DOI 10.2478/helm-2020-0007

HELMINTHOLOGIA, 57, 1: 1 – 11, 2020

The molecular profile of *Paratrajectura longcementglandatus* Amin, Heckmann et Ali, 2018 (Acanthocephala: Transvenidae) from percid fishes in the marine waters of Iran and Iraq

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Article info

Received August 20, 2019
Accepted September 3, 2019

Summary

Paratrajectura longcementglandatus Amin, Heckmann et Ali, 2018 (Transvenidae) was recently described from two species of percid fishes collected from the marine territorial waters of Iraq and Iran in the Persian Gulf. The genus *Paratrajectura* Amin, Heckmann et Ali, 2018 is a close relative to transvenid genera *Trajectura* Pichelin et Crib, 2001 and *Transvena* Pichelin et Crib, 2001. Morphologically, *Paratrajectura* is characterised by having apical proboscis cone, long, tubular cement glands, short lemnisci, prominent roots on all proboscis hooks, subterminal female gonopore, and males with long pre-equatorial testes. Molecular studies of *P. longcementglandatus* using 18S rDNA and *cox1* genes compared with available data of members of other families of Echinorhynchida showed that *P. longcementglandatus* is grouped with species of the genus *Transvena* forming a clade within the family Transvenidae.

Keywords: Spiny headed transvenid worms; 18S rDNA; *cox1*; phylogeny; Perciformes; Middle East

Introduction

Pichelin & Cribb (2001) described the family Transvenidae with two genera: monotypic *Transvena* with *T. annulospinosa* Pichelin et Cribb, 2001, and *Trajectura* with two species, *T. ikedai* (Machida, 1992) and *T. perinsolens* Pichelin et Cribb, 2001. Specimens of the two genera were recovered from wrasses (Labridae, Perciformes) in the Pacific off southern Australia and southern Japan. Lisitsyna *et al*. (2019) described two other species of the family Transvenidae, namely *Transvena pichelinae* Lisitsyna, Kudlai, Cribb et Smit, 2019, and *Pararhadinorhynchus sodwanensis* Lisitsyna, Kudlai, Cribb et Smit, 2019 from the marine fishes from the Sodwana Bay, South Africa. The other genus of this family, *Paratrajectura*, was established by Amin *et al*. (2018). It comprises one species *Paratrajectura longcementglandatus* Amin, Heckmann et Ali, 2018, which was described on the basis of worms from the Japanese threadfin bream *Nemipterus japonicus* Bloch (Nemipteridae) and the tigertooth croacker, *Otolithes ruber* Bloch et Schneider (Sciaenidae, Perciformes) caught in the marine territorial waters of Iraq and Iran, the Persian Gulf (Amin *et al*., 2018). The genus *Paratrajectura* is characterised by having apical proboscis cone, long, tubular cement glands, short lemnisci, prominent roots on all proboscis hooks, subterminal female gonopore, and males with long pre-equatorial testes.

While, several studies have been published about sequence data for acanthocephalans including two *Transvena* spp. (Westram *et al*., 2011; Garcia-Varela, *et al*., 2013; Pinacho-Pinacho *et al*., 2014; Lisitsyna *et al*., 2019), no sequence data has been published for *P. longcementglandatus* whose phylogenetic relationship with other acanthocephalans and related families was unknown. In this paper, we report the molecular profile of *P. longcementglandatus*,

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validate its generic affiliations, and explore its evolutionary relationships with related and other species and taxa based on partial 18S rDNA and *cox1* genes.

Materials and Methods

DNA extraction and PCR amplification

For extraction of genomic DNA, five adult worms of *P. longcementglandatus* were washed with sterile distilled water several times to remove the ethanol residuals. Total DNA was extracted using Qiagen DNeasy Blood and Tissue kit (Qiagen Inc., Valencia, California, USA) according to manufacturer's instructions and kept at −20 °C until use.

PCR reactions were performed in 30 µL volumes containing 2 × red PCR premix (Ampliqon, Odense, Denmark), 20 pmol of each primer and 3 µL of extracted DNA. The partial 18S rRNA gene was amplified using the forward primer (5'-AGATTAAGCCATGCATG-CGTAAG-3') and reverse primer (5'- ACCCACCGAATCAAGAAA-GAG-3'). Also, primers used for the amplification of the partial mitochondrial cytochrome oxidase subunit1 (*cox1*) gene were COI-F (5'-AGTTCTAATCATAARGATATYGG-3') and COI-R (5'-TAAACT-TCAGGGTGACCAAAAAATCA-3') (Folmer *et al*., 1994). PCR conditions for 18S rRNA gene amplification included of an initial denaturing step of 95 °C for 5 min and 35 cycles followed by denaturing step at 95 °C for 30 s, annealing step of 61 °C for 30 s, and 60 s of extension at 72 °C, and 72 °C for 7 min as a final extension. The thermal PCR profiles for *cox1* gene consisted of initial denaturation at 95 °C for 6 minutes followed by 35 cycles of 95 °C for 30 s (denaturation), 55 °C for 30 s (annealing), and at 72 °C for 60 s (extension) with a final extension of 72 °C for 6 minutes. PCR products were analysed on 1.5 % agarose gel and visualized with UV transluminator. Next, the PCR products were sequenced in both directions using the same PCR primers with ABI 3130 sequencer.

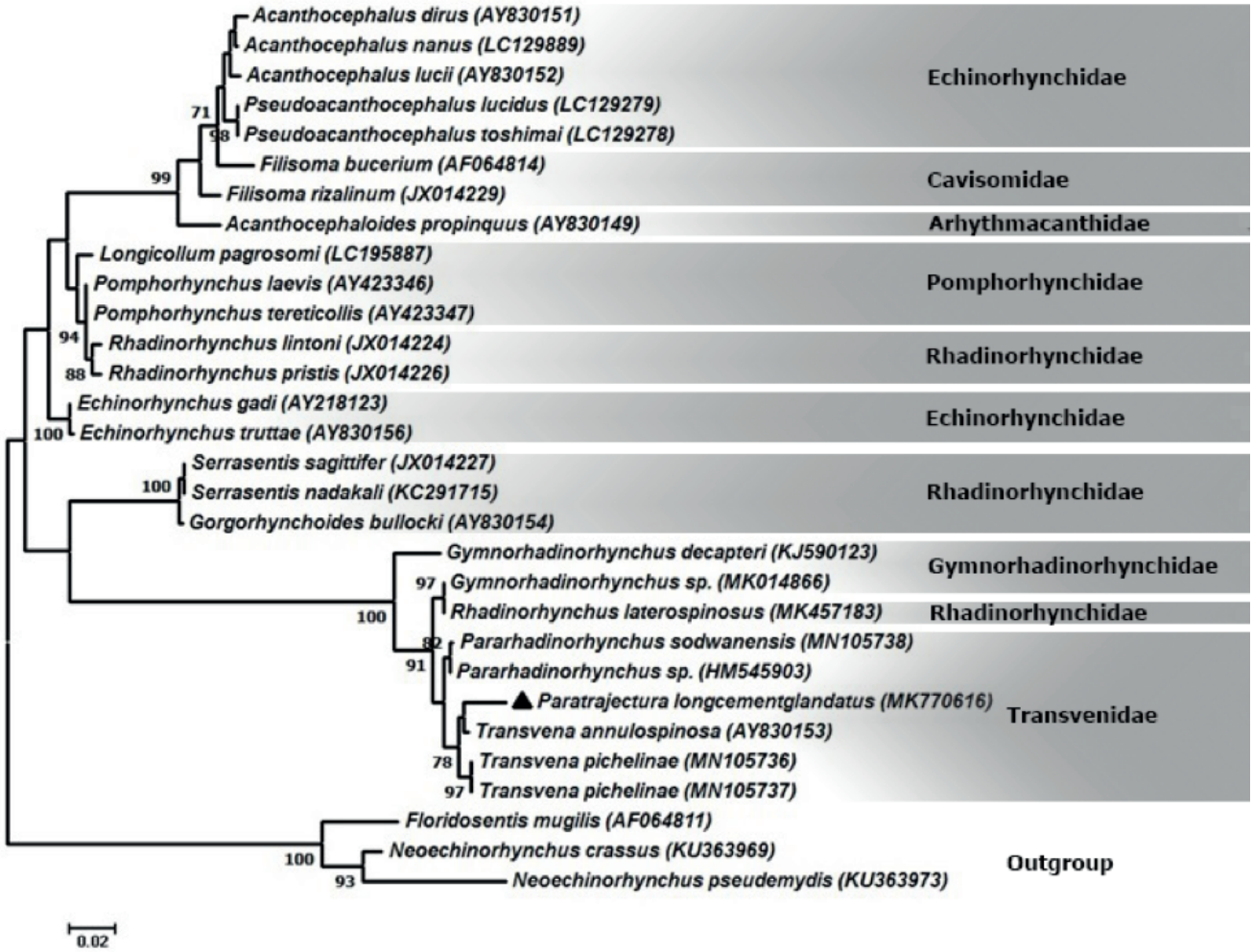

Fig. 1. Phylogenetic tree based on the Maximum likelihood analysis using 18S rDNA sequence of *Paratrajectura longcementglandatus* of current study and sequences of the closest-related members of the order Echinorhynchida deposited in the GenBank. Outgroup: *Floridosentis mugilis, Neoechinorhynchus pseudemydis* and *N. crassus*. Bootstrap values lower than 70 are omitted.

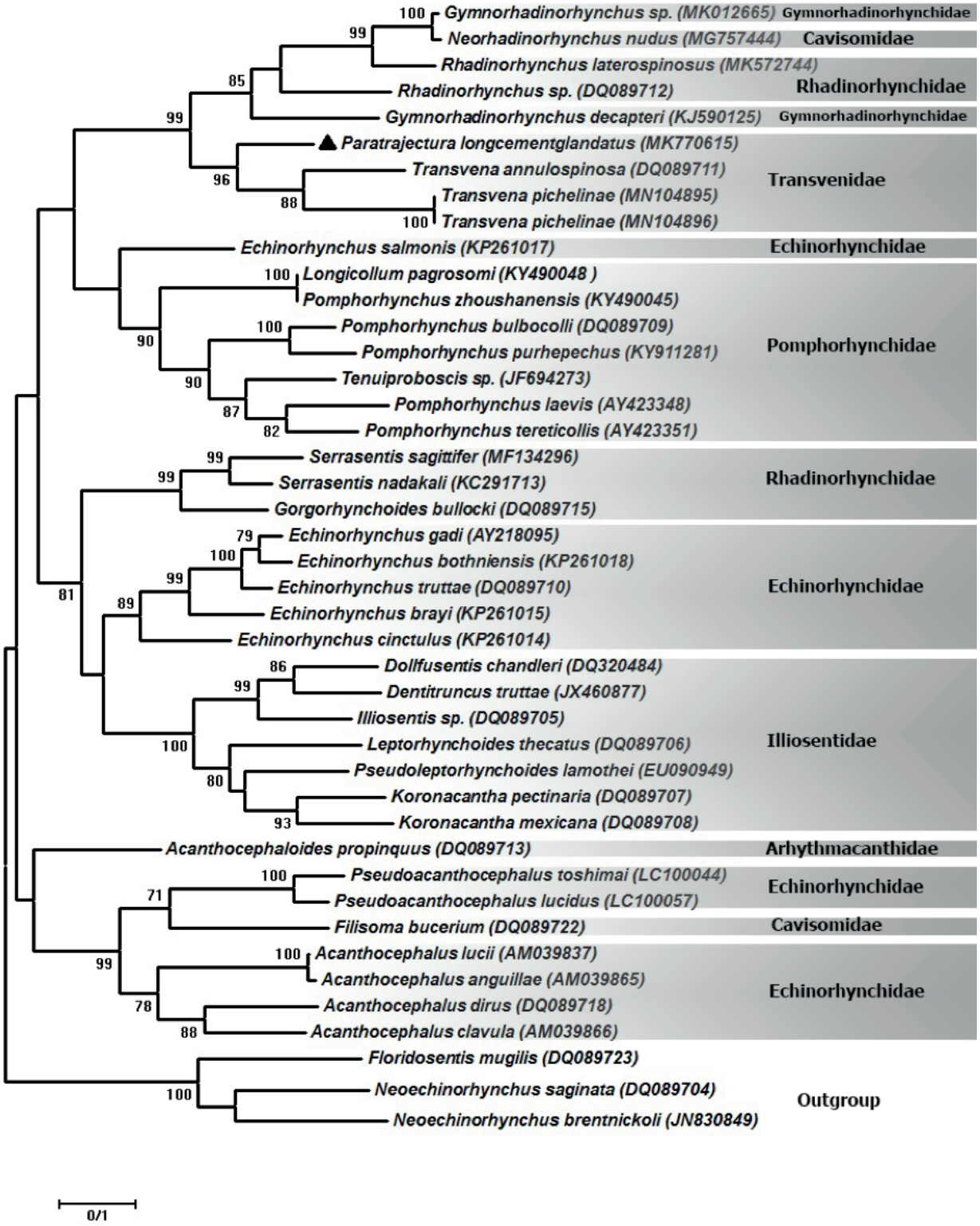

Fig. 2. Phylogenetic reconstruction based on the Maximum likelihood analysis using partial region of the *cox1* sequence of *Paratrajectura longcementglandatus* of current study and sequences of the closest-related members of order Echinorhynchida deposited in the GenBank. Outgroup: *Floridosentis mugilis, Neoechinorhynchus saginata* and *N. brentnickoli*. Bootstrap values lower than 70 are omitted.

