). High levels of widespread resistance to fenbendazole have been reported in cyathostomins worldwide (Matthews *et al.,* 2013), including in a donkey farm in Italy (Buono *et al.,* 2018). Cases of pyrantel resistance of cyathostomins have been reported in two herds of donkeys in the UK (Lawson *et al.,* 2015) and in one donkey farm in Italy (Buono *et al.,* 2018). Conversely, it has repeatedly been shown that the efficacy of ivermectin (IVM) against intestinal strongyles in donkeys is as high as 100 % (Buono *et al.,* 2018; Seri *et al.,* 2005; Imam *et al.,* 2010; Arias *et al.,* 2013; Fangama *et al.,* 2013).

The time interval between the last effective anthelmintic treatment and the resumption of significant strongyle egg shedding is called the egg reappearance period (ERP). The ERP is calculated as weeks post-treatment and this parameter differs for each drug (Nielsen *et al.,* 2019). Thus, it is important to know the ERP of each anthelmintic drug after treatment in the different equine species. However, the ERP after treatment with IVM in donkeys still remains poorly investigated. Indeed, the majority of experimental studies evaluating the efficacy of IVM in donkeys did not investigate the corresponding ERP (Seri *et al.,* 2005; Imam *et al.,* 2010; Fangama *et al.,* 2013). To the best of the authors' knowledge, only two studies have reported data about the ERP in donkeys treated with IVM so far (Buono *et al.,* 2018; Arias *et al.,* 2013). Thus, in order to give further insights, the aim of the present study was to investigate the efficacy and ERP of IVM in donkeys found naturally infected by intestinal small strongyles.

Materials and Methods

Research site and animals

The study was carried out at the Veterinary Teaching Hospital "Mario Modenato" of the University of Pisa (geographical coordinates: latitude 43° 25' 00" N, longitude 10° 43' 00" E) in Tuscany, Central Italy. The site was chosen as having animals, facilities, and management suitable to carry out this type of study. The donkeys were selected from a herd bred at the same site to minimize differences attributable to management. The herd had a history of strongyle infections with lack of clinical signs and ivermectin had been irregularly used in previous years. Ten female and 4 male donkeys of Amiata breed were randomly selected for the trial. The animals were 7 to 13 years of age (median age=10 years) and had not been treated with anthelmintics in the previous 14 months. Selected donkeys were enrolled based on confirmed natural infection with intestinal strongyles on day 0 of the study and were randomly allocated to two groups of 10 (treated group) and 4 (control group) animals, respectively. According to the classification of egg shedding given by Kaplan and Nielsen (2010) for horses, donkeys with a faecal egg per gram count (FEPGC) of 1–≤200 eggs were considered as low shedders, with a FEPGC of >200–≤500 eggs as moderate shedders, and with a FEPGC of >500 eggs as high shedders. All animals had been kept together in paddock throughout the year, but the two groups were kept in two separate pens throughout the study period. Donkeys were fed alfa-alfa hay *ad libitum* and a commercial equine feed (Equifioc®, Molitoria Val di Serchio Srl, Lucca, Italy). Drinking water was supplied ad libitum from the same source for both groups.

Collection of faecal samples

Individual faecal samples were collected from rectum when possible, otherwise a sample of freshly voided faeces was collected from the ground in the paddock (only faeces that were seen to be passed from individual donkeys were taken). Fresh faecal samples were collected from each donkey on day 0 before treatment, on day 14 post-treatment, and at weekly intervals thereafter for 11 weeks until the end of the trial (day 91). After collection, each faecal sample was placed into a separate polythene bag, labelled properly for identification, and transported to the laboratory within 1 – 2 h. All samples were stored at +5°C and examined within 48 hours after collection to reduce the effect of egg hatch, based on general recommendations suggested by Nielsen *et al.* (2010) for horse faecal samples.

Faecal egg per gram counts

The samples were examined for strongyle FEPGC using a commercial sodium nitrate solution with specific gravity of 1.200 (Coprosol®, Candioli Farmaceutici S.p.A., Beinasco (TO), Italy) and the Mini-FLOTAC technique in combination with Fill-FLOTAC which has a sensitivity of 5 EPG of faeces, as previously described (Rinaldi *et al.,* 2014; Bellaw *et al.,* 2018).

Faecal cultures

On days 0 and 70, pooled faecal samples were obtained using 10 g of faeces from each enrolled donkey and were incubated at 27°C for 7 – 10 days for larval development (MAFF, 1986). Third stage larvae were collected with the Baermann technique. One hundred larvae per culture were identified, based on the keys described by Cernea *et al*. (2008) and Kornas *et al.* (2009). If fewer than 100 larvae were present, all larvae were identified.

Treatment

After the initial FEPGC was performed on day 0, all the donkeys were weighted to obtain the body weight (BW) using an electronic scale (Meini Bilance Srl, Fornacette (PI), Italy). These donkeys were administered IVM (Eqvalan® 1.87 % paste, Merial) at the recommended dose rate of 200 mcg/kg BW. A 6.42 gr syringe is useful for treatment of horses of weight up to 600 kg. On day 0, treated donkeys were observed for approximately 5 min after treatment to verify dose retention, as all treatments were given orally, and then periodically for three times in about three hours to record adverse reactions. Animals in the control group remained untreated and were kept in the trial until the end of the study.

Data analysis

Individual FEPGCs and arithmetic means (AMs) of FEPGCs were determined at each sampling time (from day 0 to day 91) both for the treated and the control group. Differences between the AMs of FEPGCs in the two groups were compared by Student's t test and a P value ≤0.05 was considered statistically significant.

In accordance with guidelines recommended by Matthews and Burden (2013) for the control of common helminth infections in donkeys, on day 14 after treatment the efficacy of IVM was evaluated by individual and mean faecal egg count reduction tests (FECRTs) for the treatment group. The following formula was used: FECRT (%) = FEPGC before treatment – FEPGC after treatment / FEPGC before treatment x 100. According to methods proposed by the American Association of Equine Practitioners (AAEP) for parasite control in horses (Nielsen *et al.,* 2019), the results of FECRTs were interpreted as follows: FECRT >98 % = efficacy, FECRT between 95 and 98 % = suspected resistance, FECRT <95 % = resistance. To assess the persistence of efficacy of the treatment with IVM and its ERP in donkeys, individual and mean FECRTs were then scheduled at weekly intervals from day 21 till the end of the study (day 91). For the purpose of this study, the ERP was calculated by the method recommended by guidelines of the AAEP to assess the emergence of anthelmintic resistance (AR) to IVM in horses (Nielsen *et al.,* 2019). Thus, the ERP was calculated as the first week post-treatment when the FECRT falls below a cutoff value of 90 % efficacy (Nielsen *et al.,* 2019).

Ethical Approval and/or Informed Consent

For this study, formal consent is not required. The research related to animals complied with all the relevant national regulations and institutional policies for the care and use of animals.

Results

On day 0 of the study, all the 14 selected donkeys resulted to be coprologically positive for stongyle eggs and were classified as low (n=2), moderate (n=7), or heavy (n=5) shedders. Their FEPGCs ranged from 180 to 770 eggs in faeces of treated donkeys and from 210 to 640 eggs in untreated ones with AMs of 446 and 385 eggs, respectively (Table I).

Neither adverse drug reactions nor other clinical signs were observed in any of the donkeys treated with IVM during the study period. Table 1 shows individual and mean FEPGCs with percent of efficacy of IVM as determined by results of FECRTs at each sampling time. Overall, IVM showed FECRT of 100 % and, thus, 100 % efficacy in each of the treated donkeys from day 14 to day 42 (6 weeks) post-treatment. Later, the efficacy of IVM started to slightly decrease but, nonetheless, the drug still continued to be highly effective (i.e. efficacy >95 %) during four additional weeks. Mean FECRTs varied as follows: 97.7 % (individual FECRT range = from 97.9 to 100 %), 97.4 % (from 89.6 to 100 %), 97.5 % (from 94.8 to 100 %), and 96.7 % (from 93.2 to 99.2 %) on days 49, 56, 63, and 70 after treatment, respectively. Thereafter, IVM progressively lost its efficacy since the mean percent of FECRTs drastically dropped to 89.8 % (from 75 to 95.1 %), 80.9 % (from 64.6 to 93.9 %), and finally 70.8 % (from 38.8 to 85.4 %) on days 77, 84, and 91, respectively. On day 49, 2/10 of the treated donkeys were found coprologically positive and then the number of donkeys becoming coprologically positive slowly increased to 6/10 on day 56, 8/10 on day 63, and finally 10/10 from day 70 onwards. The ERP after treatment was determined to be 11 weeks, as the mean percent of FECRT decreased below the ERP-threshold chosen in the present study, i.e. a cutoff value of 90 % efficacy, on day 77 of faecal sampling.