The obtained sequence results were manually edited and trimmed using Chromas software v.2.01 (Technelysium Pty Ltd., Brisbane, Queensland, Australia). Next, generated sequences were compared with GenBank submitted sequences using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih. gov/). Also, Clustal W method of Bioedit software v.7.0.9 was used for multiple sequence alignment (Larkin *et al*., 2007). The sequences of 18S rRNA and *cox1* genes were submitted to GenBank database (Accession Numbers: MK770616 for 18S rRNA and MK770615 for *cox1*)

Phylogenetic analysis

The phylogenetic tree was constructed using Maximum-Likelihood model and Tamura-3-parameter model by Molecular and Evolution Genetic Analysis software v.6 (MEGA 6). The reliability of topology of the tree was supported with Bootstrap value based on 1000 replications. The whole scientific names of acanthocephalan species, names of host species, *localities,* and GenBank accession numbers used in the phylogenetic analysis are listed in Table 1.

Ethical Approval and/or Informed Consent

The authors declare compliance with all relevant ethical standards.

Results

The specimens of *P. longcementglandatus* successfully presented amplifications of about 1234 bp for the 18S rDNA gene and 664 bp for the *cox1* gene. Comparisons of the18S rDNA and *cox1* sequences from this parasite with other available acanthocephalan sequences in GenBank, using multiple sequence alignment, showed that it had the highest similarity with *T. annulospinosa* based on18S rDNA (98 %*)* and *cox1* (77 %) genes. The 18S rDNA dataset (1129 nt) included 26 sequences for species of seven families within the Echinorhynchida and the novel sequence of *P. longcementglandatus*. The *cox1* dataset (538 nt) included 39 sequences for species of nine families of Echinorhynchida and the sequence of *P. longcementglandatus*.

The phylogenetic reconstruction based on the partial sequence spanning the 18S rDNA showed that our sequence of *P. longcementglandatus* is clustered with *Transvena annulospinosa* (AY830153), *T. pichelinae* (MN105736 and MN105737), *P. sodwanensis* (MN105738) and an unidentified species of *Pararhadinorhynchus* (HM545903) with strong support forming a clade of the family Transvenidae. Also, the species of *Gymnorhadinorhynchus* sp. (MK014866) (Gymnorhadinorhynchidae) and *Rhadinorhynchus laterospinosus* (MK457183) (Rhadinorhynchidae) are very closely related with the family Transvenidae in the tree with 100 % of bootstrap support. The sequence of *Gymnorhadinorhynchus decapteri* (KJ590123) (Gymnorhadinorhynchidae) is located at the basal position to the members of the clade. Other families of the order Echinorhynchida including Rhadinorhynchidae, Pomphorhynchidae, Cavisomidae, Arhythmacanthidae and Echinorhynchidae located in a major sister clade (Fig. 1). Inter-generic differences are noted between *P. longcementglandatus* and *T. annulospinosa, T. pichelinae, P. sodwanensis* and *Pararhadinorhynchus* sp. from Transvenidae based on partial 18S rDNA sequence were 2.4 % (18 nt), 2.8 % (21 nt), 2.9 % (22 nt) and 2.9 % (22 nt), respectively. According to phylogenetic analyses based on the *cox1* gene, our sequence of *P. longcementglandatus* (MK770615) is grouped with *T. annulospinosa* (DQ089711) and *T. pichelinae* (MN104895 and MN104896) with strong support in a clade of the family Transvenidae. The species of *G. decapteri* (KJ590125) and *Gymnorhadinorhynchus* sp. (MK012665) (Gymnorhadinorhynchidae), *Neorhadinorhynchus nudus* (MG757444) (Cavisomidae), *R. laterospinosus* (MK572744) and *Rhadinorhynchus* sp. (DQ089712) (Rhadinorhynchidae), appear as a sister group of the family Transvenidae (Fig. 2). The interspecific divergence between *P. longcementglandatus* and *T. annulospinosa, T. pichelinae* based on partial *cox1* gene was 23.4 % (141 nt), 27.3 % (144 nt), respectively.

Discussion

Recently, molecular methods are applied for species identification, classification and phylogenetic analysis of acanthocephalan species (García-Varela *et al*., 2002). To date, molecular profile has been provided for few species of the family Transvenidae including *T. annulospinosa, T. pichelinae*, *P. sodwanensis* and *Pararhadinorhynchus* sp. (Pichelin & Cribb, 2001; Lisitsyna *et al*., 2019). In the current study, phylogenetic relationships of *P. longcementglandatus* as another genus of this family is described based on partial 18S rDNA and *cox1* genes determining relationships with other acanthocephalan families.

This study showed that the interspecific variation between *P. longcementglandatus* and species of *Transvena* based on partial 18S rDNA was 2.4 % – 2.8 % (18 – 21 nt) and between it and species of *Pararhadinorhynchus* was 2.9 % (22 nt). Also based on *cox1* gene, inter-generic variations between *P. longcementglandatus* and *T. annulospinosa* was 23.4 % (141 nt). These results illustrate that sequence differences between the genera of the family based on *cox1* gene is higher than18S rDNA and it is appropriate to consider for taxonomic studies at the generic level.

The phylogenetic analysis of the 18S rDNA sequence (Fig. 1) showed that *P. longcementglandatus* is grouped in a highly supported clade with *T. annulospinosa* (AY830153), *T. pichelinae* (MN105736 and MN105737), *P. sodwanensis* (MN105738) and *Pararhadinorhynchus* sp. (HM545903) forming a clade of the family Transvenidae. In the clade, the family Transvenidae grouped close to *R. laterospinosus* (MK457183) and *G. decapteri* (KJ590123) (Gymnorhadinorhynchidae). Our phylogenetic tree for 18S rDNA is similar to those of García-Varela *et al*. (2002) and Lisitsyna *et al.* (2019) where the family Transvenidae grouped close to different species of *Rhadinorhynchus* (Rhadinorhynchidae) and *Gymnorhadinorhynchus* (Gymnorhadinorhynchidae).

Table 1. Acanthocephalan species represented in the phylogenetic analysis with their family, host species, GenBank accession numbers, locations, and references.

Species	Host	GenBank Acc. no. 185 rDNA	GenBank Acc. no. *cox1*	Location	Reference
Gymnorhadinorhynchidae					
Gymnorhadinorhynchus sp.	*Regalecus russelii*	MK014866	MK012665	Japan	Steinauer *et al.* (2019)
Gymnorhadinorhynchus decapteri (Braicovich, Lanfranchi, Farber, Marvaldi, Luque et Timi, 2014)	*Decapterus punctatus*	KJ590123	KJ590125	Brazil	Braicovich *et al*. (2014)
Cavisomidae					
Neorhadinorhynchus nudus (Harada, 1938)	*Auxis thazard*	-	MG757444	China	Li *et al*. (2018)
Filisoma bucerium (Van Cleave, 1940)	*Kyphosus elegans*	AF064814	DQ089722	Na*	García-Varela *et al*. (2000), García-Varela and Nadler (2006)
Filisoma rizalinum (Tubangui et Masilungan, 1946)	*Scatophagus argus*	JX014229	-	Indonesia	Verweyen *et al*. (2011)
Rhadinorhynchidae					
Rhadinorhynchus laterospinosus (Amin, Heckmann et Van Ha, 2011)	*Auxis rochei*	MK457183	MK572744	Vietnam	Amin *et al*. (2019a)
Rhadinorhynchus sp.	*Sciaenidae*	AY062433	DQ089712	Na	García-Varela *et al*. (2002), García-Varela and Nadler (2006)
Serrasentis sagittifer (Linton, 1889)	*Lutjanus sebae*	-	MF134296	Australia	Barton *et al*. (2018)
Serrasentis sagittifer	*Johnius coitor*	JX014227	-	Indonesia	Verweyen *et al*. (2011)
Serrasentis nadakali (George et Nadakal, 1978)	Na	KC291715	KC291713	Na	Paul *et al*. (unpublished)
Gorgorhynchoides bullock (Cable et Mafarachisi, 1970)	*Eugerres plumieri*	AY830154	DQ089715	Na	Garcia-Varela and Nadler (2005, 2006)
Rhadinorhynchus lintoni (Cable et Linderoth, 1963)	*Selar Crumrn- opht halmus*	JX014224	-	USA	Verweyen *et al*. (2011)
Rhadinorhynchus pristis (Rudolphi, 1802)	*Selar Crumrn- opht halmus*	JX014226	-	USA	Verweyen *et al*. (2011)

Transvenidae					
Paratrajectura longcementglandatus (Amin, Heckmann et Ali, 2018)	Percid fishes	MK770616	MK770615	Marine waters of Iraq and Iran	Present study
Transvena annulospinosa (Pichelin et Cribb, 2001)	*Anampses neoguinaicus*	AY830153	DQ089711	Na	Garcia-Varela and Nadler (2005, 2006)
Transvena pichelinae sp. n. (Lisitsyna, 2019)	*Thalassoma purpureum*	MN105736, MN105737	MN104895, MN104896	South Africa	Lisitsyna *et al*. (2019)
Pararhadinorhynchus sodwanensis sp. n. (Lisitsyna, 2019)	*Pomadasys furcatus*	MN105738	-	South Africa	Lisitsyna *et al*. (2019)
Pararhadinorhynchus sp.	*Siganus fuscescens*	HM545903	-	China	Wang *et al*. (unpublished)
Echinorhynchidae					
Pseudoacanthocephalus toshimai (Nakao, 2016)	*Rana pirica*	LC129278	LC100044	Japan	Nakao (2016)
Pseudoacanthocephalus lucidus (Van Cleave, 1925)	*Rana ornativentris*	LC129279	LC100057	Japan	Nakao (2016)
Acanthocephalus lucii (Müller, 1776)	*Perca fluviatilis*	AY830152	-	Na	Garcia-Varela and Nadler(2005), Benesh *et al*. (2006)
Acanthocephalus lucii	*Perca fluviatilis*	-	AM039837	England	Garcia-Varela and Nadler (2005), Benesh *et al*. (2006)
Acanthocephalus anguillae (Müller, 1780)	*Perca fluviatilis*	-	AM039865	Austria	Benesh *et al*. (2006)
Acanthocephalus dirus (Van Cleave, 1931)	*Asellus aquaticus*	AY830151	DQ089718	Na	Garcia-Varela and Nadler (2005, 2006)
Acanthocephalus clavula (Dujardin, 1845)	*Perca fluviatilis*	-	AM039866	Ireland	Benesh *et al*. (2006)
Acanthocephalus nanus (Van Cleave, 1925)	*Cynops pyrrhogaster*	LC129889	-	Japan	Nakao (2016)
Echinorhynchus salmonis (Müller, 1784)	*Coregonus lavaretus*	-	KP261017	Finland	Wayland *et al*. (2015)
Echinorhynchus gadi (Müller, 1776)	Na	AY218123	AY218095	Na	Giribet *et al*. (2004)
Echinorhynchus bothniensis (Zdzitowiecki et Valtonen, 1987)	*Osmerus eperlanus*	-	KP261018	Finland	Wayland *et al*. (2015)

Echinorhynchus truttae (Schrank, 1788)	*Thymallus thymallus*	AY830156	DQ089710	Na	Garcia-Varela and Nadler (2005, 2006)
Echinorhynchus brayi (Wayland, Sommerville et Gibson, 1999)	*Pachycara crassiceps*	-	KP261015	Atlantic Ocean: Porcupine Seabight	Wayland *et al*. (2015)
Echinorhynchus cinctulus (Porta, 1905)	*Lota lota*	-	KP261014	Finland	Wayland *et al*. (2015)
Pomphorhynchidae					
Longicollum pagrosomi (Yamaguti, 1935)	*Pagrus major*	LC195887	-	Japan	Mekata *et al*. (unpublished)
Longicollum pagrosomi	*Oplegnathus fasciatus*	-	KY490048	China	Li *et al*. (2017)
Pomphorhynchus zhoushanensis (Li, Chen, Amin et Yang, 2017)	*Oplegnathus fasciatus*	-	KY490045	China	Li *et al*. (2017)
Pomphorhynchus bulbocolli (Linkins, 1919)	*Lepomis macrochirus*	-	DQ089709	Na	Garcia-Varela and Nadler (2006)
Pomphorhynchus purhepechus (García-Varela, Mendoza-Garfias, Choudhury et Pérez-Ponce de León, 2017)	*Moxostoma austrinum*	-	KY911281	Na	Garcia-Varela *et al*. (2017)
Tenuiproboscis sp.	*Epinephelus malabaricus*	-	JF694273	Na	Vijayan *et al*. (unpublished)
Pomphorhynchus laevis (Zoega in Müller, 1776)	*Gammarus pulex*	AY423346	AY423348	France	Perrot-Minnot (2004)
Pomphorhynchus tereticollis (Rudolphi, 1809)	*Gammarus pulex*	AY423347	AY423351	France	Perrot-Minnot (2004)
Arhythmacanthidae					
Acanthocephaloides propinquus (Dujardin, 1845)	*Gobius bucchichii*	AY830149	DQ089713	Na	Garcia-Varela and Nadler (2005, 2006)
Diplosentidae					
Sharpilosentis peruviensis (Lisitsyna, Scholz et Kuchta, 2015)	*Duopalatinus cf. peruanus*	-	KP967562	Peru	Lisitsyna *et al*. (2015)
Illiosentidae					
Dollfusentis chandleri (Golvan, 1969)	Na	-	DQ320484	Na	Baker and Sotka(unpublished)
Dentitruncus truttae (Sinzar, 1955)	*Salmo trutta*	JX460865	JX460877	Croatia	Vardić Smrzlić *et al*. (2013)
Illiosentis sp.	Na	AY830158	DQ089705	Na	Garcia-Varela and Nadler (2005, 2006)

Leptorhynchoides thecatus (Linton, 1891)	*Lepomis cyanellus*	AF001840	DQ089706	Na	Near *et al*. (1998), Garcia-Varela and Nadler (2006)
Pseudoleptorhynchoides lamothei (Salgado-Maldonado, 1976)	*Ariopsis guatemalenis*	EU090950	EU090949	Na	Near *et al*. (1998), Garcia-Varela and Nadler (2006)
Koronacantha pectinaria (Van Cleave, 1940)	*Microlepidotus brevipinnis*	AF092433	DQ089707	Na	García-Varela and Nadler (2005, 2006)
Koronacantha Mexicana (Monks et Pérez-Ponce de León, 1996)	*Haemulopsis leuciscus*	AY830157	DQ089708	Na	García-Varela and Nadler (2005, 2006)
Neoechinorhynchidae (Outgroup)					
Neoechinorhynchus brentnickoli (Monks, Pulido-Flores and Violante-González, 2011)	*Dormitator latifrons*	-	JN830849	Na	Pinacho-Pinacho *et al*. (2012)
Neoechinorhynchus saginata (Van Cleave & Bangham, 1949)	Na	-	DQ089704	Na	García-Varela and Nadler (2006)
Floridosentis mugilis (Machado Filho, 1951)	Na	AF064811	DQ089723	Na	García-Varela and Nadler(2006), García-Varela *et al*. (2000)
Neoechinorhynchus crassus (Van Cleave, 1919)	Na	KU363969	-	Iran	Dadar and Adel (unpublished)
Neoechinorhynchus pseudemydis (Cable and Hopp, 1954)	*Capoeta aculeata*	KU363973	-	Iran	Dadar and Adel (unpublished)

*Na = not available

Our phylogenetic analysis of the *cox1* gene (Fig. 2) confirmed that *P. longcementglandatus* is grouped with *T. annulospinosa* (DQ089711) and *T. pichelinae* (MN104895 and MN104896) which made the clade of the family Transvenidae with good statistical support. Also, the families Rhadinorhynchidae, Gymnorhadinorhynchidae and Cavisomidae appear as a sister group with the clade of family Transvenidae. Other families of Echinorhynchida such as Pomphorhynchidae, Echinorhynchidae, Cavisomidae, Illiosentidae, Rhadinorhynchidae, Gymnorhadinorhynchidae, Diplosentidae and Arhythmacanthidae are well separated in the later clade.

In the present study, the higher level of variation in *cox1* gene compared to the 18S rDNA gene provides better resolution of the relationships within closely related taxa. While Amin *et al*. (2019a) presented relationships in their analysis of *Rhadinorhynchus* based on *cox1* sequences, it was not clearer than 18S rDNA due to the lack of sufficient sequences of this gene in GenBank.

One of the most commonly used molecular markers for classification of acanthocephalans is the small subunit from RNA ribosomal gene or 18S rRNA. This gene displays a slow evolution rate and is highly conserved. It was used to infer phylogenetic relationships among the major classes of Acanthocephala (García-Varela & Pérez-Ponce de León, 2015). Most of phylogenetic studies of acanthocephalans similar to this research showed that 18S rDNA sequences appear to be suitable marker for phylogenies among acanthocephalans (García-Varela *et al.,* 2000; Near, 2002; Herlyn *et al*., 2003; Verweyen *et al*., 2011; Amin *et al*., 2019b). Also, *cox1* gene is commonly used for phylogenetic studies and to recognize and establish species limits in acanthocephalans (Guillen-Hernández *et al*., 2008; Alcántar-Escalera *et al*., 2013; García-Varela, *et al*., 2013). The present study confirmed that this gene has high genetic diversity among genera of the family and other families of Echinorhynchida which would be more particularly useful for phylogenetic analysis.

Finally, the genetic data collected in the current study provide a better understanding of the taxonomic status of *P. longcementglandatus*. Sequence variations within the family Transvenidae and among other families of Echinorhynchida based on *cox1* gene is higher than18S rDNA that can be useful for achieving a proper assessment of biodiversity. More sequence data from other geographical isolates using more gene targets will be useful for exploring the phylogenetic relationships among species. On the other hand, using of molecular tools for identification of acanthocephalan species is still scarce due to the lack of sequences of different genera of acanthocephalans in GenBank (Amin *et al*., 2013; Salgado-Maldonado, 2013; Weaver & Smales, 2013; Amin *et al*., 2014; Smales, 2014; Gomes *et al*., 2015; Steinauer & Nickol, 2015). More molecular studies are recommended in order to elucidate acanthocephalans classification.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

This project was supported by the Department of Biology, Brigham Young University (BYU), Provo, Utah and by an Institutional Grant from the Parasitology Center, Inc. (PCI), Scottsdale, Arizona.

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DOI 10.2478/helm-2020-0002

HELMINTHOLOGIA, 57, 1: 12 – 18, 2020

Prevalence of intestinal parasitic infections among the Bulgarian population over a three year period (2015 – 2017)

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Article info

Received June 14, 2019
Accepted October 9, 2019

Summary

The aim of the present study is to perform a retrospective analysis of the prevalence of intestinal parasitic infections among the Bulgarian population over the period 2015 – 2017. The study involved all Bulgarians and foreign nationals residing in the country who had been tested for intestinal protozoa and helminths. A total of 23,785 infections have been revealed, of which 17,712 (74.47 %) were helminth and 6,073 (25.53 %) protozoan invasions. Enterobiasis was found to be the most prevalent among patients infected with intestinal helminths (81.75 %), while giardiasis (62.05 %) was the most common among those diagnosed with protozoan infections. In spite of improved living conditions and increasing public health awareness, parasitic diseases in general, and intestinal parasitic infections in particular, still represent a significant part of the overall morbidity in Bulgaria, thus posing a major issue for the public health care system.
Keywords: protozoa; helminths; prevalence; intestinal parasitic infections

Introduction

Parasitic diseases caused by intestinal protozoa and helminths are among the most common illnesses and one of the major causes of increased morbidity and mortality among people in developing countries (Speich *et al*., 2016). It is generally acknowledged that parasitic diseases are the most common among children under the age of five as they are more vulnerable to soil-transmitted helminthic infections than adults. Nutritional disorders caused by the parasites may lead to iron deficiency anemia, malnutrition and they may have a negative impact on growth and cognitive development of a child (Bethony *et al.,* 2006; Pullan *et al.,* 2011). Despite all the medical and pharmaceutical advance as well as progress in sanitary engineering, intestinal parasitic infections remain the most prevalent in the world, especially in developing countries struggling with water scarcity, poor hygiene and lack of adequate health care services. Furthermore, it is difficult to control intestinal parasitoses in these regions due to the high cost of infrastructure improvements and lack of educational projects offered to the population (Speich *et al*., 2016; Ostan *et al.,* 2007; Mehraj *et al.,* 2008). Water is essential to life, but it is also an important vehicle for pathogen dissemination, and many invasive helminth eggs and larvae, and protozoan cysts are distributed via water in the environment. Protozoa such as *Giardia lamblia* and *Cryptosporidium* spp. are recognized as important waterborne pathogens, causing in some cases severe gastrointestinal disease (Baldursson & Karanis, 2011; Kumar *et al.,* 2014). It has been well documented that the conventional treatment of drinking and waste water is not always sufficient for a complete destruction of protozoan oocysts and helminth eggs (Betancourt *et al.,* 2004; Savioli *et al.,* 2006;

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Hatam-Nahavandi *et al.,* 2015). Incorrect landfilling of human and animal feces is also identified as a possible source of contamination of water sources (Smith *et al.,* 2007) and recreational waters such as swimming pools, water parks and lakes (Savioli *et al.,* 2006). Sometimes sewage overflows also contribute to pollution of surface water and farmland, which leads to the possibility of potential human infections. Food contamination can also occur during food processing, directly (through contaminated equipment or washing water) or indirectly (through contaminated irrigation water) (Dawson, 2005).

Cases of intestinal parasitoses in humans, both autochthonous and imported from other endemic regions of the world, are reported annually in Bulgaria. The aim of this study was to perform a retrospective analysis of the intestinal parasitic infections prevalence in the Bulgarian population for the period 2015 – 2017.

Materials and Methods

Examined groups

The study involved all persons, Bulgarians as well as foreign citizens residing in the country, tested for intestinal protozoa and helminths from January 2015 to December 2017. The patients were tested in the Independent Medical Diagnostic Laboratories (IMDL), Parasitological Laboratories at the Regional Health Inspectorates (RHI) and University Hospitals, and at the National Reference Laboratory for Diagnosis of Parasitic Diseases (NRL) at the National Centre of Infectious and Parasitic Diseases (NCIPD).

Methods for parasitological diagnosis

Fecal samples were investigated for intestinal protozoans and helminths using various methods such as: microscopic examination of direct wet smear, staining with Lugol's iodine, enrichment helmintho-ovoscopic methods (sedimentation, flotation, formalin-ether concentration method), helmintho-larvoscopy by the methods of Berman and Harada-Mori, a perianal tape test for detection of *Enterobius vermicularis* eggs, staining, culture and rapid immunochromatographic methods for intestinal protozoa.

Statistical methods

It was used descriptive statistics. The mean, standard deviation and confidence interval of the mean have been calculated in the study.

Ethical Approval and/or Informed Consent

For the purposes of the present study no informed consent was required from the study participants.

Results

Soil-transmitted helminth infections

Of this group two nematode infections i.e., ascariasis and trichuriasis have local transmission in Bulgaria and cases are recorded on the territory of the whole country. Table 1 shows the data on the number of persons tested and diagnosed with ascariasis or trichuriasis.

Community-acquired parasitic diseases

This group includes three intestinal parasitic diseases: enterobiasis, giardiasis and hymenolepiasis. During the study period a large group of individuals (children, adolescents and adults) was screened for community-acquired parasitic diseases; the tests were performed for prophylactic, epidemiological or clinical indications The data are presented in Table 2.

Table 1. Distribution of the cases of ascariasis and trichuriasis for the period 2015 – 2017.

Year	Number of persons examined for ascariasis	Number positive	Prevalence (%)	Number of persons examined for trichuriasis	Number positive	Prevalence (%)
2015	637 543	805	0.13	623 288	90	0.01
2016	499 729	678	0.14	493 063	71	0.01
2017	576 159	706	0.12	576 159	110	0.02
Total	1 713 431	2 189		1 692 510	271	
Mean ± Standard deviation	571144 ± 69044	729.7 ± 66.73	0.13 ± 0.01	564170 ± 65935	90.33 ± 19.50	0.01333 ± 0.005774
Lower limit of the 95% confidence interval of the mean	399630	563.9	0.1052	400379	41.89	-0.001009
Upper limit of the 95% confidence interval of the mean	742657	895.4	0.1548	727961	138.8	0.02768

Table 2. Distribution of the cases of enterobiasis, giardiasis and hymenolepiasis for 2015 – 2017.

Year	Number of persons examined for enterobiasis	Number positive / prevalence (%)	Number of persons examined for giardiasis	Number positive / prevalence (%)	Number of persons examined for hymenolepiasis	Number positive / prevalence (%)
2015	479149	4604 / 0.96	481197	1245 / 0.26	388364	126 / 0.03
2016	388971	4403 / 1.13	471537	1367 / 0.29	363359	474 / 0.13
2017	451466	5472 / 1.21	438379	1156 / 0.26	327567	120 / 0.04
Total	1319586	14479/1.10	1391113	3768 / 0.27	1079290	720 / 0.07
Mean ± Standard deviation	439862 ± 46195	4826 ± 568.1	463704 ± 22458	1256 ± 105.9	359763 ± 30558	240.0 ± 202.7
Lower limit of the 95% confidence interval of the mean	325107	3415	407916	992.9	283854	-263.5
Upper limit of the 95% confidence interval of the mean	554617	6238	519493	1519	435672	743.5

Taeniasis

A total of 53 individuals were diagnosed with taeniosis caused by beef tapeworm (n = 18 for 2015, n = 19 for 2016 and n = 16 for 2017) during the study period, with the mean annual incidence over the period of 0.25 per 100,000 (0.25 / 100,000 in 2015, 0.26 / 100,000 in 2016 and 0.23 / 100,000 in 2017). Infections with *Taenia solium* have not been revealed.

Blastocytosis and cryptosporidiosis

The data on the prevalence of the two intestinal protozoan infections are presented in Table 3.

Discussion

Parasitic infections of the gastrointestinal tract have a cosmopolitan distribution. The analysis of our survey data (2015 – 2017) shows that a significant proportion of the country's population were screened for the soil-transmitted helminth infections with local transmission (ascariasis and trichuriasis). A total of 1 713 431 persons were examined for ascariasis; the mean annual prevalence of the disease was 0.13 %. We found that the absolute number of people infected by *Ascaris* remained steady over the present study period, (approx. 730 persons per year). As for the age of the asca-

Table 3. Distribution of blastocystosis and cryptosporidiosis for the period 2015 – 2017.

Year	Number of persons examined for blastocystosis	Number positive	Prevalence (%)	Number of persons examined for cryptosporidiosis	Number positive	Prevalence (%)
2015	345549	660	0.19	352	0	0.00
2016	358697	769	0.21	479	4	0.84
2017	328315	858	0.26	231	14	6.06
Total	1032561	2287		1062	18	
Mean ± Standard deviation	344187 ± 15237	762.3 ± 99.17	0.22 ± 0.04	354 ± 124	6 ± 7.21	2.3 ± 3.28
Lower limit of the 95% confidence interval of the mean	306337	516.0	0.1304	45.94	-11.91	-5.856
Upper limit of the 95% of the confidence interval of the mean	382037	1009	0.3096	662.1	23.91	10.46

riasis patients, our findings are in agreement with previous studies of our and other authors (Harizanov *et al.,* 2013; Dudlova *et al.*, 2016) – 86.8 % (n = 1190) of the infected are children, and the age groups 1 – 4 and 5 – 9 years were most often affected. In most cases the infection is asymptomatic and the diagnosis is made after spontaneous passage of the parasite during defecation or following prophylactic tests. This can be explained by the fact that when the parasite burden is low, infection is often asymptomatic, while high-concentration invasions result in acute abdominal pain and ileus from mechanical obstruction of the small intestine, especially in children (Brooker & Bundy, 2014). Clinically prominent and severely progressing forms of ascariasis may be seen in highly endemic countries where the worm burden may be high and ascariasis is often complicated by malabsorption syndrome, growth retardation, cognitive disorders, impaired immune regulation, and increased risk of secondary infections (Lamberton & Jourdan, 2015).