All the donkeys of the untreated control group were constantly found coprologically positive for strongyle eggs throughout the study period, with individual FEPGCs ranging from 195 to 1575 eggs and mean FEPGCs ranging from 285 to 1043.7 eggs (Table I). No one of them showed clinical signs referable to intestinal strongylosis or to any other pathologic condition. Statistical analysis by Student's t test showed that differences between the AMs of FEPGCs in the treated group and those in the control group after treatment were extremely significant (P=0.0000) at each sampling time.

Results of pre-treatment (day 0) and post-treatment (day 70) faecal cultures and morphological identification showed that only small strongyle larvae were present in samples from all the enrolled donkeys, both from those assigned to the treated group and from those assigned to the control group.

Discussion

Results of our study provide information on the efficacy of IVM against small strongyle infection in donkeys. The Mini-FLOTAC technique in combination with Fill-FLOTAC was used for FEPGCs in the present study. This technique can be considered as the most accurate egg counting method nowadays available in Veterinary Medicine (Bosco *et al.* 2014) and, thus, it allowed an accurate interpretation of the results of FEPGCs which were very reliable. Our findings show that IVM efficacy was very high (100 %) against

Table 1. Efficacy of Ivermectin (200 mcg/kg BW) in donkeys naturally infected with small strongyles. Results are presented as individual and mean faecal egg per gram counts (FEPGCs) on treatment day (day 0) as well as individual FEPGC with faecal egg count reduction test (FECRT %) in the same cell, mean FEPGCs, and mean FECRTs in treated donkeys (I to X) examined at weekly intervals after treatment (days 14 to 91). In addition, individual and mean FEPGCs in untreated control donkeys (XI to XIV) at the same sampling times are also presented.

Days	Treated donkeys												Control donkeys				
	I	II	III	IV	V	VI	VII	VIII	IX	X	Mean FEPGCs	Mean FECRT	XI	XII	XIII	XIV	AM of FEPGCs
0	370	630	410	200	770	700	580	240	180	380	446	–	280	210	410	640	385
14	0	0	0	0	0	0	0	0	0	0	0 [A]	100%	365	280	460	400	376.2 [A]
	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%							
21	0	0	0	0	0	0	0	0	0	0	0 [B]	100%	275	400	525	355	388.7 [B]
	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%							
28	0	0	0	0	0	0	0	0	0	0	0 [C]	100%	465	355	525	485	457.5 [C]
	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%							
35	0	0	0	0	0	0	0	0	0	0	0 [D]	100%	655	310	525	615	526.2 [D]
	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%							
42	0	0	0	0	0	0	0	0	0	0	0 [E]	100%	205	335	325	275	285 [E]
	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%							
49	5	0	0	0	0	0	0	5	0	0	1 [F]	99.7%	845	790	985	855	868.7 [F]
	98.6%	100%	100%	100%	100%	100%	100%	97.9%	100%	100%							
56	10	0	5	0	45	25	0	25	0	5	11.5 [G]	97.4%	505	520	770	660	613.7 [G]
	97.3%	100%	98.8%	100%	94.1%	96.4%	100%	89.6%	100%	98.7%							
63	5	5	10	0	40	25	5	5	0	15	11 [H]	97.5%	475	550	960	1010	748.7 [H]
	98.6%	99.2%	97.6%	100%	94.8%	96.4%	99.1%	97.9%	100%	96%							
70	25	5	5	5	35	20	15	15	5	15	14.5 [I]	96.7%	680	490	785	755	677.5 [I]
	93.2%	99.2%	98.8%	97.5%	95.4%	97.1%	97.4%	93.7%	97.2%	96%							
77	60	45	20	35	75	60	45	40	45	30	45.5 [L]	89.8%	360	390	195	300	311.2 [L]
	83.8%	92.8%	95.1%	82.5%	90.2%	91.4%	92.2%	83.3%	75%	92.1%							
84	80	150	25	35	140	145	85	85	55	50	85 [M]	80.9%	1575	1165	675	760	1043.7 [M]
	78.4%	76.2%	93.9%	82.5%	81.8%	79.3%	85.3%	64.6%	69.4%	86.8%							
91	195	195	60	40	160	125	175	125	110	115	130 [N]	70.8%	715	505	340	625	546.2 [N]
	47.3%	69%	85.4%	80%	79.2%	82.1%	69.8%	47.9%	38.8%	69.7%							

A, B, C, D, E, F, G, H, I, L, M, N Extremely statistically significant differences (P=0.0000)

Cyathostominae larvae in all orally treated donkeys. These findings are supported by the high FEPGCs constantly detected in untreated control donkeys which rule out any influence on the worms' burden in the examined donkeys caused by season or weather conditions occurred during the study period. The present findings are in agreement with those of other reports where an efficacy of 100 % was stated in donkeys at 14 days after administration of a dose of 200 mcg/kg of IVM both in oral (Buono *et al.*, 2018; Imam *et al.*, 2010; Arias *et al.*, 2013) and in injectable formulation (Seri *et al.*, 2005; Fangama *et al.*, 2013). Similar findings were also detected in horses (McFarlane *et al.*, 2010; Larsen *et al.*, 2011). Nevertheless, lower efficacy and complete AR of cyathostomins to IVM are important reasons for concern in horses (Canever *et al.*, 2013; Molena *et al.*, 2018). Moreover, donkey breeders and equine veterinary practitioners working with donkeys have a reduced number of anthelmintics to use, which can lead to continuous and frequent use of a single drug, subjecting cyathostomins to higher selection pressure. This inappropriate approach is considered responsible for the rapid appearance of AR.

The control of small strongyles in donkeys depends largely on the routine administration of anthelmintic drugs at specific times of the year to remove adult parasites, to prevent or minimize contamination of the environment with eggs and larvae, and thereby to disrupt the seasonal cycle of transmission. Knowing the ERP of each anthelmintic drug is of pivotal importance for parasite control in equines, including donkeys, since anthelmintic treatments at intervals equal to or shorter than ERP can provide a selective advantage to resistant strongyle populations (Saeed *et al.*, 2019). Indeed, the frequent exposure of small strongyles to an individual anthelmintic may result not only in the elimination of susceptible adult worms but also in the lack of parasite stages in refugia (encysted larvae, free-living larval stages in environment) that escape the effects of the treatment, though they are still susceptible to the anthelmintic. The refugia subpopulation is not under selection for AR because it is not exposed to the drug at the time of treatment. This subpopulation of worms does not have genes for AR, as such it may dilute and delay the proliferation of those worm populations having resistance alleles and allow the effectiveness of an anthelmintic drug to be prolonged by providing a reservoir for drug-susceptible genes (Saeed *et al.*, 2019). Therefore, treatment intervals with any anthelmintic should be based on its ERP and a shortened ERP is the first indicator of the development of resistance to an anthelmintic drug (Nielsen *et al.,* 2019). Accurate knowledge of the ERP of an anthelmintic is also essential to assess the potential for shedding and contamination of eggs by each animal within any group of adult equines. For this purpose, indeed, individual fecal samples should be examined by FEPGC a minimum of 4 weeks beyond the ERP for the last drug used (Nielsen *et al.,* 2019). Unfortunately, ERP definitions have been reported exclusively for horses but researchers are not in agreement with a common threshold beyond which animals should be retreated, not even in horses. In fact, the criterion determining the ERP varies between authors. Some authors have calculated the ERP as the first week after treatment when eggs reappear in the stool (Little *et al.,* 2013). Other authors have considered the ERP as the week after treatment when the AM of FEPGCs is equal to or greater than a fixed threshold such as 100 (Van Doorn *et al.,* 2012) or 200 (Mercier *et al.,* 2001) eggs. Unfortunately, using all these methods, results are highly biased by the pre-treatment FEPGC levels, since some imply that an anthelmintic must reduce the FEPGC of 100 % to be considered effective, whilst others do not take into consideration that low shedder donkeys can take longer to reach the fixed threshold or may never reach the threshold. A third group of authors have defined the ERP based on results of FECRTs. The ERP is thereby measured by performing FECRTs at weekly intervals and is calculated as the first week after treatment when the mean percent FECRT is lower than a predetermined cutoff level of 80 % (Tarigo-Martinie *et al.,* 2001) or 90 % (Larsen *et al.,* 2011) efficacy. In the present study, the ERP was considered as the week when a <90 % FECRT was observed. This definition was chosen among the different methods reported in literature to calculate ERP because we agree with other authors (Larsen *et al.,* 2011) that it represents a reasonable and reliable approach. Indeed, as the ERP is a measure of the efficacy of an anthelmintic during the weeks after treatment, this method is adapted to the drug under evaluation and sets the cutoff level approximately 10 % lower than the expected 99 – 100 % efficacy of IVM (Larsen *et al.,* 2011). The present study was performed for a 13-week period to determine the ERP of IVM in the treated donkey group as ERP rates of 9 – 13 weeks for IVM have been reported in horses (Nielsen *et al.,* 2019), and the ERP was determined to be 11 weeks. Thus, we found no evidence of shortened small strongyle ERP in donkeys as the ERP we achieved for IVM is consistent with guidelines of the AAEP for parasite control in horses. Moreover, our study with IVM indicates a much longer ERP for cyathostomins in donkeys than the range of ERP (6 – 8 weeks) usually reported in horses in the last ten years, when the drug results to be effective (Nielsen *et al.,* 2019). The present cyathostomin ERP for IVM in donkeys falls within the range of rates previously reported in other studies. One study relied on the first post-treatment positive coprological flotation and showed that the ERP after treatment with IVM plus Praziquantel was 2 and 3 months in a group of 6 European donkeys and 6 African donkeys kept in a zoological park in Spain, respectively (Arias *et al.,* 2013). Another study estimated the ERP based on FECRT calculation and reported that the ERP for IVM was 8 or 12 weeks in two groups of 6 farmed donkeys each in Italy (Buono *et al.,* 2018). Therefore, combining results of the present study with those of similar studies previously reported (Buono *et al.,* 2018; Arias *et al.,* 2013), it can be argued that the ERP after treatment with IVM in donkeys range from 8 to 12 weeks. This is very close to ERP rates (9 – 13 weeks) documented for IVM when the drug was first introduced in horses (Nielsen *et al.,* 2019). However, it is somewhat difficult to compare our results with those of other studies assessing the ERP after treatment with IVM in donkeys due to the lack of