Trichuriasis is one of the most common helminth diseases worldwide (around 800 million infected individuals), with the highest incidence in warm and humid areas (Dori *et al.,* 2011). The highest prevalence rate is recorded in Central Africa, South India and South East Asia (Alum *et al.,* 2010). In Bulgaria, the disease is with local distribution and only a limited number of cases are recorded annually. Over the three-year study period were registered 271 cases of trichuriasis in the country, and the mean annual prevalence was 0.01 %. It should be pointed out that trichuriasis was recorded primarily among persons living in social institutions (e.g. for children deprived of parental care and adults with mental disabilities), which is in line with our previous findings (Harizanov *et al.,* 2013; Rainova *et al.,* 2018). As regards the age structure of the infected, the prevalence of trichuriasis was higher in the age group over 20 years, 89.3 % (n = 242), and significantly lower among children and adolescents 10.7 % (n = 29). This can be explained by the characteristics of patients staying in mental health facilities and the difficulties in maintaining good hygiene in such institutions, as well as the fact that the number of children's homes or similar institutions has significantly decreased in recent years. However, it needs to be stressed that some of the social care establishments are active foci of soil transmitted helminth infections and greater efforts by the public health authorities are needed with regard to control measures related to diagnosis, timely treatment, environmental remediation. The spread of soil transmitted helminth infections, and ascariasis and trichuriasis in particular, is directly proportional to the population density in the region, education level, health protection measures for wastewater management, use of fertilizers, personal hygiene, available health care and socio-economic country status (Das, 2014). In this regard, the WHO Regional Office for Europe developed a strategy entitled "Framework Program for the Control and Prevention of Soil Transmitted Helminth Infections in the WHO European Region, 2016 – 2020" aiming at reducing the incidence of these parasitic diseases to an extent that they will no longer pose a problem for the public healthcare (WHO, 2016).

The community acquired parasitic diseases are of particular health importance in Bulgaria, because they most often affect children in organized facilities. Of these, enterobiasis continue to be the most common community acquired parasitic infection as its prevalence remains high. During the period of our study 14,479 cases of enterobiasis were recorded, of which 7,922 (54.7 %) in children attending organized children's institutions. In this respect our data are similar to the data in available literature. Enterobiasis is a common disease occurring globally, including in countries in temperate zones. It most often affects children. The prevalence among the pediatric populations in different regions of the world varies from 4 to 28 % (Dori *et al.,* 2011). The average prevalence among the subjects within the study period was 1.10 %, while the average prevalence in the general pediatric population in the country was found to be 1.59 %; however, it should be taken into consideration that the study group involved pre-school children attending child care institutions where annual parasitological testing is obligatory. The second most common of the community acquired parasitic diseases in our study was giardiasis with 3,768 reported cases. *G. intestinalis* is the most commonly detected intestinal parasite in the world (Choy *et al*., 2014). In developing countries, the prevalence is 20 – 40 %, and infection mainly affects the pediatric population (Vandenberg *et al.,* 2006). In developed countries, the prevalence is 2 to 5 %. In the European continent, giardiasis is the most prevalent in Eastern Europe and Turkey (Alum *et al.,* 2010). Although the prevalence of giardiasis in Bulgaria shows a downward trend (Table 2), it still remains a major public health issue.

Hymenolepiasis is a zoonotic disease caused by the *Hymenolepis nana* (dwarf tapeworm) and *H. diminuta* (rat tapeworm). The disease is endemic in Asia, Southern and Eastern Europe, Central and South America and Africa. Epidemiological studies have shown that *H. nana* is more commonly reported as a cause of human disease than *H. diminuta*. More than 175 million human cases of hymenolepiasis caused by *H. nana* have been reported worldwide and in contrast, only a few hundred people have been described as infected with *H. diminuta*. In general, human cases of hymenolepiasis occur without symptoms. However, in some cases, mild clinical manifestations may be observed, mainly diarrhea, abdominal pain, anorexia and other nonspecific gastrointestinal symptoms (Yang *et al.,* 2017). Hymenolepiasis is a community acquired parasitic disease, rarely recorded in Bulgaria (Rainova *et al.,* 2018). Over the three-year study period a total of 720 cases were registered (mean prevalence 0.07 %).

As regards taeniasis, cases caused by beef tapeworm have been sporadically recorded in the country, they are mainly seen in areas with livestock production. The causes of the disease are mainly a lack of awareness of the disease, slaughtering farm animals at home without the proper veterinary supervision. No cases of pork tapeworm infection were recorded during the study period and we were unable to access the official data on the number of cases of cysticercosis in swine.

Protozoa infections of the gastrointestinal tract are recorded worldwide. The most common outbreaks caused by protozoa are generally associated with *Giardia intestinalis* and *Cryptosporidium parvum*, respectively 40.6 % and 50.8 % (Dudlova *et al.,* 2016). In contrast to the literature data, epidemic outbreaks of *G. intestinalis* or *C. parvum* infections in humans have not been recorded in Bulgaria as yet. Although tests for giardiasis are routinely conducted in all diagnostic parasitology laboratories, it is not the case with the diagnosis of cryptosporidiosis. The tests to diagnose that particular protozoan infection (microscopy of stained slides and rapid immunochromatographic tests) are performed in a relatively small number of diagnostic parasitology laboratories. During the study period, a total of 1062 individuals were examined for cryptosporidiosis, of whom 18 (1.69 %) proved to be positive. Although there is evidence for the presence of *C. parvum* in some water sources in the country (Karanis *et al.,* 2006), the control measures during epidemic outbreaks of diarrheal syndrome primarily focus on bacterial pathogens rather than protozoa.

Cases of *Blastocystis* spp. infections are often diagnosed in developed countries. Its role in the etiology of gastrointestinal symptoms remains unclear. According to some authors, it is a conditionally pathogenic protozoa (Scanlan *et al.,* 2014). According to others, clinical symptoms associated with the presence of *Blastocystis* spp. include nausea, loss of appetite, abdominal pain or chronic diarrhea, often associated with chronic gastrointestinal disease of unknown etiology and irritable bowel syndrome (Yakoob *et al.,* 2010; Fletcher *et al.,* 2014). Studies conducted in Africa showed that *B. hominis* can be a major cause of diarrhea where poor adherence to personal and household hygiene has been described (Graczyk *et al.,* 2005).In our study, the mean prevalence of the infection was 0.22 % in all age groups. Regarding clinical manifestations, there are cases of asymptomatic infections, and when symptoms are present, they are no different from those described in the literature.

The extent of the damage caused by intestinal parasites depends on: (a) the type of the parasite; (b) the parasitic load and clinical course of the disease; (c) the nature of the interactions between the parasitic species and the presence of concomitant infections; (d) the nutritional and immunological status of the population; and e) socio-economic factors. All of the above factors may additionally be affected by seasonal and climatic conditions. It is usually difficult to measure the suffering caused by infectious diseases, and in the case of intestinal parasitic infections, this is even more difficult, as many of the cases are asymptomatic and therefore remain undetected (WHO, 1987). In Europe, intestinal protozoan and helminth infections predominantly spread in Southeast Europe and Turkey where the living conditions are poorer and the socioeconomic status is lower (Hotez & Gurwith, 2011). Over 165 million people (more than 20 % of the European population) live below the poverty line and about 2 % of the European population lives in absolute poverty. This has a significant impact on the incidence and prevalence of parasitic diseases (Stuckler *et al.,* 2009).

Some population groups require particular attention because they are more vulnerable and susceptible to infections and parasitic diseases. One of these is the Roma population, which comprises between 7 and 9 million people living in Central and Eastern Europe. The largest numbers of the Roma people in Europe reside in Romania, Bulgaria, North Macedonia and Slovakia, where they account for over 8 % of the population (Rechel *et al.,* 2009). Of these, 70 % to 80 %, mainly in Bulgaria and Romania, live in poverty, have poor housing conditions, poor hygiene habits and often suffer from malnutrition. Their susceptibility to intestinal parasitic diseases, bacterial and viral infections is very high (Rechel *et al.,* 2009). Other population risk groups in Europe include immigrants, especially migrants from Africa, who often suffer from the so-called "neglected tropical diseases", as well as the children deprived of parental care living in social institutions (Norman *et al.,* 2010).

Conclusions

In Bulgaria, the existing regulations for surveillance and control of parasitic diseases provide for relatively high detection rates of parasitic infections. Many of the infected individuals are asymptomatic hosts of parasites. High detection rates can be attributed to annual prophylactic parasitological screening of children (attending children's establishments/ institutions), adults (working in the food industry, retail outlets, catering establishments, etc.) and migrants. This allows timely treatment of the infected persons who might potentially become the source of infection for other individuals.

Conflicts of interest

Authors declare no conflict of interest.

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sciendo

DOI 10.2478/helm-2020-0001

HELMINTHOLOGIA, 57, 1: 19 – 27, 2020

Urinary schistosomosis in patients of rural medical health centers in Kwale county, Kenya

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Article info

Received February 22, 2019
Accepted October 10, 2019

Summary

Urinary schistosomosis is a serious public health problem prevalent in low-income rural regions of sub-Saharan Africa, including coastal part of Kenya. Praziquantel administration to school-aged children is the prevailing tool of schistosomosis control in these regions. The aim of our study was to find out if this control strategy can lead to interruption of parasite trasmission and disease elimination. During February and March 2018, the occurrence of urinary schistosomosis in volunteers of primary health care facilities in Kwale County, Kenya was examined and the occurrence of infected intermediate hosts *Bulinus globosus* in local water resources was monitored. Participants completed a questionnaire concerning source of water for household purposes, type of housing and health status and were asked to provide urine samples. Diagnosis of urinary schistosomosis was established by detection of *Schistosoma haematobium* eggs in urine specimens microscopically, using filtration method. Infected *B. globosus* snails were detected using cercaria shedding tests. From the hemolymph of snails, prepatent period of infection was identified by polymerase chain reaction (PCR). The presence of urinary schistosomosis was detected in 15.07 % (69 out of 451) of study participants. Cercaria shedding test was positive in 2 particular sites of river Pengo and Tsanganyiko. Genetic material (haemolymph) of 68 *B. globosus* snails tested by DraI PCR revealed 7 *Schistosoma* spp. positive samples. Six of seven DraI positive snails were infected by *S. haematobium,* as it was detected by Sh110/SmS1 PCR. The study revealed, that the disease was still present in the region studied and the transmission was not interrupted. The rate of infection was significantly influenced by the water supplies used for household purposes and the type of housing.
Keywords: *Schistosoma haematobium*; urinary schistosomosis; parasite transmission

Introduction

Urinary schistosomosis (known also as snail fever or bilharzia), caused by parasitic blood trematode *S. haematobium*, is prevalent in regions with lack of clean water and sanitation systems in the home, often in rural regions of sub-Saharan Africa (Bruun and Aagaard-Hansen, 2008). Humans become infected when they come in contact with contaminated water sources infested by *S. haematobium* cercariae that are released from intermediate hosts – freshwater snails of the genus *Bulinus.*

Urinary schistosomosis causes a serious public health problem in coastal parts of Kwale county, Kenya. Regular praziquantel administration to school-aged children over the past years might lower the disease prevalence and reduce morbidity. It seems that interruption of transmission might be essential for the successful elimination of the disease. The intermediate host – freshwater snail of

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the genus *Bulinus* – releases cercarial larvae in the water. If they come into contact with human skin, they penetrate it within minutes and migrate via the blood circulation to the portal vein. In this place they develop into the tiny immature flukes called schistosomulae. In the portal vein schistosomulae mature, copulate, and the constantly paired couples migrate to perivesicular venous plexus. Fertilised females lay eggs, which may move through the vessels and enter the cavity of the bladder to be excreted. Ciliated miracidia released from the eggs after hatching move actively and seek for their intermediate host, thus completing the life cycle. However, some of the eggs may be retained in tissues of the hosts inducing immune response and inflammation process, which may lead to various pathological consequences. Untreated infection can lead into painful urination, suprapubic discomfort, haematuria, inflammatory and granulomatous lesions in the male and female urinary systems. Anaemia, bladder cancer, kidney failure as well as co-infection with some viruses, bacteria, or parasites are another possible complications of disease in the later stages (El Ridi and Tallima, 2013). In spite of the fact, that people with mild worm burdens can have minimal or no symptoms, the health implications of the disease may be far-reaching (Gryseels *et al.*, 2006). Efforts to control morbidity of schistosomosis is increasing in countries of sub-Saharan Africa, including endemic region parts of Kenya. In these areas*, S. haematobium* infection control is focused on regular administration of single dose of praziquantel to school-aged children, referred to as mass drug administration (MDA) (N'goran *et al.,* 2003; Muhumuza *et al.,*2014).

The main objective of our study was to evaluate the impact of the control strategies on the transmission of schistosomiasis in the area studied. Therefore, during February and March 2018, the occurrence of urinary schistosomosis in patients of primary health care facilities was examined and at the same time the occurrence of infected intermediate hosts *Bulinus globosus* in selected local water resources was monitored.

Materials and Methods

Specimens of urine and questionnaire answers were obtained from individuals/patients of cooperating primary health care facilities in Kwale County, Kenya – Mwachiga Dispensary (Kinango Constituency, Kinango Location, Dumbule Sub Location), Mwaluphamba Dispensary (Matuga Constituency, Mwaluphamba Location, Mlafyeni Sub Location), Bilashaka Dispensary (Matuga Constituency, Tsimba Golini Location, Tsimba Golini Sub Location) a Mbuwani Dispensary (Msambweni Constituency, Diani Location, Msambweni Sub Location). Snails were collected from the selected places of water bodies localised on rivers Mbeto, Kombo, Bora, Ndugunane, Jarumani and Ramoyo in the surroundings of Mwachinga Dispensary; rivers Bechone Swabirina, Mbadzi, Tsanganyiko, Bangoni, Komanazilale, Mzizima in the surroundings of Mwaluphamba Dispensary as well as rivers Buburu, Kivumiro, Chimambani and Mbararani in the surroundings of Bilashaka Dispensary (Fig.1).

All of these areas are endemic only for urinary schistosomiasis caused by *S. haematobium*. Inhabitants of these remote villages work mostly as peasant farmers or casual workers. Most of them declare an acces to sanitation system in the form of pit latrines and access to clean water - *e.g.,* in Mwachinga Japan International Cooperation Agency constructed water kiosk for villagers. However, they still use water from lakes, rivers and ponds for watering animals, bathing or washing clothes, as was shown by questionnaire results. In the framework of deworming programme, in Mwaluphamba, Bilashaka and Mbuwani, co-administration of praziquantel (against urinary schistosomiasis) and albendazole (against soil-transmitted helminth infections) to community and school children was performed once a year in follwoing years: 2004, 2005,2007, 2009 and 2012. To date, praziquantel is applied in these areas to school-children once a year. In Mwachinga, praziquantel was administered to both school children and community yearly from 1998 – 2007. From 2010 to date, it is applied to school-children once a year.

Inclusion/exclusion criteria of the study participants

The aim of our study was to monitor *S. haematobium* infection in volunteers of different ages to be able to evaluate their contribution to the disease transmission. Therefore, all patients of primary health care facilities who were willing to complete a questionnaire and sign informed consent were enrolled into the study, except menstruating female volunteers. The urine samples were analyzed the same day in the laboratory section (corner/room) of these cooperating health care centers (dispensaries).

Informed consent, questionnaire, and urine sample collection

From February 19th – March 16th, 2018 randomly selected individuals/patients of primary health care facilities were contacted by local health officials who explained to them about voluntary participation and objectives of the study. Parents/care-takers were asked to give their ageement on behalf of participating minors. After acceptance to be enrolled in the study, participants (or parents/care-takers) were asked to sign informed consent and complete a short questionnaire concerning health status, source of water used, previous history of praziquantel administration and awareness of disease transmission. Samples of urine were assigned with the identification number and processed the same day in the laboratory section of health facilities. In urine specimens, macrohematuria was detected visually. For the presence of microhaematuria in the urine samples, diagnostic strips Hemophan (Erba Lachema Ltd., Brno, Czech Republic) working on the basis of chemical reaction of hemoglobin with chemicals on the reagent pad of the strip were used. Then, 10 ml of urine sample was passed through the Nucleopore membrane filter (25 mm, pore size 8 μm). The filter was then placed on a microscope glass slide and the eggs of *S. haematobium* were detected under the microscope at a magnification of x 400. The intensity of infection was expressed as light (L), medium (M) or heavy (H) according to the number of eggs per 10 ml

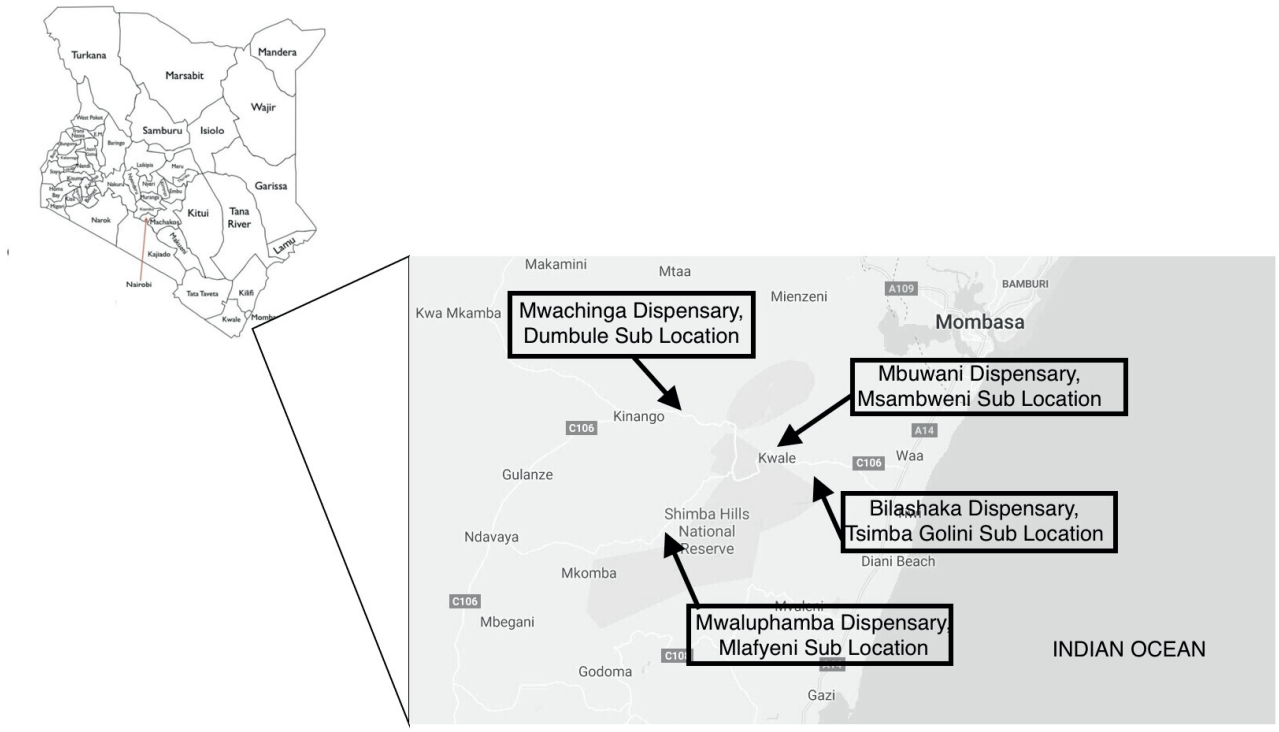

Fig.1. Map showing the location of areas included in survey, Kwale County, Kenya.

of urine (light: 1 – 4 eggs, medium: 5 – 49 eggs, heavy: ≥ 50 eggs). Participants found positive for *S. haematobium* infection throughout the study were treated with praziquantel in cooperation with primary health care facility workers.

Field procedures

Cercariae of infected freshwater snails were detected with the assistance of NUITM-KEMRI workers using cercaria shedding test. Snails were collected from the selected places of water bodies localised in the surroundings of cooperating health facilities, as described earlier. For this purpose scoop from steel sieves was used. Snails were then placed into a collecting pot containing water from the habitat, and supporting data such as date and time of collection, water and air temperature as well as site and weather conditions were recorded. After identification of snails based on shell morphological characteristics, cercaria shedding test was performed. The snails were placed in 24-well culture plates individually and exposed to indirect sunlight for 2 hours to induce cercaria shedding. The wells of the plates were then examined for the presence of cercariae under a binocular microscope (magnification 40x). Visually identified cercariae were then captured in 2 µl of fresh water and pipetted onto sample areas of Whatman Elute FTA micro cards.

Moreover, Whatman FTA Elute cards were used to trap the genetic material of the snails (hemolymph), keeping it stable and safe at room temperature and easily transportable. The presence of prepatent stadium of parasite in *B. globosus* snails was then tested by polymerase chain reaction (PCR).

Table 1. Basic characteristic of study group.

	n (%)
Female	323 (71.62%)
Male	128 (28.32%)
Age	
Less than 10 years	7 (1.55%)
10 – 15 years	106 (23.50%)
16 – 20 years	65 (14.41%)
21 – 26 years	57 (12.64%)
27 – 35 years	98 (21.73%)
36 and more years	118 (26.16%)
Study area	
Mwachinga Dispensary	117 (25.94%)
Mwaluphamba Disp.	124 (27.49%)
Bilashaka Dispensary	122 (27.05%)
Mbuwani Dispensary	88 (19.51%)

Table 2. Presence of *S. haematobium* eggs in urine by gender and study area, n=451.

Gender	Negative	Positive	*p-value*; χ^2
Female			**0.483168; 0.4917***
Mwachinga Dispensary	74 (22.91 %)	7 (2.17 %)	
Mwaluphamba Disp.	66 (20.43 %)	18 (5.57 %)	
Bilashaka Disp.	73 (22.60 %)	21 (6.50 %)	
Mbuwani Disp.	63 (19.50 %)	1 (0.31 %)	
Total (n=323)	**276 (85.45 %)**	**47 (14.55 %)**	
Male			
Mwachinga Dispensary	29 (22.66 %)	7 (5.47 %)	
Mwaluphamba Disp.	31 (24.22 %)	9(7.03 %)	
Bilashaka Disp.	22 (17.19 %)	6 (4.69 %)	
Mbuwani Disp.	24 (18.75 %)	0 (0.00 %)	
Total (n=128)	**106 (82.81 %)**	**22 (17.19 %)**	
Study area			
Mwachinga	103 (88.03 %)	14 (11.97 %)	
Mwaluphamba	97 (78.23 %)	27 (21.77 %)	
Bilashaka	95 (77.87 %)	27 (22.13 %)	
Mbuwani	87(98.86 %)	1(1.14 %)	
Total	**382 (84.70 %)**	**69 (15.30 %)**	
Mwachinga Dispensary			
Less than 10 years	0 (0.00 %)	1 (100 %)	
10 – 15 years	23 (79.31 %)	6 (20.69 %)	
16 – 20 years	13 (86.67 %)	2 (13.33 %)	
21 – 26 years	9 (81.82 %)	2 (18.18 %)	
27 – 35 years	27 (93.10 %)	2 (6.90 %)	
36 and more years	31 (96.88 %)	1(3.12 %)	
Total	**103 (88.03 %)**	**14 (11.97 %)**	
Mwaluphamba Dispensary			
Less than 10 years	5 (83.33 %)	1 (16.67 %)	
10 – 15 years	31 (75.61 %)	10 (24.39 %)	
16 – 20 years	23 (79.31 %)	6 (20.69 %)	
21 – 26 years	12 (80.00 %)	3 (20.00 %)	
27 – 35 years	11 (78.57 %)	3 (21.43 %)	
36 and more years	15 (78.95 %)	4 (21.05 %)	
Total	**97 (78.23 %)**	**27 (21.77 %)**	
Bilashaka Dispensary			
Less than 10 years	0 (0.00 %)	0 (0.00 %)	
10 – 15 years	16 (69.57 %)	7 (30.43 %)	
16 – 20 years	9 (75.00 %)	3 (25.00 %)	
21 – 26 years	14 (82.35 %)	3 (17.65 %)	
27 – 35 years	28 (82.35 %)	6 (17.65 %)	
36 and more years	28 (77.78 %)	8 (22.22 %)	
Total	**95 (77.87 %)**	**27 (22.13 %)**	
Mbuwani Dispensary			
Less than 10 years	0 (0.00 %)	0 (0.00 %)	
10 – 15 years	13 (100.00 %)	0 (0.00 %)	
16 – 20 years	9 (100.00 %)	0 (0.00 %)	
21 – 26 years	13 (92.86 %)	1(7.14 %)	
27 – 35 years	20 (100.00 %)	0 (0.00 %)	
36 and more years	32 (100.00 %)	0 (0.00 %)	
Total	**87 (98.86 %)**	**1 (1.14 %)**	

**p-value* and χ^2 is related to differences between males and females altogether

PCR detection of S. haematobium DNA from B. globosus

For DNA extraction a 3 mm disc containing the sample was punched out of the Whatman FTA Elute cards (GE Whatman, Maidstone, Kent, United Kingdom). The discs were then placed into a microcentrifuge tubes containing 500 µl of PCR water and vortexed 3 times for 5 seconds at high speed. After removal of excess wash water from the tubes, 35 µl of PCR water was added to the punch. Samples were incubated at 95 °C for 30 min and then centrifuged to separate the matrix from the eluate containing DNA. Using sterile pipette tips, the discs were removed from the tubes and discarded.

Primers for two PCR assays were designed on the basis of previously published sequence information (Amarir *et al.*, 2014). DraI PCR was performed using commercially available ready-to use mastermix My Taq™Mix, 2x (Bioline, London, UK), DraI primers (forward: GATCTCACCTATCAGACG, reverse: GTCAC-CAATAATATGAAAC), and target DNA.

To distinguish *S. haematobium* from other relative species - *e.g., S. bovis,* which is sympatric with *S. haematobium* in many endemic areas - the DNA of DraI positive snails were analysed by Sh110/SmSl PCR that was unique for *S. haematobium*. The primer combination used included Sh110 primer: 5'- TTC CTC CAA CTA CCA TCT TAT CTC-3'and Sm-Sl primer: 5'- AAC CGT CAC GGT TTT ACT CTT GTG-3'. PCR conditions for both assays were set at 95 °C for 5 min for initial denaturation, followed by 35 cycles of 95 °C for 1 min (denaturation), 35 cycles of 55 °C for 1 min (annealing) and 35 cycles of 75 °C for 1 min (extension). Using UV trans-illumination, the amplified products then were visualized on 1.5 % agarose gel stained with fluorescent nucleic acid dye GelRed™.

Data analysis

Basic descriptive analysis was performed, differences in proportions were compared by the Chi square ($\chi 2$) test using R software, version 3.4.0. Statistical significance was set at a p -value of 0.05.

Ethical Approval

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

Results

From February 19 – March 16, 2018 a total of 451 participants (323 women and 128 men) were involved into the study. The research team examined 117 samples in Mwachiga Dispensary, 124 in Mwaluphamba Dispensary, 122 in Bilashaka Dispensary and 88 in Mbuwani Dispensary. The basic characteristic of the study group is presented in Table 1.

The presence of *S. haematobium* eggs was detected in urine specimens of 15.30 % (69 out of 451) of study participants altogether; 11.97 % (14 out of 117) in urine samples of participants in Mwachinga Dispensary, 21.77 % (27 out of 124) in Mwaluphamba Dispensary and 22.13 % (27 out of 122) in Bilashaka Dispensary. In Mbuwani Dispensary we detected the presence of *S. haematobium* eggs only in one urine sample (1.14 %).