a uniform method to calculate ERP. Moreover, additional reasons for different results may be due to level of parasite burden and age of donkeys as well as to season and weather conditions, since intestinal strongyles are known to reduce the egg shedding when environmental conditions are less favorable for their transmission.

Conclusion

Small strongyles can be a threat to health and welfare of donkeys. The findings of the present study (i.e. 100 % efficacy in all treated donkeys on day 14 after treatment and ERP as long as 11 weeks) show that a single oral dose of IVM paste formulation administered at an estimated dose rate of 200 mcg/kg BW was highly effective and safe to control naturally acquired small strongyle infection in donkeys and ruled out any emerging resistance to IVM in the studied donkey group bred in Italy. This corroborates results of previous studies on efficacy of IVM and its ERP after treatment against small strongyles in donkeys. Therefore, our findings may be useful to veterinary practitioners and breeders as they show that IVM can be still considered as a valuable pharmacological tool to use for deworming programmes in donkeys. Given the increased number of reports of the emergence of AR worldwide (Tarigo-Martinie *et al.,* 2001; Kaplan and Nielsen, 2010; Van Doorn *et al.,* 2012; Canever *et al.,* 2013; Little *et al.,* 2013; Matthews *et al.,* 2013; Molena *et al.,* 2018), including cases in donkeys (Trawford *et al.,* 2005; Lawson *et al.,* 2015 ; Buono *et al.,* 2018), and mostly given the lack of new anthelmintics, our findings are a strong reminder that all strategies needed to prevent and minimize the development of AR to IVM in donkeys must be undertaken. Furthermore, since ERP may be an early indicator of suspected AR, it is advisable that researchers and practicing veterinarians reach an agreement on the definition of ERP in donkeys.

Conflict of Interest

Authors state no conflict of interest.

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Research Note

Preliminary study on the prevalence of endoparasite infections and vector-borne diseases in outdoor dogs in Bulgaria

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Summary

The present work was designed to evaluate the prevalence of gastrointestinal parasites and some vector-borne pathogens in dogs in Bulgaria. A total of 172 owned dogs, keeping outside, were included in the study. Fecal samples were examined using standard flotation and sedimentation methods. Blood samples were processed by Knott's technique, SNAP™ 4Dx Plus Test (IDEXX) and Angio Detect™ Test (IDEXX). The overall prevalence of gastrointestinal parasites was 64.5%. Eggs of hookworms (*Ancylostoma* sp. and *Uncinaria* sp.) were the most frequently detected (54.1%), followed by *Trichuris vulpis* (15.1%), *Capillaria* sp. (11.0%), *Toxocara canis* (6.4%), *Cystoisospora* sp. (4.1%), *Sarcocystis* sp. (2.3%), *Toxascaris leonina* (1.7%), *Taenia* sp. (1.2%) and *Linguatula serrata* (0.6%). In addition, hookworms were the most commonly involved in the cases of single infection (20.3%). Combinations between *Capillaria* sp./hookworms and *T. vulpis*/hookworms were the most common co-infections (4.1% and 2.9%, respectively). Blood samples revealed the presence of antibodies against *Ehrlichia* sp. (13.4%), *Anaplasma* sp. (13.4%) and *Borrelia burgdorferi* (1.7%). Antigens of *Dirofilaria immitis* and *Angiostrongylus vasorum* were detected in 10.5% and 0.6% of the samples tested, respectively. Microfilariae of *Dirofilaria repens* were found in 5.8% of the blood samples. Additionally, the prevalence of *D. immitis* and *Ehrlichia* sp. was significantly higher in adult than in young dogs ($p<0.05$). In contrast, the gender was not considered as a risk factor contributing to the occurrence of infections.

Keywords: prevalence; dogs; gastrointestinal parasites; *Angiostrongylus vasorum*; Bulgaria

Introduction

Dogs are still the most common companion animals establishing more frequent and closer contact with humans than any other pets. Furthermore, various canine parasites are involved in the epidemiology of many parasitic diseases affecting a wide range of domestic and wild herbivorous and omnivorous. Also, the canids act as reservoirs and sources of several zoonotic parasites posing a serious threat to the human health (Xhaxhiu *et al.,* 2011). The children are generally at higher risk of acquiring infections with some parasites (e.g. *Toxocara canis*) than adults due to the habit of placing their fingers in the mouth after playing with dogs or after contact with contaminated soil. In contrast, human hydatid disease is mainly associated with persons, practicing hunting and sheep farming. This dangerous and life-threatening infection is caused by the larval stage of *Echinococcus granulosus*, a cestode, inhabiting the small intestines of various canids as final hosts (Gillespie & Bradbury, 2017).

The prevalence of parasitic infections in dogs depends on several factors such as the lifestyle, deworming frequency, climate

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conditions, and contacts with stray dogs or wildlife (Roussel *et al.,* 2019). Additionally, some owners neglect the prophylaxis of their dogs resulting in contamination of environment with infective parasite stages of high tenacity. Also, the shepherd dogs often have no prophylactic therapy, and the occurrence and intensity of parasite infections, especially with tapeworms, usually results from scavenging sheep carcasses (Vasileiou *et al*., 2015; Rehbein *et al*., 2016).

Canine parasitic fauna has been a purpose of many studies conducted all around the world over the last few decades. Numerous Bulgarian investigations on the same focus have also been carried out, including different categories of dogs, originating from many regions of the country. In addition, gastrointestinal (GI) parasitism of *Trichuris vulpis* and *Capillaria* sp., hookworms (*Ancylostoma caninum*, *Uncinaria stenocephala*), ascarids (*T. canis*, *Toxascaris leonina*), cestodes (*Dipylidium caninum*, *Mesocestoides lineatus*, *Taenia hydatigena*, *Taenia ovis*, *Taenia multiceps*, *Taenia pisiformis*, *E. granulosus*), protozoans (*Cystoisospora* sp., *Sarcocystis* sp., *Giardia* sp.) has been recorded in studies based on necropsy (Kamenov *et al*., 2009); combined necropsy/coproscopy (Georgieva *et al*., 1999; Lalkovski & Sabev, 2009) or coproscopy (Kirkova *et al*., 2006; Kirkova *et al.,* 2013; Kanchev *et al*., 2014; Radev *et al*., 2016; Iliev *et al*., 2017).

Canine vector-borne diseases (CVBDs) constitute an important group of illness caused by a diverse range of pathogens, which are transmitted to both animals and humans by different blood-sucking arthropods (Dantas-Torres, 2008). Those diseases are usually endemic in tropics and subtropics, but an increasing prevalence has also been recognized in temperate regions (Dantas-Torres, 2008; Beugnet & Chalvet-Monfray, 2013). The prevalence of CVBDs in Bulgaria has been frequently reported over the past few years, including infections with *Dirofilaria immitis*, *Dirofilaria repens*, *Ehrlichia canis*, *Anaplasma* sp., and *Borrelia burgdorferi* (Georgieva *et al*., 2001; Tsachev *et al*., 2006; Tsachev *et al*., 2006a; Tsachev *et al*., 2008; Panayotova-Pencheva *et al*., 2016; Radev *et al*., 2016; Iliev *et al*., 2017). Considering the data mentioned above as well as the trend towards increase in canine population in the country focused our attention on performing this epidemiological work. We aimed both at corroborating previously published findings and providing updated information on the prevalence of GI parasites as well as some arthropod-transmitted pathogens in outdoor dogs in Bulgaria.

Materials and Methods

Animals and study areas

This study was performed from July 2016 to September 2017 on 172 owned dogs (115 males and 57 females) aged from 1 month to 12 year, reared outdoor. The animals were recruited from outskirts of Rousse (43º50'N, 25º57'E), Razgrad (43º32'N, 26º32'E), Sofia (42º41'N, 23º19'E) (Northern Bulgaria), Stara Zagora (42º25'N, 25º38'E), and Plovdiv (42º8'N, 24º44'E) (Southern Bulgaria).