Of 451 participants (323 women and 128 men) who were examined for the presence of eggs in urine samples, 47 women and 22 men were detected as schistosomosis positive. Men (17.19 %) had a slightly higher rate of infection than women (14.55 %), but this difference was not statistically significant ($\chi^2 = 0.48$; p = 0.49) (Table 2).

Out of 451 of study participants 53 (11.75 %) reported access to borehole water, the rest of them used ponds, lakes, rivers, backwaters, or water from tanks as a source of household water or water for animals. The significantly higher infection rate was found among participants who used environmental water sources such as rivers, ponds, or backwaters (14.6%) in comparison to those, who reported access to the borehole water (0.6%) for household purposes or as a source of water for animals ($\chi^2 = 4.99$; OR = 5.04; 95 % CI 1.19 – 21.24; p = 0.025). Similarly, participants living in traditional houses made of mud showed a significantly higher number of *Schistosoma* infection cases than those living in concrete houses ($\chi^2 = 5.18$; OR = 2.18; 95 % CI 95 % 1.13 – 4.22; p = 0.02).

The comparison between haematuria and infection status revealed, that 47 individuals (out of 69) with detected *S. haematobium* eggs in the urine samples were screened positive for haematuria, whereas the rest (n = 22) had no sign of haematuria. Inversely, 97 of the participants were found to be *S. haematobium* infection negative, although they demonstrated haematuria and 284 were both infection and haematuria negative.

The presence/absence of *S. haematobium* eggs in the urine with/without haematuria is shown in Table 3.

Table 3. The presence/absence of *S. haematobium* eggs in the urine with/without haematuria.

	Eggs pos.	Eggs neg.	Total
Haematuria pos.	47 (10.42 %)	97(21.5 %)	144
Haematuria neg.	22 (4. 87 %)	285(63.19)	307
Total	69	382	451 (100 %)

Sensitivity rate = 68 %, specificity rate = 74 %, positive predictive value = 32 %, negative predictive value = 92 %, validity = 74 %.

Table 4. Presence of *B. globosus* and cercaria shedding test results in selected local water resources.

Date of collection	Locality/area	Water source	*B. globosus* (n)	Cercaria shedding test
23.02.18	Mwachinga	Mbeto river	0	_
23.02.18	Mwachinga	Kombo river	0	_
26.02.18	Mwachinga	Bora river	31	neg.
27.02.18	Mwachinga	Ndugunane river	10	neg.
28.02.18	Mwachinga	Jarumani river	13	neg.
01.03.18	Mwachinga	Ramoyo river	21	neg.
02.03.18	Mwaluphamba	Bechone Swabirina river	27	neg.
02.03.18	Mwaluphamba	Pengo river	23	pos.
05.03.18	Mwaluphamba	Mbadzi river	0	_
05.03.18	Mwaluphamba	Bangoni river	11	pos.
06.03.18	Mwaluphamba	Tsanganyiko river	30	pos.
07.03.18	Mwaluphamba	Komanazilale	3	neg.
08.03.18	Mwaluphamba	Mzizima river	0	_
12.03.18	Bilashaka	Buburu river, 1. site	28	neg.
12.03.18	Bilashaka	Buburu river, 2. site	15	neg.
13.03.18	Bilashaka	Kivumiro river, site 1	5	neg.
13.03.18	Bilashaka	Kivumiro river, site 2	5	neg.
14.03.18	Bilashaka	Chimambani river	32	neg.
15.03.18	Bilashaka	Mbararani river, 1. site	11	neg.
15.03.18	Bilashaka	Mbararani river, 2. site	24	neg.

However, after stratification of the intensity of *S. haematobium* infection by light (n = 50), medium (n = 10) and heavy (n = 9), it was seen that all cases of heavy infections (i.e., 50 or more eggs/10 ml of urine) were connected with documented haematuria in the urine samples.

Nine of 69 infection-positive and 36 of infection-negative participants reported treatment with praziquantel in the past 6 months. There was no significant difference between infection positivity of treated and un-treated group of patients (χ^2 = 0.85; OR = 1.44; 95 % CI 0.66 – 3.15; p = 0.36). Nine infection-positive participants belonged to the age group 10 – 15 years; 29 infection positive participants were in age group 16 – 20 years, 11 in age group 20 – 25 years, 10 in age group 26 – 35 years, 9 were older than 36 years and 1 positive participant was 6 years old.

Intermediate host of infection in these regions – *B. globosus* – was found in 10 out of 16 screened water resources. Cercaria shedding test was positive in 2 particular sites of river Pengo and Tsanganyiko (Table 4).

Genetic material of 68 *B. globosus* snails tested by DraI PCR revealed 7 positive samples. Confirmation by Sh110/SmSl PCR showed that 6 of 7 DraI positive snails were infected by *S. haematobium*. The positive snails came from the rivers Mbararani, Buburu, Kiwumiro and Bangoni.

Discussion

Efforts to control morbidity of schistosomosis as well as other helminthosis is increasing in many countries of sub-Saharan Africa, including Kenya – School Based Deworming Programme is one of the main tool of control focused on regular administration of a single dose of anthihelminth drugs to school-aged children. At the end of the twentieth century the prevalence of *S. haematobium* infection was >70% among school-aged children in Kwale County, Kenya (King *et al.*, 1988, Sato *et al., 1988*). Recent publications refer that more than 30 % of schoolchildren could be infected with *S. haematobium* in this part of Kenya (Bustinduy, 2013, Chadeka, 2017, Njenga, 2014). In the framework of deworming programme, in Mwaluphamba, Bilashaka and Mbuwani, co-administration of praziquantel (against urinary schistosomiasis) and albendazole (against soil-transmitted helminth infections) to community and school children was performed once a year in follwoing years: 2004, 2005,2007, 2009 and 2012. To date, praziquantel is applied in these areas to school-children once a year. In Mwachinga, praziquantel was administered to both school children and community yearly from 1998 – 2007. From 2010 to date, it is applied to school-children once a year.

However, this regular treatment approach cannot interrupt schis-

tosomosis transmission, as is indicated by a number of authors (Njenga *et al.,* 2014; Adenowo *et al.,* 2015; Sokolow *et al.,*2016). This is congruent also with our study conducted in Kwale County, Kenya in February-March, 2018. Our survey revealed some areas with a higher proportion of study participants infected with *S. haematobium* (i.e., the catchment areas of Mwachinga, Mwaluphamba and Bilashaka Dispensaries with 11.96 %, 20.96 % and 22.13 % of infected individuals, respectively), while the other locality surveyed (catchment area of Mbuwani Dispensary) showed only a very slight proportion of infected subjects (1.13 %). The focal character of schistosomosis transmission was documented by a number of relevant publications (Brooker, 2002; Clennon *et al.,* 2006; Meurs *et al.,* 2013; Chadeka *et al.,* 2017), suggesting a public health problem in geographically restricted localities. The difference between men and women in the prevalence of schistosomosis was not statistically significant (17.18 % and 14.55 %, respectively; p = 0.48). However, there was a higher sex ratio in favor of women among the participants in our study, which was caused by the proportion of patients in primary health care facilities, focusing mainly on reproductive, maternal and child health care services.

The results of studies dealing with gender effect on the prevalence of urinary schistosomosis are inconsistent, moreover, the majority of them were evaluating data collected from school children or preschool children, in contrast to our study, in which also older age categories were included (Nkegbe *et al.,* 2010; Ekpo *et al.,* 2011; Kayuni *et al.,*2017). Males may be involved in water-contact activities such as cattle watering, preparing materials for construction of buildings or farming, while females in laundry, household cleaning and other domestic works – with no diffefence in exposure to the risk factor. The significantly higher infection rate was found among participants who used environmental water sources as rivers, ponds, or backwaters in comparison to those, who reported access to the borehole water for household purposes or as a source of water for animals (χ^2 = 4.99; OR = 5.04; 95 % CI 1.19 – 21.24; p = 0.025). This is consistent with Singh *et al.* (2016), who in Sokoto, Nigeria, reported higher occurrence of urinary schistosomosis among those using dam water as a water supply for drinking (75.24 %) in comparison to those who used water from boreholes (17.64 %). Also Grimes *et al.* (2014) who carried out a systematic meta-analysis of studies reporting *S. haematobium* infection rate suggested, that safe water supplies were associated with significantly lower odds of schistosomosis. In contrast to these findings, Kholy *et al.* (1989) state that borehole well introduction had minimal impact on transmission of schistosomosis in 3 endemic villages in Kenya. Also Mutuku *et al.*(2011) indicate, that residents in some infection areas might prefer pond and river water for laundry and bathing over the hard water from borehole wells.

The significantly higher rate of detected schistosomosis in participants living in traditional mud houses found in our study (χ^2 = 5.18; OR = 2.18; 95 % CI 95 % 1.13 – 4.22; p = 0.02)

may be connected with a lower socioeconomic status of their inhabitants rather than the construction material itself. Sady *et al.* (2013) indicated, that low household monthly income was one of the key factors significantly associated with schistosomosis among children in rural areas in Yemen. An association between schistosomosis and socioeconomic conditions was pointed out also by study of Ximenes *et al.* (2006). Socioeconomic development connected with implementation of adequate water supply, sewage system, sanitation facilities and health education could have permanent positive effect on the control of schistosomosis (Ximenes, 2006; Sady, 2013).

In highly endemic areas, detection of hematuria could serve as a proxy indicator for *S. haematobium* infection identification (Anosike *et al*. 2001; French *et al.* 2007; Houmsou *et al.* 2011). The validity of haematuria as a diagnostic criterion for urinary schistosomosis screening was discussed in a number of publications (King*et al*., 2013; Krauth *et al.* 2015; Ochodo*et al*., 2015; Knopp *et al*., 2018). In our study, dipstick test sensitivity and specificity for detection of egg-positive urine were estimated at 68 % and 74 %, respectively (Table 3). However, after stratification of the intensity of *S. haematobium* infection by light, medium and heavy, we observed a substantial increase in the sensitivity rate of the dipstick test in heavy infections. This is in agreement with the results of other studies (King *et al*.,2013; Krauth *et al*., 2015), which found that the sensitivity of the hematuria test is reduced in groups with light intensity infections.

Nine of 69 infection-positive participants reported medication with praziquantel in the past 6 months. All of these 9 participants belonged to the age group 10 – 15 years, so they were involved in the programme of MDA to school-aged children. However, after a treatment with praziquantel, they might get re-infected during their contact with water infested with *S. haematobium* positive intermediate hosts. Infected study participants excluded from deworming programme may contribute to water infestation via excreted parasite ova.

In order to estimate disease transmission, suspected water bodies localised in the surroundings of cooperating health facilities were monitored. After scooping, snails of the genus *Bulinus* were morphologically identified and cercaria shedding test was performed. The intermediate host of infection – *B. globosus* – was found in 10 out of 16 screened water resources. The cercaria shedding test was positive in 2 particular sites of river Pengo and Tsanganyiko. Moreover, from the genetic material (haemolymph) trapped on FTA cards, the presence of prepatent stages of *S. haematobium* in *B. globosus* was detected by two PCR assays (DraI PCR and Sh110/Sm-SI PCR) using primers according to Amarir *et al.* (2014). These reactions, which enabled differentiation of *Schistosoma haematobium* DNA from DNA of related *Schistosoma* spp. (*e.g., S. bovis*, which is sympatric with *S. haematobium* in many endemic areas), revealed infected snails also at the river Buburu, Mbararani, Kiwumiro and Bangoni.

The results of our study indicate that the treatment strategy focusing only on MDA to school age children cannot interrupt the transmission of the disease. Since most affected people live in

simple dwellings without running water, they will inevitably come into contact with water contaminated by cercariae when bathing, washing clothes or watering animals. Recently, therefore, many authors have emphasized the need for integrated control measures, including regulation of the number of intermediate hosts, access to clean water and health education as a complement to MDA (Sokolow, 2016; Ross, 2017). Furthermore, the World Health Assembly (WHA) in its resolution called for the implementation of complementary, non-pharmaceutical control strategies to eliminate this disease (WHA, 2012).

In conclusion, schistosomosis was still present in the study area, although the majority of positive participant had light form of infection. Transmission of diseases was not interrupted and continued to take place in some areas of reasearch. The rate of infection was significantly influenced by type of housing and water supplies used for indoor and outdoor household purposes. Preventive measures should consider that MDA to school children as well as implementation of adequate water supply, sewage system, sanitation facilities and health education could have positive impact on the control of schistosomosis.

Acknowledgements

Our appreciation goes to all participants of the study. We also thank to the Ministry of education, science, research and sport of the Slovak Republic for the funding of the project and the head of Specific Department of FHSaSW TU in Kenya in Kwale region, Kenya, Dr. Zuzana Kráľová, PhD., for management of cooperation with KEMRI-NUIT workers, Kwale, Kenya. We are also gratful to Mrs. Viera Mišurová and Mr. Moses Whakungu Machimbo for excelent technical assistance and to Dr. Mark Steven Taylor for his linguistic review of the manuscript. In addition, we acknowledge the support offered by grant KEGA no. 013TTU-4/2019.