Table 1. Overall prevalence (%) of the pathogens in dogs (n=172)

Species	Southern Bulgaria (n=124)	Northern Bulgaria (n=48)	Total (n=172)		Test applied
	Positive (%)		Positive (%)	95% CIs	
D. repens	4 (3.2)	6 (12.5)	10 (5.8)	2.3 - 9.3	Knott's test
D. immitis	14 (11.3)	4 (8.3)	18 (10.5)	5.9 – 15.0	Serology
A. vasorum	1 (0.8)	-	1 (0.6)	0 – 1.7	
T. vulpis	14 (11.3)	12 (25.0)	26 (15.1)	9.8 – 20.5	
Capillaria sp.	10 (8.1)	9 (18.8)	19 (11.0)	6.4 – 15.7	
T. canis	9 (7.3)	2 (4.2)	11 (6.4)	2.7 – 10.1	Flotation
Ancylostoma/Uncinaria spp.	57 (46.0)	36 (75.0)	93 (54.1)	46.6 – 61.5	
T. leonina	1 (0.8)	2 (4.2)	3 (1.7)	0 – 3.7	
Taenia sp.	1 (0.8)	1 (2.1)	2 (1.2)	0 – 2.8	
L. serrata	-	1 (2.1)	1 (0.6)	0 – 1.7	Sedimentation
Cystoisospora sp.	6 (4.8)	1 (2.1)	7 (4.1)	1.1 – 7.0	Flotation
Sarcocystis sp.	4 (3.2)	-	4 (2.3)	0.1 – 4.6	
Anaplasma sp.	16 (12.9)	7 (14.6)	23 (13.4)	8.3 – 18.5	
Ehrlichia sp.	22 (17.7)	1 (2.1)	23 (13.4)	8.3 – 18.5	Serology
B. burgdorferi	2 (1.6)	1 (2.1)	3 (1.7)	0 – 3.7	

Table 2. Cases of single infection in dogs (n=172).

Species	Positive (%)	95% CIs
D. immitis	2 (1.2)	0 – 2.8
Ehrlichia sp.	6 (3.5)	0.7 – 6.2
T. vulpis	6 (3.5)	0.7 – 6.2
T. canis	5 (2.9)	0.4 – 5.4
Ancylostoma/Uncinaria spp.	35 (20.3)	14.3 – 26.4
Anaplasma sp.	6 (3.5)	0.7 – 6.2
D. repens	2 (1.2)	0 – 2.8
Cystoisospora sp.	1 (0.6)	0 – 1.7
A. vasorum	1 (0.6)	0 – 1.7
T. leonina	1 (0.6)	0 – 1.7
B. burgdorferi	1 (0.6)	0 – 1.7

Sampling and assaying

Fecal samples were obtained manually from *ampulla recti*, placed into plastic bags, stored at 4ºC, and processed (within 24 hours) by following methods: direct smear for detection of motile trophozoites or cysts of protozoa; flotation technique, using 3 grams feces and saturated sodium chloride (sp. gr. 1.20), for extraction of lighter helminth eggs and coccidian oocysts or sporocysts; routine sedimentation, using 3 grams feces, for recovering heavier helminth and pentastomid eggs.

Blood samples were collected by venipuncture of *v. cephalica antebrachii*, from each animal, into vacutainers. After clotting the samples, the sera were stored at 4ºC and assayed within 24 hours for detection of *D. immitis* antigens; antibodies against *Anaplasma* sp., *Ehrlichia* sp., and *B. burgdorferi* (SNAP® 4Dx Plus Test, IDEXX) as well as *A. vasorum* antigens (Angio Detect™ Test, IDEXX). Both assays were performed according to manufacturer's instructions. Additional blood samples were collected into sterile tubes with anticoagulant (K_2EDTA) and processed by Knott's technique for detection of filariid first stage larvae. Isolated microfilariae were identified on basis of their morphometrical characteristics (Zajac & Conboy, 2012).

Statistical analysis

All data were analysed by means of MedCalc v.10.2.0.0, MedCalc Software (Belgium). The prevalence and its 95 % confidence interval (CI) were calculated for each parasitic species, including co-infections. The difference of prevalence among groups (regarding to the gender and age) was evaluated by Chi-square (χ^2) test and was considered significant at $P \leq 0.05$.

Ethical Approval and/or Informed Consent

Approvals for using animals in the current study were obtained from the Bulgarian Food Safety Agency (registration of the permits: №85/09.01.2014 and №138/28.06.2016). The research has been complied with all the relevant national regulations and institutional policies for the care and use of animals.

Results

The overall prevalence of GI parasites was 64.5 %. Eggs of hookworms were the most commonly observed in feces (54.1 %), followed by *T. vulpis* (15.1 %), *Capillaria* sp. (11.0 %), *T. canis* (6.4 %), *Cystoisospora* sp. (4.1 %), *Sarcocystis* sp. (2.3 %), *T. leonina* (1.7 %) and *Taenia* sp. (1.2 %) (Table 1). Eggs of the nasal pentastomid *Linguatula serrata* were also detected (0.6 %). Cases of single infection were found in 38.4 % of dogs sampled (Table 2). Mixed infections with two or more species were observed in 20.3 % and 18.0 % of dogs, respectively (Tables 3). The most frequently detected co-infections were with *Capillaria* sp./hookworms (4.1 %) and *T. vulpis*/hookworms (2.9 %).

The serum analysis identified antigens of *D. immitis* and *A. vasorum* (10.5 % and 0.6 % of the samples, respectively) and antibodies against *Anaplasma* sp. (13.4 %), *Ehrlichia* sp. (13.4 %) and *B. burgdorferi* (1.7 %). *Dirofilaria repens* microfilariae were found in 5.8 % of the dogs (Table 1).

The age was identified as a risk factor for *D. immitis* infection (χ^2=4.358). The highest prevalence was observed in dogs above 12 months of age and no case was recognized in younger dogs. Similarly, antibodies against *Ehrlichia* sp. were more commonly detected in the adult animals (χ^2=3.740). Statistical analysis showed no significant association between the gender and prevalence of infections.

Discussion

The findings of this study showed that more than half of the dogs (64.5 %) were infected by at least one species of GI parasite.

Table 3. Cases of mixed infections in dogs (n=172)

Combinations	Positive (%)	95% CIs
Ancylostoma/Uncinaria spp. + *Anaplasma* sp.	2 (1.2)	0 – 2.8
Ancylostoma/Uncinaria spp. + *D. immitis*	5 (2.9)	0.4 – 5.4
Ancylostoma/Uncinaria spp. + *Ehrlichia* sp.	2 (1.2)	0 – 2.8
D. immitis + *Ehrlichia* sp.	1 (0.6)	0 – 1.7
Capillaria sp. + *Ancylostoma/Uncinaria* spp.	7 (4.1)	1.1 – 7.0
Ancylostoma/Uncinaria spp. + *Cystoisospora* sp.	2 (2.1)	0 – 2.8
Ancylostoma/Uncinaria spp. + *Sarcocystis* sp.	1 (0.6)	0 – 1.7
Cystoisospora sp. + *Ehrlichia* sp.	1 (0.6)	0 – 1.7
T. vulpis + *Ancylostoma/Uncinaria* spp.	5 (2.9)	0.4 – 5.4
D. immitis + *T. vulpis*	1 (0.6)	0 – 1.7
T. vulpis + *Anaplasma* sp.	1 (0.6)	0 – 1.7
D. repens + *Ancylostoma/Uncinaria* spp.	4 (2.3)	0.1 – 4.6
T. canis + *Ancylostoma/Uncinaria* spp.	1 (0.6)	0 – 1.7
Ancylostoma/Uncinaria spp. + *T. leonina*	1 (0.6)	0 – 1.7
L. serrata + *Ancylostoma/Uncinaria* spp.	1 (0.6)	0 – 1.7
T. vulpis + *Capillaria* sp. + *Ehrlichia* sp.	2 (1.2)	0 – 2.8
D. immitis + *Anaplasma* sp. + *Ehrlichia* sp.	1 (0.6)	0 – 1.7
D. immitis + *Ancylostoma/Uncinaria* spp. + *Ehrlichia* sp.	1 (0.6)	0 – 1.7
D. immitis + *T. vulpis* + *Ancylostoma/Uncinaria* spp.	2 (1.2)	0 – 2.8
Ancylostoma/Uncinaria spp. + *Anaplasma* sp. + *Ehrlichia* sp.	2 (1.2)	0 – 2.8
D. immitis + *Capillaria* sp. + *Ancylostoma/Uncinaria* spp.	1 (0.6)	0 – 1.7
Cystoisospora sp. + *Ancylostoma/Uncinaria* spp. + *Sarcocystis* sp.	1 (0.6)	0 – 1.7
D. immitis + *Ancylostoma/Uncinaria* spp. + *Sarcocystis* sp.	1 (0.6)	0 – 1.7
T. canis + *Ancylostoma/Uncinaria* spp. + *Sarcocystis* sp.	1 (0.6)	0 – 1.7
T. vulpis + *Capillaria* sp. + *Ancylostoma/Uncinaria* spp.	3 (1.7)	0 – 3.7
Capillaria sp. + *Ancylostoma/Uncinaria* spp. + *Anaplasma* sp.	1 (0.6)	0 – 1.7
D. repens + *Capillaria* sp. + *Ancylostoma/Uncinaria* spp.	1 (0.6)	0 – 1.7
T. vulpis + *Ancylostoma/Uncinaria* spp. + *Anaplasma* sp.	3 (1.7)	0 – 3.7
D. immitis + *T .canis* + *Ancylostoma/Uncinaria* spp. + *Anaplasma* sp.	1 (0.6)	0 – 1.7
B. burgdorferi + *Anaplasma* sp. + *Ehrlichia* sp.	1 (0.6)	0 – 1.7
D. immitis + *D. repens* + *Ancylostoma/Uncinaria* spp. + *Ehrlichia* sp.	1 (0.6)	0 – 1.7
Ancylostoma/Uncinaria spp. + *Taenia* sp. + *Anaplasma* sp. + *Ehrlichia* sp.	1 (0.6)	0 – 1.7
D. repens + *B. burgdorferi* + *Capillaria* sp. + *Ancylostoma/Uncinaria* spp.	1 (0.6)	0 – 1.7
D. repens + *T. vulpis* + *Capillaria* sp. + *Ancylostoma/Uncinaria* spp.	1 (0.6)	0 – 1.7
T. vulpis + *Capillaria* sp. + *Ancylostoma/Uncinaria* spp. + *Taenia* sp. + *Ehrlichia* sp.	1 (0.6)	0 – 1.7
T. vulpis + *T. canis* + *Ancylostoma/Uncinaria* spp. + *Anaplasma* sp. + *Ehrlichia* sp.	1 (0.6)	0 – 1.7
T. canis + *Ancylostoma/Uncinaria* spp. + *Cystoisospora* sp. + *Anaplasma* sp. + *Ehrlichia* sp.	1 (0.6)	0 – 1.7
T. canis + *Ancylostoma/Uncinaria* spp. + *T. leonina* + *Anaplasma* sp. + *Ehrlichia* sp.	1 (0.6)	0 – 1.7
D. immitis + *Capillaria* sp. + *Ancylostoma/Uncinaria* spp. + *Cystoisospora* sp. + *Anaplasma* sp.	1 (0.6)	0 – 1.7

Numerous recent surveys performed on the Balkans have disclosed comparable results indicating the presence of GI parasitism in 67.1 % of dogs in Romania (Ursache *et al*., 2016), 75.4 % in Serbia (Sommer *et al*., 2017), between 48.1 % and 64.9 % in Croatia (Brezak *et al*., 2017), 26 % in Greece (Papazahariadou *et al*., 2007), 30.4 % in Turkey (Senlik *et al*., 2006) and 40.7 % in Albania (Shukullari *et al*., 2015). All the mentioned results were obtained by coprological exaninations. According to the regional reports, between 24.8 % and 65 % of dogs in Bulgaria harbor several species of endoparasites including ascarids, hookworms, whipworms, tapeworms and coccidians (Kirkova *et al*., 2006; Lalkovski & Sabev, 2009; Kirkova *et al*., 2013; Radev *et al*., 2016), which seems to be in general agreement with our finding. The differences between aforementioned results are not unexpected and could be due to various factors. Those investigations include either well- or not well-cared dogs of different ages and categories (e.g. hunting, shepherd, military, pet, shelter and stray dogs) with different deworming frequency (e.g. regular or not), clinically healthy or under veterinary care (with GI disorders). Taking into consideration the above, it can be argued that the combined influence of the age and purpose of dogs, the general hygiene, and the access to regular deworming may exert a marked effect on the prevalence and species variety of GI parasites among the canine population (Shukullari *et al*., 2015). In addition, the strong influence of the geographic location and climate conditions is confirmed by the results of a recent study, demonstrating lower prevalence of GI parasitic infections (9.4 %) even in stray and not well-cared dogs in Germany (Becker *et al*., 2012).

In this study, hookworms were recognized as the most common enteric pathogens, which were found in the fecal samples of more than half of dogs (54.1 %) In similar investigation conducted in Stara Zagora (Bulgaria), the prevalence of *A. caninum* and *U. stenocephala* in stray dogs was even higher, reaching 90 % and 60 %, respectively (Georgieva *et al*., 1999). Other local investigations have indicated that between 6.18 % and 37.8 % of the dogs harbor hookworms (Kirkova *et al*., 2013; Iliev *et al*., 2017). Those findings were expected, taking into consideration the modes of infection transmission. Also, it should not be underestimated the zoonotic potential of hookworms. Once entered the body, the infective larvae of *A. caninum* may induce two serious conditions known as human gut disease (eosinophilic enteritis) and cutaneous larva migrans (CLM) or creeping eruption (Katagiri & Oliveira-Sequeira, 2008). The relationship between *U. stenocephala* and CLM remains unclear and still debated (Villeneuve *et al*., 2015).

The canine whipworm, *T. vulpis*, was the second most frequent GI parasite detected in this study (15.1 %). Our results are in general agreement with those of comparable surveys in dogs in Bulgaria (Kirkova *et al*., 2006; Lalkovski & Sabev, 2009; Kirkova *et al*., 2013; Radev *et al*., 2016; Iliev *et al*., 2017).

The overall number of dogs infected with *T. canis* and *T. leonina* in this study was substantially low (6.4 % and 1.7 %, respectively). As observed in similar Bulgarian investigations, the prevalence of those ascarids ranged from 6.8 % to 17.8 % for *T. canis* and from 0.78 % to 3.1 % for *T. leonina* (Kirkova *et al*., 2006; Kirkova *et al.,* 2013; Kanchev *et al*., 2014; Iliev *et al*., 2017). More importantly, *T. canis* is better recognized as the most common causative agent of visceral and ocular larva migrans in people; both syndromes might lead to severe damages of different tissues, especially in children (Villeneuve *et al*., 2015).

The results of our study showed that 1.2 % of dogs are infected with *Taenia* sp., which is in general agreement with findings of Kirkova *et al*. (2006) and Iliev *et al.* (2017). Those authors also reported a substantially low prevalence of taeniid infections (0.8-1.16 %) in dogs in Bulgaria.

Numerous studies regarding to the prevalence of aforementioned helminth species have been conducted in closer geographical regions, including Croatia (Brezak *et al*., 2017); Romania (Ursache *et al*., 2016), Serbia (Sommer *et al*., 2017); Greece (Papazahariadou *et al*., 2007); Albania (Shukullari *et al*., 2015) and Turkey (Senlik *et al*., 2006). Summarized results indicate the presence of GI parasitism ranging from 1.2 % to 41 % for hookworms, from 2.9 % to 9.6 % for *T. vulpis*, from 3 % to 34.8 % for *T. canis*, from 0.7 % to 21.8 % for *T. leonina* and from 0.3 % to 1.5 % for *Taenia* sp.

Our results showed *Capillaria* sp. infection, which is probably caused by the capillariid lungworm *Eucoleus aerophilus*. We detected eggs of such helminth in 11 % of the fecal samples. That value is very likely to be lower than real percentage because of the eggs may originate not only from adult lungworms, but also after passing through the alimentary tract following ingestion of contaminated food or after coprophagy (Shukullari et al., 2015). The prevalence of *E. aerophilus* ranges from 0.2 % to 2.8 % in European and Balkan countries (Traversa *et al*., 2010; Shukullari *et al*., 2015; Ursache *et al*., 2016; Brezak *et al*., 2017) and from 2 % to 6.8 % in Bulgaria (Kirkova *et al*., 2006; Kirkova *et al.,* 2013).

One case of nasal linguatulosis due to the pentastomid *L. serrata* was recorded here. The adult parasites reside in nasal cavities in dogs but the eggs pass from the respiratory system to intestines and release into the environment through feces. The total prevalence of *L. serrata* in dogs in Bulgaria reaches to 0.7 % (Kirkova *et al*., 2013). However, nymphs of this parasite (known as *Pentastomum denticulatum*) have been recovered from the lungs, liver and mesenteric lymph nodes in Bulgarian goats as intermediate hosts (Ivanov *et al*., 2012). This pentastomid is considered responsible for important zoonotic disease. Furthermore, visceral linguatulosis (pentastomosis) in Bulgaria was reported in a 9-year-old boy in Pleven (Mateva *et al*., 2013).

Our investigation presents the first serologically proven case of angiostrongylosis due to the cardiopulmonary nematode *A. vasorum*. In contrast, several studies have shown such infection in canids in the European countries (Traversa *et al*., 2010; Elsheikha *et al*., 2014) and on the Balkans (Papazahariadou *et al*., 2007; Shukullari *et al*. 2015; Ilie *et al*., 2016).