Conflict of Interest

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

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DOI 10.2478/helm-2020-0011

HELMINTHOLOGIA, 57, 1: 28 – 36, 2020

Copro-prevalence and risk factor assessment of gastrointestinal parasitism in Indian domestic pigs

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Article info

Received February 2, 2019
Accepted December 23, 2019

Summary

The aim of the present study was to determine copro-prevalence of gastrointestinal parasites and their associated potential risk factors in pigs of Punjab (India). A total of 839 faecal samples were collected from pigs of all age group and sex from different agro-climatic zones of Punjab covering all seasons and subjected to qualitative and quantitative examination. Among the samples examined, 28.4 % were positive for gastrointestinal parasites and their respective prevalences were *Ascaris suum* (11.1 %), coccidia (9.41 %), *Trichuris suis* (6.43 %), *Balantidium coli* (4.5 %), amphistome (3.33 %), strongyle (2.14 %) and *Ascarops strongylina* (1.78 %). Upon sporulation of coccidian positive samples, 8 species of *Eimeria* were recorded (*Eimeria polita*, *E. spinosa*, *E. scabra*, *E. perminuta*, *E. suis*, *E. debliecki*, *E. neodebliecki* and *E. porci*). Among the various risk factors analysed, season, agro-climatic zones and managemental practices had a significant ($p<0.05$) effect on gastrointestinal parasitism of pigs. Quantification of the infection levels in various seasons and age groups revealed the highest mean egg per gram in rainy season (1966. 6± 1146.5) and grower pigs (1457.1 ± 500.4). Coproculture analysis revealed the presence of larvae of *Hyostrongylus rubidus* and *Oesophagostomum* species. The results of the current study would be of immense help in formulation and implementation of control strategies for effective control of gastrointestinal parasitism in pigs.
Keywords: coprology; India; prevalence; gastrointestinal parasites; pig; risk factors

Introduction

Internal parasites are the major biological constraints to efficient pig production but they are often overlooked as the clinical symptoms are rarely apparent. In pigs, parasites cause 5 % and 31 %, reduction of the daily feed intake and average daily growth, respectively and an average 17 % higher Feed Conversion Ratio compared to the parasite-free fattening pigs (Ózsvári, 2018). In India, majority of pigs are raised under free range system where they feed upon raw garbage, kitchen waste and faecal matter, making them more prone to parasitic infections (Tiwari *et al.,* 2009). Moreover, the close association between pigs and humans enables cross-infection with a range of zoonotic parasites like *Taenia solium, Trichinella spiralis* and *Toxoplasma gondii*, all of which contribute deleteriously to human health. Pigs are infected with wide range of gastrointestinal (GI) parasites with reports from all corners of the world (Permin *et al.,* 1999; Tamboura *et al.*2006; Lai *et al.,* 2011; Navarro-Gonzalez *et al.,* 2013; Yui *et al.,* 2014;Alynne *et al.,* 2015; Junhui *et al.,* 2015; Kabululu *et al.,* 2015;) including India (Laha *et al.,* 2014; Dadas *et al.,* 2016; Joute *et al.,* 2016;

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Krishna Murthy *et al.,* 2016; Singh *et al.,* 2017; Patra *et al.,* 2019). Age, sex of animal, management practices and geographical location are the major risk factors associated with GI parasites in pigs (Permin *et al.,* 1999; Geresu *et al.,* 2015; Roesel *et al.,* 2017). It is predicted that in next ten years, the total consumption of meat in India will double from its present numbers and hence more emphasis need to be given for the efficient and economical pig husbandry. The growing demand for pork due to the growth of hotel industry and proliferation of fast food chains in the country has prompted farmers in Punjab to go in for pig farming in a big way. In preview of the significance of GI parasites as one of the most important causes of economic losses to pig industry (Roesel *et al.,* 2017) and lack of data from Indian states, it was justified to reassess the prevalence and its associated risk factors from Punjab state.

Materials and Methods

Sampling procedure

As per the 19th Livestock Census (2012) of India, population of the pigs in Punjabis approximately 32,221 (DAHP, 2016). . The sample size for each category according to demography and management was calculated by employing software EpiTool (http://epitools.ausvet.com.au). The state population of 32221 heads with an expected prevalence of 50 % for GI parasites, margin of error as 3.1 % (≤5 %), and a 95 % confidence interval (CI) resulted in sample size of approximately 320. However, additional convenient sampling was done to include more samples in the study. Thereafter, cluster sampling was employed where each of five agro-climatic zones namely sub-mountain undulating zone (I), undulating plain zone (II), central plain zone (III), western plain zone (IV) and western zone (V) formed a single cluster each and a systematic sampling comprising of villages representing each cluster was done. As the pig population was unevenly distributed, sampling from the villages which were representative of each cluster was done. Thus, total of 839 faecal samples of pigs from 18 districts comprising 32 villages representing 5 agro-climatic zones were collected. A total of 36 commercial and backyard farms were sampled throughout the year covering all the seasons viz. summer (March to June), rainy (July to October) and winter (November – February) and a proportional sampling scheme was adopted. Faecal samples were collected from the suckling and weaner piglets (<4months), growers (4 – 8months) and adults (>8months) from both the sexes.

Data collection

Information regarding various risk factors hypothesized to be associated with the prevalence of GI infection i.e. type of farm management (organised/ unorganised), water consumption (controlled/ uncontrolled), deworming (present/absent) and cleanliness status of the farm (present/absent) based on the scores like regular removal of waste material from sheds and drainage etc. was collected through predesigned questionnaire.

Sample processing and examination

The faecal samples were collected immediately after defecation, and subjected to qualitative faecal examination techniques (concentration-flotation and sedimentation technique) as per standard protocols (Soulsby, 1982). Quantification of strongyle infection was done by McMaster technique (MAFF,1986). Faecal cultures were prepared by incubating 5 – 10 g of faeces at 26 – 28˚C for 5 – 7 days after which infective larvae were isolated using a modified Baermann technique as described by Roberts & O'Sullivan (1950).

Data handling and statistical analysis

The analyses were conducted using IBM SPSS Statistics for Windows, Version 22.0 statistical software (released 2013. © 2013, Armonk, NY: IBM Corp). The bivariate association between each hypothesized risk factor and GI parasitic infection in pigs was evaluated using the Pearson chi-square test for categorical and continuous variables. The factors significantly associated with the risk of infection with GI parasites were then subjected to regression analysis. A binary logistic regression analysis model was developed and the relationship between the prevalence of infections and independent variables was analysed using coprological status (positive/negative) as dependent variable. The effect of each risk factor on the likelihood of infection was measured by the odds ratio (OR) along with their 95 % CI was computed as the exponent of the respective regression coefficient. Cluster analysis was performed to identify parasites that were more likely to cluster or coexist, by applying an unsupervised hierarchical cluster analysis algorithm, available in Pvclust package (Suzuki & Shimodaira 2006) in R statistical software (R Core Team, 2005). A binary method of similarity was used between the dichotomous variables for each of the parasites and the unweighted pair group method with arithmetic mean (UPGMA), as the dissimilarity measure between clusters. This analysis was run for 1,000 iterations using bootstrap resampling techniques which provided with approximately unbiased probability values (AU *P*-value) for clusters; clusters with AU *P* value of >95 % indicated significant clusters.

Ethical Approval and/or Informed Consent

This study was reviewed and approved by the Dean of Post Graduate studies, Guru Angad Dev Veterinary and Animal Science University (GADVASU), Ludhiana, Punjab, India. More detailed ethical review was not required since no invasive sampling was undertaken.

Results

Prevalence of GI parasites in pigs

Out of the 839 pig faecal samples analysed, 238 (28.4 %) were found positive for one or more GI parasites. *Ascaris suum* was the predominant species with a prevalence of 11.1 %. The other GI parasites recorded were coccidia (9.41 %), *Trichuris suis* (6.43 %),

Supplementary Table 1. Agro-climatic Zone-wise prevalence of gastrointestinal parasites in pigs in Punjab.

Agro-climatic Zone	Number of samples			Number of positive samples (%)						
	Examined	Positive (%)	Mixed Infection (%)	*Ascaris suum*	*Balantidium coli*	Amphistome	Coccidia	Strongyle	*Trichuris suis*	*Ascarops strongylina*
Sub-mountain undulating	130	14(10.8)	1(0.8)	2 (1.5)	0	2(1.5)	4(3.1)	0	7(5.4)	0
Undulating plain	110	22(20.0)	7(6.4)	6(5.5)	6(5.5)	2(1.8)	6(5.5)	0	7(6.4)	2(1.8)
Central plain	342	122(35.7)	34(9.9)	50(14.6)	20(5.8)	11(3.2)	39(11.4)	12(3.5)	15(4.4)	8(2.3)
Western plain	31	11(35.5)	5(16.1)	8(25.8)	2(6.5)	2(6.5)	7(22.6)	0	3(9.7)	0
Western	226	69(30.5)	16(7.1)	27(11.9)	10(4.4)	11(4.9)	23(10.2)	6(2.7)	22(9.7)	5(2.2)
Total	839	238(28.4)	63(7.5)	93(11.1)	38(4.5)	28(3.3)	79(9.4)	18(2.1)	54(6.4)	15(1.8)
p value		0	0.005	0.0001	0.09	0.322	0.003	0.05	0.123	0.44

Supplementary Table 2. Prevalence of gastrointestinal parasites in pigs of different age groups.

Age	Number of samples			Number of positive samples (%)						
	Examined	Positive (%)	Mixed Infection (%)	*Ascaris suum*	*Balantidium coli*	Amphistome	Coccidia	Strongyle	*Trichuris suis*	*Ascarops strongylina*
Piglet (<4m)	341	101 (29.6)	25 (7.3)	40(11.7)	18(5.3)	0	44(12.9)	5(1.5)	20(5.9)	6(1.8)
Grower (4-8m)	228	66 (28.9)	18 (7.9)	28 (12.3)	12(5.5)	17(7.5)	16(7.0)	4(1.8)	15(6.6)	3(1.3)
Adult (>8m)	270	71 (26.3)	20 (7.4)	25 (9.3)	8(3.0)	11(4.07)	19(7.0)	9(3.3)	19(7.0)	6(2.2)
Total	839	238 (28.4)	63 (7.5)	93(11.3)	38(4.6)	28 (3.4)	79(9.4)	18(2.1)	54(6.6)	15(1.8)
p value		0.66	0.966	0.50	0.32	0.01	0.01	0.25	0.85	0.74

Supplementary Table 3. Sex-wise prevalence of gastrointestinal parasites in pigs in Punjab.

Sex	Number of samples			Number of positive samples (%)						
	Examined	Positive (%)	Mixed Infection (%)	*Ascaris suum*	*Balantidium coli*	Amphistome	Coccidia	Strongyle	*Trichuris suis*	*Ascarops strongylina*
Male	341	97 (28.4)	15 (4.4)	39 (11.4)	13 (3.8)	8 (2.3)	25 (7.3)	6(1.8)	21(6.2)	6(1.8)
Female	498	141 (28.3)	48(9.6)	54(10.8)	25(5.0)	20(4.0)	54(10.8)	12(2.4)	33(6.6)	9(1.8)
Total	839	238 (28.4)	63(7.5)	93(11.3)	38(4.6)	28 (3.4)	79(9.4)	18(2.1)	54(6.6)	15(1.8)
p value		0.97	0.005	0.78	0.41	0.19	0.08	0.52	0.78	0.95

Supplementary Table 4. Seasonal prevalence of various gastrointestinal parasites of pigs in Punjab.

Season	Number of samples			Number of positive samples (%)						
	Examined	Positive (%)	Mixed Infection (%)	*Ascaris suum*	*Balantidium coli*	Amphistome	Coccidia	Strongyle	*Trichuris suis*	*Ascarops strongylina*
Rainy	397	104 (26.2)	35(8.8)	31(7.8)	25(6.3)	11(2.8)	48(12.1)	7(1.8)	28(7.1)	9(2.3)
Winter	121	56(46.3)	10(8.3)	28(23.1)	0	3(2.5)	21(17.4)	6(5.0)	11(9.1)	0(0)
Summer	321	78(24.3)	18(5.6)	34(10.6)	13 (4)	14(4.4)	10(3.1)	5(1.6)	15(4.7)	6(1.9)
Total	839	238(28.4)	63(7.5)	93(11.1)	38(4.5)	28(3.3)	79(9.4)	18(2.1)	54(6.4)	15(1.8)
p value		0.001	0.25	0.0001	0.012	0.42	0	0.07	0.19	0.25

Table 1. Prevalence of various gastrointestinal parasites of pigs under different management systems in Punjab.

Management system		Number of samples			Number of positive samples (%)						
		Examined	Positive (%)	Mixed Infections (%)	*Ascaris suum*	*Balantidium coli*	Amphistome	*Coccidia*	Strongyle	*Trichuris suis*	*Ascarops strongylina*
Type of farm	Organised	372	91(24.5)	20(5.4)	32(8.6)	16(4.3)	7(1.9)	32(8.6)	3(0.8)	15(4)	1(0.3)
	Unorganised	409	143 (34.9)	39 (9.5)	61(14.9)	22(5.4)	18(4.4)	42(10.3)	15(3.7)	33(8.1)	14(3.4)
	p value		0.001	0.02	0.007	0.48	0.04	0.42	0.01	0.02	0.002
Deworming	Absent	348	135(38.8)	33(9.5)	60(17.2)	21(6)	15(4.3)	41(11.8)	10(2.9)	35(10.1)	9(2.6)
	Present	418	93(22.2)	24(5.7)	32(7.7)	14(3.3)	8(1.9)	33(7.9)	8(1.9)	11(2.6)	6(1.4)
	p value		0.000	0.04	0.00	0.08	0.05	0.07	0.39	0.00	0.25
Cleanliness	Present	375	94(25.1)	20(5.3)	34(9.1)	16(4.3)	8(2.1)	31(8.3)	3(0.8)	16(4.3)	1(0.3)
	Absent	391	134(34.3)	37(9.5)	58(14.8)	19(4.9)	15(3.8)	43(11)	15(3.8)	30(7.7)	14(3.6)
	p value		0.000	0.02	0.01	0.01	0.17	0.201	0.006	0.05	0.001
Water consumption	Controlled	675	188 (27.9)	47(7)	69(10.2)	33(4.9)	20(3)	61(9)	14(2.1)	42(6.2)	11(1.6)
	Uncontrolled	41	17(41.5)	7(17.1)	10	0	2(4.9)	10(24.4)	4(9.8)	1(2.4)	0
	p value		0.04	0.02	0.005	0.14	0.49	0.001	0.002	0.322	0.41

Balantidium coli (4.5 %), amphistome (3.33 %), strongyle (2.14 %) and *Ascarops strongylina* (1.78 %). The morphometric studies of the coccidian oocysts revealed eight species of *Eimeria* in pigs of Punjab, India *viz. Eimeria polita*, *E. scabra*, *E. porci*, *E. debliecki*, *E. spinosa*, *E. suis*, *E. neodebliecki* and *E. perminuta*. Faecal cultures followed by modified Baermann technique revealed presence of larvae of *Hyostrongylus rubidus* and *Oesophagostomum* species.