Protozoa infections were less often identified in this study than

helminth infections (4.1 % for *Cystoisospora* sp. and 2.3 % for *Sarcocystis* sp.). The prevalence of GI protozoa in dogs in Bulgaria varies from 1.97 % to 7.72 % for *Cystoisospora* sp. and from 0.39 % to 0.5 % for *Sarcocystis* sp. (Kirkova *et al*., 2006; Lalkovski & Sabev, 2009; Radev *et al*., 2016; Iliev *et al*., 2017). Our findings are in general agreement with those reported in closer geographical region (Croatia, Romania, Albania, Serbia, Greece), where the overall prevalence ranges from 3 % to 16.1 % for *Cystoisospora* sp. and from 0.3 % to 4.5 % for *Sarcocystis* sp. (Papazahariadou *et al*., 2007; Shukullari *et al*. 2015; Ursache *et al*., 2016; Brezak *et al*., 2017; Sommer *et al*., 2017).

Almost half of the dogs (44.8 %) were positive for vector-borne parasites and bacteria such as *D. immitis*, *D. repens*, *Ehrlichia* sp., *Anaplasma* sp. and *B. burgdorferi*. The prevalence of those pathogens usually depends on several factors, but the age of dogs is considered as an important parameter as we found for *D. immitis* and *Ehrlichia* sp. in current study. Our findings showed that 10.5 % and 5.8 % of dogs were infected with *D. immitis* and *D. repens*, respectively. According to other Bulgarian researchers, between 7.4 % and 16.2 % of clinically healthy dogs and 34.33 % of dogs with cardiopulmonary disorders and under veterinary care are infected with *D. immitis* (Georgieva *et al*., 1999; Georgieva *et al*., 2001; Pantchev *et al*., 2015; Radev *et al*., 2016; Iliev *et al*., 2017). The prevalence of canine heartworm disease due to *D. immitis* is also reported on the Balkans and ranges from 0.7 % to 17.9 % in Greece, from 7.2 % to 22.01 % in Serbia, from 1 % to 27 % in Turkey and from 8 % to 16 % in Croatia (Morchon *et al*., 2012).

Our results also showed infections with *Anaplasma* sp. (13.4 %), *Ehrlichia* sp. (13.4 %) and *B. burgdorferi* (1.7 %). Data of several researches regarding the overall seroprevalence of those tick-borne pathogens among canine population in Bulgaria have been previously reported, indicating occurrence of infections ranging from 21 % to 37.5 % for *E. canis*, from 3.5 % to 46.1 % for *A. phagocytophilum* (Tsachev, 2006; Tsachev *et al*., 2006; Tsachev *et al*., 2006a; Pantchev *et al*., 2015) and from 22.74 % to 74.5 % for *B. burgdorferi* (Angelov *et al*., 1993; Zarkov & Marinov, 2003). Those findings are much higher and are not in agreement with our results. One reason could be that the authors present data obtained from dogs originated from enzootic regions or under veterinary care. However, our results are similar with the findings published by Pantchev *et al*. (2015) who also found a low seroprevalence (2.4 %) of *B. burgdorferi* among dogs in Bulgaria.

The results obtained here indicated that elder dogs (irrespective of sex) were commonly affected by *D. immitis* and *Ehrlichia* sp. Similar findings have also been published earlier (Villeneuve *et al*., 2011; Volgina *et al*., 2013; Hamel *et al*., 2016; Pantchev *et al*., 2015). Other authors have reported that elder dogs, kept outdoor, were more commonly infected by *D. immitis* (Yildirim *et al*. 2007; Cardoso *et al*. 2012; Mircean *et al*. 2012). According to Hamel *et al*. (2016), the occurrence of CVBDs is significantly higher in dogs over one year of age. The gender of animals included in our work was not considered as a risk factor for *Ehrlichia* sp. and *Anaplasma* sp., which coincides with findings reported from other authors (Solano-Gallego *et al*., 2006; Tsachev *et al*., 2006; Tsachev *et al*., 2006a; Villeneuve *et al*., 2011; Miro *et al*., 2013).

Conclusion

This study presents an overview of the prevalence of GI parasites and some vector-borne helminths and bacteria in outdoor dogs in Bulgaria as well as the first report of *A. vasorum*. Our findings demonstrate a wide variety of endoparasites and high prevalence rates of parasitism, suggesting environmental contamination with infective stages of parasites and presence of arthropods carrying different pathogens. Therefore, both the arthropods and infected dogs could be responsible for occurrence of several zoonotic diseases; particularly *T. canis*, *Taenia* sp. (refers to *E. granulosus*), *B. burgdorferi* and hookworms. This statement thus should increase the efforts of veterinarians and owners on performing a regular and proper prophylaxis of dogs against ecto and endoparasites resulting in lower levels of parasitism in both animals and humans.

Conflict of Interest

Authors state no conflict of interest.

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Research Note

Crenosoma striatum in lungs of European hedgehogs (*Erinaceus europeus*) from Portugal

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Article info

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Summary

Crenosoma striatum is a host-specific metastrongiloid nematode causing respiratory tract disease in hedgehogs (*Erinaceus europaeus*). Since few studies have reported *C. striatum* in hedgehogs and little genetic data is available concerning this lungworm, this study aimed to determine the occurrence of *C. striatum* in a population sample of hedgehogs from Portugal, additionally providing morphological, histological and molecular data. From 2017 to 2018 a survey of infection was carried out in 11 necropsied hedgehogs. Worms were extracted from fresh lung tissues and microscopically evaluated. Molecular characterization of partial mitochondrial (12S rRNA) and nuclear (18S rRNA) genes was performed. The presence of lungworms in pulmonary tissues of five hedgehogs (45.5%) was detected. Morphological and histopathological analyses evidenced adult forms of nematodes consistent with *C. striatum*. Molecular characterization of 18S rRNA genes confirmed the classification as C. striatum. Also, novel genetic data characterizing the mitochondrial (12S rRNA) gene of *C. striatum* is presented.

This is the first report of *C. striatum* infection in hedgehogs of Portugal. The findings here reported provide new insights regarding the geographic distribution and the molecular identification of this lungworm species.

Keywords: *Crenosoma striatum;* hedgehog; lungworm; nematode

Introduction

European hedgehogs (*Erinaceus europaeus*) are hosts for a wide variety of parasites namely ticks, fleas, mites and helminths (Pfaffle *et al*., 2014). Several studies demonstrated various species of helminths parasitizing hedgehogs such as *Crenosoma striatum*, *Physaloptera clausa* and *Hymenolepis erinaceid* (Naem *et al*., 2015; Pavlovic & Savic 2017; Raue *et al*., 2017). *Crenosoma* (Nematoda: Metastrongyloidea) is a genus of lungworms of the family Crenosomatidae, and C*renosoma striatum* is a species known to invade trachea, bronchi, and alveolar ducts of hedgehogs (Beck 2007).

The European hedgehog is a synanthropic nocturnal species in Europe that feeds on gastropods like slugs and snails, and which act as definitive or paratenic host of several agents that pose a considerable risk for morbidity and mortality, particularly *C. striatum* (Gaglio *et al*., 2010; Hoseini *et al*., 2014; Riley & Chomel 2005). After a prepatent period of 21 days the worms become sexually mature and the first stage larvae (L1) can be found in the feces (Beck 2007). Hedgehog crenosomosis is generally characterized by weight loss, nasal discharge, increased respiratory effort, cough, and in severe cases death (Hoseini *et al*., 2014).

A growing body of literature have documented the presence of *C. striatum* in hedgehogs, namely in Turkey (Cirak *et al*., 2010),

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Poland (Mizgajska-Wiktor *et al*., 2010), Iran (Hoseini *et al*., 2014; Naem *et al*., 2015), Italy (Manzocchi *et al*., 2016), Serbia (Pavlovic & Savic 2017), Greece (Liatis *et al*., 2017) and Germany (Raue *et al*., 2017).

The aim of the present report was to evaluate the occurrence of *C. striatum* in a population sample of hedgehogs from Portugal., This study also describes the histological features associated with *C. striatum* pulmonary occurrence and provides the molecular characterization of partial 12S and 18S rRNA genes of *C. striatum*. To the best of our knowledge, no study identifying and characterizing *C. striatum* has ever been done in the Iberian Peninsula, and no characterization of the mitochondrial (12S rRNA) ribosomal gene has ever been performed.

Materials and Methods

From January 2017 to October 2018, all deceased hedgehogs (N=11) that were housed at a Rescue and Rehabilitation Center (RRC) in Porto, Portugal, were necropsied at the Veterinary Pathology Laboratory of ICBAS-UP. These animals had been collected from several Portuguese municipalities and sent for the RRC for rehabilitation. During the necropsy examination, representative samples of all macroscopic alterations detected, as well as others from apparently healthy tissues were collected, in order to identify the eventual cause of death and to evaluate the health status of the animals.

For parasitology, nematodes were extracted from fresh lung tissues for identification. Parasites were suspended in sterile saline (0.9 % NaCl) and microscopically examined under glass coverslips for morphological identification.

Nematode-infected animals were subjected to histological examination. Lung tissue samples were fixed in 10 % phosphate-buffered formalin (pH 7.0) for 24 h, routinely processed, embedded in paraffin wax, cut into 3 – 4-µm sections, and stained with hematoxylin and eosin (H&E). Slides were then analyzed using a Nikon Eclipse E600 microscope and tissues photomicrographs and measurements of the parasites for morphologic identification were taken using a digital image processing system (Nikon Digital DS-5M).

Genomic DNA from adult worms (one from each animal with lungworms) was extracted using a commercial kit (GRS Genomic DNA Kit – Tissue, Grisp, Portugal) in accordance with the manufacturer's instructions. Partial fragments of mitochondrial 12S rRNA (330 bp) and nuclear 18S rRNA (1700 bp) genes were amplified using two sets of primers (12SF: 5'-CGGGAGTAAAGTTTTGTTTAAACCG-3' and 12SR: 5'-CATTGACGGATGGTTTGTACCAC-3'; NC18SF1: 5'-AAAGATTAAGCCATGCA-3' and NC5BR: 5'-GCAGGTTCACCTACAGAT-3', respectively) (Latrofa *et al*., 2015). The PCR amplification was performed using KAPA Taq DNA polymerase (Kapabiosystems, Massachusetts, USA). Genomic fragments were amplified using the following conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 60 s; 55 °C for 60 s, 72 °C for 60 s; and a final extension at 72 °C for 7 min. The amplicons were purified (GRS PCR & Gel band purification kit, Grisp, Portugal) and bidirectionally sequenced, using the same primers as for PCR, employing the BigDye ® Terminator v3.1 Cycle Sequencing Kit [Applied Biosystems] in an automated sequencer (3130XL Genetic Analyzer, Applied Biosystems). Sequences were compared, using Basic Local Alignment Search Tool (BLAST – http://blast.ncbi.nlm.nih.gov/Blast.cgi), with those available in the GenBank database.

In order to investigate the phylogenetic relationship with other metastrongyloids, the sequences of mitochondrial and nuclear genes herein generated were aligned, using ClustalW, with those available in the GenBank database. Phylogenetic trees based on ribosomal 12S rRNA and 18S rRNA were constructed using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model and the Neighbor-Joining method based on the

Table 1. Characterization of necropsied hedgehogs lung lesions and cause of death.

Case nr	Gender	Season	Lung lesions	Concurrent lungworm	Cause of death
1	Male	Spring	Diffuse subacute bronchopneumonia	Yes	Parasitic pneumonia
2	Female	Autumn	Pulmonary edema	No	Heart chronic failure
3	Female	Winter	Hyperemia and pulmonary edema	No	Heart chronic failure
4	Male	Spring	Subacute multifocal to diffuse pneumonia	No	Pneumonia
5	Female	Spring	Multifocal chronic interstitial pneumonia	No	Chronic pneumonia
6	Male	Spring	Fibrinopurulent pleuropneumonia	No	Pleuropneumonia
7	Male	Spring	Diffuse subacute bronchopneumonia	Yes	Parasitic pneumonia
8	Male	Autumn	Diffuse subacute bronchopneumonia	Yes	Parasitic pneumonia
9	Male	Autumn	Diffuse subacute interstitial pneumonia	No	Pneumonia
10	Male	Autumn	Diffuse subacute bronchopneumonia	Yes	Parasitic pneumonia
11	Female	Autumn	Diffuse subacute bronchopneumonia	Yes	Parasitic pneumonia

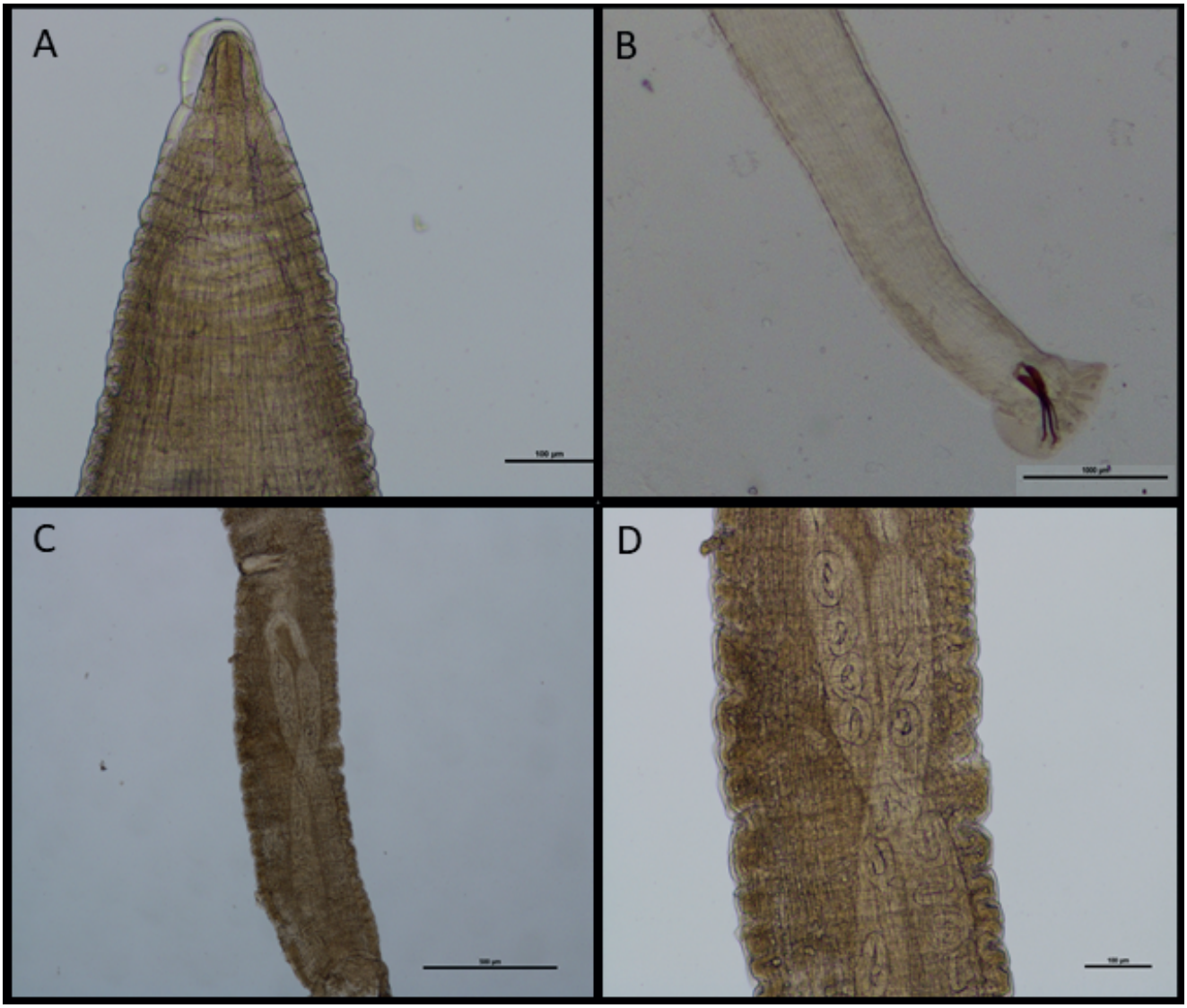

Fig. 1. Morphological characteristics for the identification of *Crenosoma striatum*.
A - rudimentary buccal capsule with a cross striation body cutile, B – copulatory bursa with two spicules, C – median vulva, D – eggs with ovoid shape that contain L1.

Kimura 2-parameter model, respectively, and bootstrap values are based on 5000 replicates, using MEGA X software (Kumar *et al*., 2018). For each analysis, the bootstrapped confidence interval was based on 5000 replicates.

Results and Discussion

During the aforementioned period, all the 11 necropsied hedgehogs displayed macroscopically pronounced and then microscopically confirmed, pulmonary changes (Table 1). Out of the 11 corpses, 5 (45.5 %) presented macroscopic evidence of nematodes with long, thin, simple and whitish colour bodies of approximately 6 mm in length. Microscopically, these presented bilaterally symmetrical bodies surrounded by a non-cellular and cross-striated cuticle, rudimentary buccal capsule, copulatory bursa with two spicules and a median vulva. Females depone a large number of ovoid eggs, that contained the first larval stage when being excreted (Fig. 1). Histologically, the presence of several worms within the bronchi and bronchiole surrounded by mucous coexisted with moderate to severe inflammatory reaction, with mixed inflammatory cell infiltrate, including neutrophils, macrophages, lymphocytes, plasma cells and variable number of eosinophils (Fig. 2). Hyperplasia of the bronchial epithelium and pulmonary oedema was also noticed. All together, these features were suggestive of crenosomosis.
PCR amplification of each target region from individual DNA samples resulted in amplicons of the expected size for both studied regions. Only a 12S and a 18S rRNA sequence from one nematode were retrieved and further compared with those available in

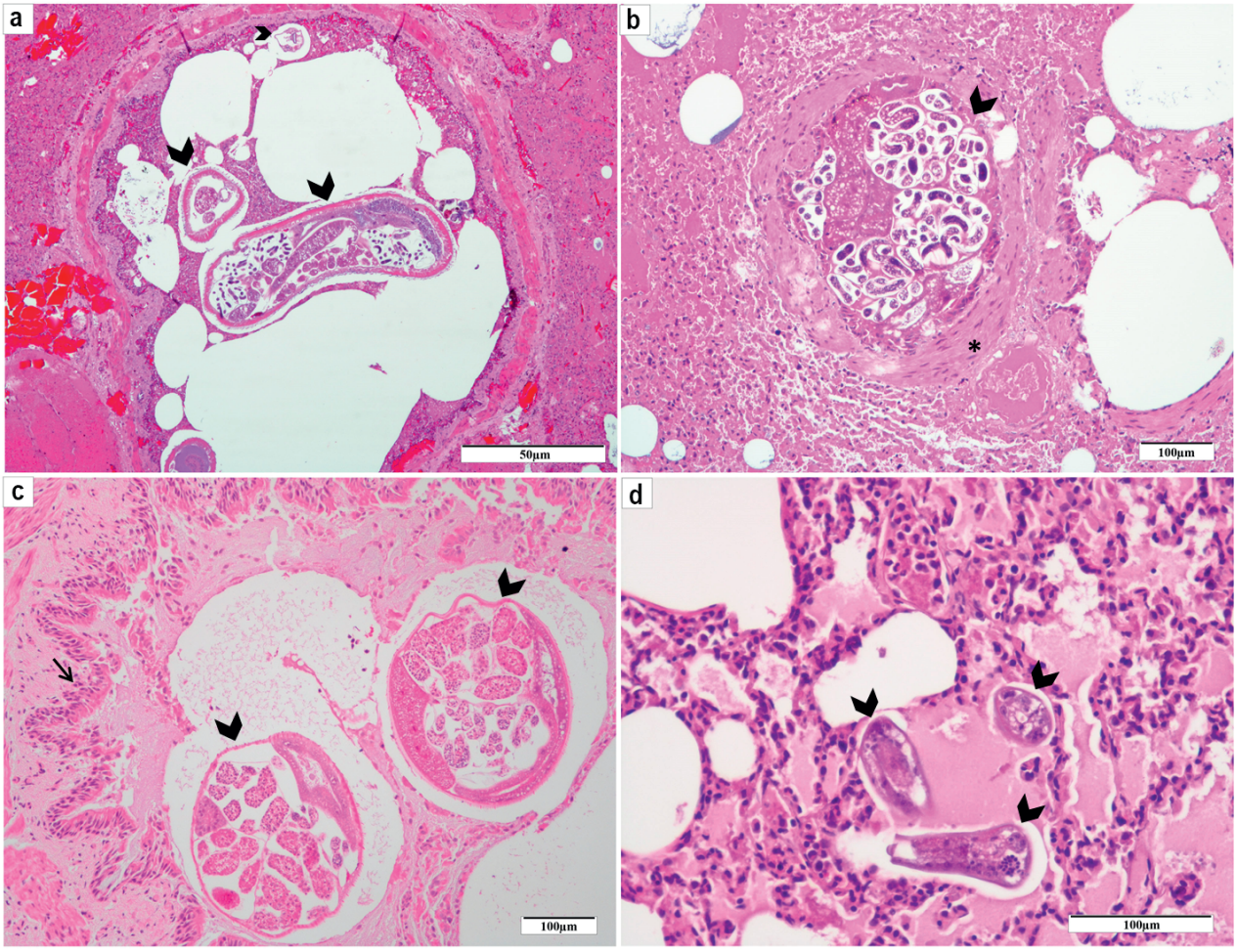

Fig. 2. a) and b) Presence of worms (arrowhead) within the bronchi and bronchioles. The alveoli are oedematous and contain abundant mixed inflammatory infiltrate and smooth-muscle hyperplasia (*) around the bronchiole is observed (case 7). c) Hyperplasia of the bronchial epithelium (arrow). Presence of intraluminal worms mixed with mucus and increased number of macrophages (case 1). d) Alveoli are filled with worms, proteinaceous material (oedema) and inflammatory infiltrate composed of neutrophils, eosinophils, macrophages, lymphocytes and plasma cells (case 8).

GenBank dataset by BLAST analysis. From the 12S rRNA target PCR, a 177 nucleotide stretch sequence was obtained and showed the highest BLAST nucleotide identity with that of *C. vulpis* (i.e. 83.73 %, KU641458), since no 12S rRNA of *C. striatum* has ever been deposited in GenBank.

The phylogenetic analysis of the 12S rRNA sequence herein obtained and those of other metastrongyloids showed a cluster with *C. vulpis* (Fig. 3).

From the 18S rRNA target PCR analysis, a 321 nucleotide sequence was retrieved showing the highest BLAST nucleotide identity with that of *C. striatum* (i.e. 99.69 %, KP941434), found in hedgehogs from Germany (Lange *et al*., 2018). Phylogenetic tree based on 18S rRNA also showed that the obtained sequence clustered with *C. striatum* (Fig. 3). Sequences were deposited in GenBank with accession numbers MN149537 (12S rRNA) and MN097947 (18S rRNA).

C. striatum is a host-specific nematode with a high infection prevalence amongst hedgehogs (Pfäffle 2010). Lung disease associated with this nematode is one of the most observed reasons for health support in these mammals and it is associated with respiratory distress up to cardiac failure as a direct result of heavy worm burdens (Hoseini *et al*., 2014). The present report identifies *C. striatum* in *E. europeus* hedgehogs rescued from various Portuguese municipalities and strengthens the phylogenetic interpretation of this species. The genetic data here described corroborates the morphological classification of *C. striatum* and provides a first nucleotide sequence for a partial 12S rRNA gene of this nematode. To broaden the knowledge on the biology of this parasite, particularly its life cycle, further molecular studies are also needed on intermediate hosts.

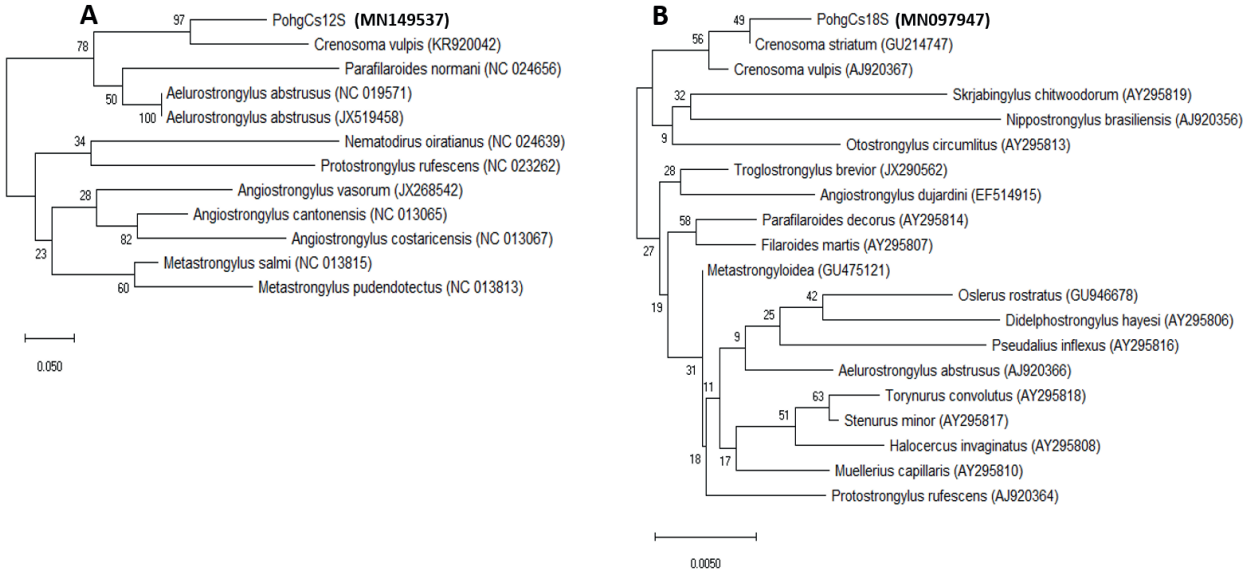

Fig. 3. Phylogenetic trees based on ribosomal 12S rRNA and 18S rRNA were constructed using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model (A) and the Neighbor-Joining method based on the Kimura 2-parameter model (B), and bootstrap values are based on 5000 replicates.

Conflict of Interest

Authors state no conflict of interest.

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