Zone wise prevalence of GI parasites

Among different agro-climatic zones of Punjab, a significant difference (P<0.05) in prevalence of GI parasites was observed. Central plain zone (OR= 2.24; CI 95 %:1.41 – 3.557) and undulating plain zone (OR=1.11, CI 95 %:0.606 – 2.05) had the increased odds of GI parasitic infection as compared to western zone (reference zone) (Supplementary Table1).

Age wise and sex wise prevalence of GI parasites

The age of host had a non-significant (P>0.05) effect on prevalence rates (Supplementary Table 2). The higher proportion of pigs (81.25 %) had moderate (500 – 2000 EPG) infection with strongyles. Further, the highest intensity of infection was recorded in growers (1457.1 ± 500.4) followed by piglets (900.2 ± 161.2) and adults (466.6 ± 176.3). Further, statistically non-significant (P>0.05) difference and almost similar prevalence of GI parasites was recorded in males (28.4 %) and female (28.3 %) pigs (Supplementary Table 3).

Seasonal prevalence of GI parasites

A significant (P<0.05) seasonal variation was recorded with the highest prevalence in winter season (46.3 %) followed by rainy (26.2 %) and lowest in the summer (24.3 %) (Supplementary Table 4). The results indicated that pigs had more odds of acquiring the infection during winter (OR 4.36; CI 95 %: 1.58 – 9.51) and rainy (OR: 1.16; CI 95 %: 1.11 – 2.34) season. Further, quantification of the infection levels by McMaster technique revealed the highest mean EPG in rainy season (1966.6 ± 1146.5) followed by summer (1876.6 ± 1124.4) and winter (560.6 ± 146.9).

Risk factor assessment

The results revealed that the uncontrolled water consumption from unidentified water sources such as streams and drains (OR=3.88 [1.58 – 9.51]), unorganised farms (OR=5.201 [1.69 – 15.92]) and absence of deworming (OR=1.16 [1.11 – 2.34]) significantly increased the odds of infection with GI parasites in pigs (Table 1,). The location (OR=3.27 ([0.51 – 4.49]) and absence of deworming (OR=2.24 [1.41 – 3.59]) increased odds of infection with *A. suum*. The absence of deworming (OR= 2.604 [1.28 – 5.27]) was significantly associated with the infection with *B. coli* whereas, season, deworming and uncontrolled water consumption increased the odds for infection with *Trichuris suis.*

Discussion

Prevalence rates in range of 11 – 38 % have been reported in pigsfrom various geographical locations in India (Kumari *et al*., 2002; Deka *et al*., 2005; Borthakur *et al.,* 2007; Godara & Sharma 2010; Singh *et al*., 2017). Kaur *et al*.,2017 have recorded an overall higher prevalence of 56.5 % in pigs of more than 1year age from Punjab, India. This might be due to the differences in the sampling criteria as they targeted scavenging pigs and had included only a few pigs from organised farms. Similarly, infection of pigs with GI parasites with prevalence rates varying between 13.2 to 96.4 % has been widely reported from all corners of world (Roepstorff & Jorsal 1989; Roepstorff *et al*., 1998;; Tamboura *et al* ., 2006; Tiwari *et al*., 2009; Ismail *et al*., 2010; Lai *et al*., 2011; Obonyo *et al*., 2013; Dey *et al*., 2014; Okorafor *et al*., 2014; Alynne *et al*., 2015; Lipendele *et al*.,2015; Atawalna *et al*., 2015; Nonga & Paulo, 2015; Junhui *et al*., 2015; Roesel *et al*., 2017; Chilundo *et al.,* 2017; Kouam *et al*., 2018) .The parasite spectrum was similar to that of previous studies from other tropical or subtropical countries (Tamboura *et al.,* 2006; Nissen *et al.,* 2011; Chilundo *et al.,* 2017). Within Punjab state, India, Central plain zone and undulating plain zone have more humid conditions as compared to other zones, thus facilitating survival, development and propagation of the pre-parasitic stages of the GI parasites, hence higher prevalence rates.

Ascaris suum (11.1 %) was recorded as the predominant GI parasite of pigs which is in agreement with the findings of Yadav &Tandon (1989from sub-tropical regions of India. The prevalence rate of *Trichuris suis* (6.43 %) was low and was similar to the reports from other tropical countries like Ghana (4.6 %) and Zimbabwe (4.2 %), by Permin *et al.,* (1999) and Marufu *et al.,* (2008), respectively. Ismail *et al.* (2010) from Korea, Giarratana *et al.* (2012) from Italy and Dey *et al.* (2014) from Bangladesh reported higher prevalence rate of *B. coli* as 64.7 %, 36.66 % and 40 %, respectively which is contrary to the findings of the current study (4.5 %).

Variation in prevalence of various species of GI parasites observed in the current study and other regions of the world, suggested that geographical location along with management practices including hygiene and deworming are the major determinants responsible. Nonetheless, not only the macroclimate, but the microclimate of the pens *i.e.* flooring and drainage facilities provide optimum conditions including moisture and temperature for the propagation and existence of pre-parasitic stages in the contaminated feed and surroundings. In addition to these, differences in the basic biological requirements of the pre-infective developmental stages, transmission characteristics and immunogenicity of the different worm species are the contributors. Availability of clean water, practice of open defecation with easy access of pigs, housing, inadequate feed to the pigs, health status as well as inherent characteristics such as host immunity are the other important determinants.

The result of present study suggested that sex of the pigs did not have direct bearing on the prevalence of GI parasites. Similar ob-

servations has been reported by other researchers (Yadav &Tandon, 1989; Tamboura *et al.,* 2006; Kumsa & Kifle, 2014; Okarafor, 2014). The highest intensity of infection with strongyles (EPG) was recorded in growers followed by piglets and adults. This can be ascribed to the fact that growers get exposed to the infective stages while they forage on grasses after weaning. The infection rates in piglets are associated to their immune status, level of pre-exposure and nutritional status while adults develop resistance against re-infections.

A significant (P<0.05) seasonal variation was recorded with highest intensity of infection during rainy season followed by winter with large proportion of animals having moderate infection. This finding can be attributed to the fact that suitable environmental conditions like temperature and humidity during this period for development of preparasitic stages makes maximum availability of infective stages to the host. The infection in the winter season may be ascribed to the reduced immuno-tolerance of animals and long prepatent periods of some of the GI parasites. Similar seasonal variations have been reported by Muraleedharan (2005) and Kagira *et al*.(2010).

It was found that the factors like unorganised farms, absence of deworming and pigs consuming water from unknown sources significantly increased the odds of infection with GI parasites. This could be credited to the habit of coprophagy and scavenging by the pigs which make them more prone to parasitic infections as pigs reared under unhygienic conditions have free access to garbage and contaminated feed and water. Analogous were the findings of Kagira *et al.* (2010) who have considered confinement or housing as a protective factor against GI parasitism in Western Kenya. There was less evidence of GI parasites in pigs with effective deworming programmes and better farm hygiene. Similar were the findings of a survey conducted in Nigeria by Weka & Ikeh (2009) who found a negative correlation between the prevalence of intestinal parasites and routine deworming of the pigs. However, this is in contrast to the findings of Roesel *et al.* (2017) who have reported that administering anthelminthic drugs have no significant impact on the prevalence of GI parasites in pigs.

Cluster analysis of the parasites did not identify any significant clusters in the present data. The three parasitic infections (*B. coli*, *T. suis* and mixed infection) were closer to each other as compared to other parasites. There have been earlier reports of chronic diarrhoea associated with *Balantidium* and *Trichuris* concurrent infection in canines (Ewing & Bull 1966). However, the importance of this co-occurrence in pigs which although was non-significant, needs to be explored further, especially in diarrhoeic animals.

Thus, our findings revealed that the pigs get infected by wide array of GI parasites. Due to their behaviour of coprophagy, pigs are likely to ingest infective stages from the environment. The unorganised farm management system, water consumption from unknown sources and lack of deworming schedule makes them more prone to the risk of GI parasitism.

Conclusion

Gastrointestinal parasites are prevalent in pigs of Punjab state, India with a prevalence rate of 28.4 % and *Ascaris suum* as the predominant parasite. Among the various risk factors analysed season, agro-climatic zones and managemental practices had a significant (p<0.05) effect on GI parasitism of pigs. Therefore, managemental practices *viz*. routine removal of manure and litter from pig pens, anthelmintic treatment during rainy and summer season and the use of disinfectants in the pens can be helpful in effective control of GI parasites of pigs.

Conflict of Interest

Authors state no conflict of interest.

Acknowledgments

The authors acknowledge The Dean, Post Graduate Studies, Guru Angad Dev Veterinary and Animal Science University, Ludhiana, Punjab, India for providing facilities to carry out the research work. Thanks are also due to the farmers who generously gave their time and hospitality.

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DOI 10.2478/helm-2020-0005

HELMINTHOLOGIA, 57, 1: 37 – 42, 2020

On the first occurrence of *Xiphinema santos* in Brazil

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Article info

Received August 19, 2019
Accepted September 17, 2019

Summary

Based on morphology, measurements of juveniles and female specimens and sequences of the D2/D3 expansion 28S rDNA gene and ITS1 analysis by DNA barcode technique, a *Xiphinema americanum* group species associated with olive trees from state of Sao Paulo, Brazil was identified as *X. santos*. This is the first report of *X. santos* in Southern Hemisphere and outside the European and African continents, thus extending its geographic range.
Keywords: DNA sequence; Longidoridae; *Xiphinema americanum*-group; distribution; olive

Introduction

Xiphinema americanum sensu lato includes vectors of several important plant pathogenic viruses that cause significant damage to a wide range of agricultural crops. This group is considered to be a complex of many species (Lamberti & Bleve-Zacheo, 1979; Lamberti *et al.*, 2000) comprising 61 putative species (OEPP/EPPO, 2017). Recently, substantial progress has been achieved on the accurate determination of species belonging to the *X. americanum* group (Gutiérrez-Gutiérrez *et al*., 2012; Archidona-Yuste *et al*., 2016; Lazarova *et al*., 2019), however the validity of some species is uncertain. Traditionally, morphological characters have been used to distinguish and identify different species of *X. americanum* group. However, some species like *X. brevicolle* and *X. diffusum* are distinguished by only minute morphometric or morphological differences (Brown & Halbrendt, 1997), and considerable taxonomic expertise is required for accurate determination of individual species. As the number of skilled taxonomists decrease, development of molecular-based diagnostic protocols is increasingly a viable alternative to nematode identification (Roberts *et al*., 2016). Although *Xiphinema* originated in Africa and Latin America (Taylor & Brown, 1997), few investigations on taxonomic studies of *X. americanum* group, has been done in Latin America (Oliveira & Neilson, 2016). For example, currently only five *X. americanum*-group species: *X. brevicolle*, *X. diffusum*, *X. luci*, *X. oxycaudatum* and *X. peruvianum* (Oliveira *et al*., 2003; Silva *et al*., 2008) has been recorded from Brazil.

Recently, during a survey for plant-parasitic nematodes in São Paulo State, Brazil, it was detected a population of *X. americanum*- group species from a root zone soil sample of olive trees, *Olea europea*. Thus, the objective of this study was to identify and characterize this *X. americanum*-group species based on both morphological characters of females and juveniles and DNA sequences of D2/D3 expansion segment of 28S rDNA.

* – corresponding author

Materials and Methods

Longidorid specimens were obtained from three soil samples collected from olive tree plantation from Bom Sucesso de Itararé, SP Brazil (S22°38.738; W45°40.685; 1514m). Each sample consisted of approximately 1.5 – 2.0 kg of moist soil collected around actively growing young roots, to a depth of 40 cm. Soil was placed in a polythene bag, stored in a cooled box (15 – 20ºC) and immediately transported to the laboratory.

Nematodes were extracted from a 1 kg sub-sample of soil by a modified decanting and sieving technique (Ploeg & Brown, 1997). Nematodes were examined under a stereoscopic microscope and longidorid specimens were removed for morphological study. Longidorid nematodes were heat-killed at 60 ºC, fixed in a 1 % formalin/glycerol mixture, and processed to anhydrous glycerine using a slow method (Hooper, 1986). Species identification and measurements were made using a high power microscope. To identify the *X. americanum*-group species, taxonomic keys proposed by Lamberti *et al*. (2000) and OEPP/EPPO (2017) standard diagnostic protocol were used, as well as original descriptions of the species from the literature.

Principal component analysis of X. santos populations

Principal Components Analysis (PCA) was performed on the correlation matrix of the set of 11 measurements [length of body (L), length of both odontostyle and odontophore, tail length, body diameter at anus, largest body diameter, position of the vulva in relation to the anterior end of the body expressed as a percentage of the length of the body (V %), body length divided by largest body diameter (a), body length divided by oesophageal length (b), tail length divided by body diameter at the anus (c') and ratio of the length of the body and tail (c)] of females taken in the present study and data sourced from the literature of *X. santos* populations from Portugal, Egypt and Spain (Lamberti *et al*., 1993; 1994; 1996; Gutiérrez-Gutiérrez *et al*., 2012) using Community Analysis Package (PISCES Conservation Ltd, Lymington UK).

Molecular study

DNA from single female specimens was extracted with Lysis Buffer Holterman [(HLB) (800 ug proteinase K/ml, β-mercaptoethanol 1 % (v/v), 0.2M NaCl and 0.2M Tris HCl pH 8)] (Holterman *et al*., 2006). A total of 25 µL of HLB was diluted in 25 µL of ultrapure water totaling 50 µL in a 0.2 mL Eppendorff tube. A drop of this solution (5 µL) was placed on a glass slide, where the nematodes were individually cut into three parts and placed in the same 0.2 mL tube. The 45 µL remaining solution was used for wash the

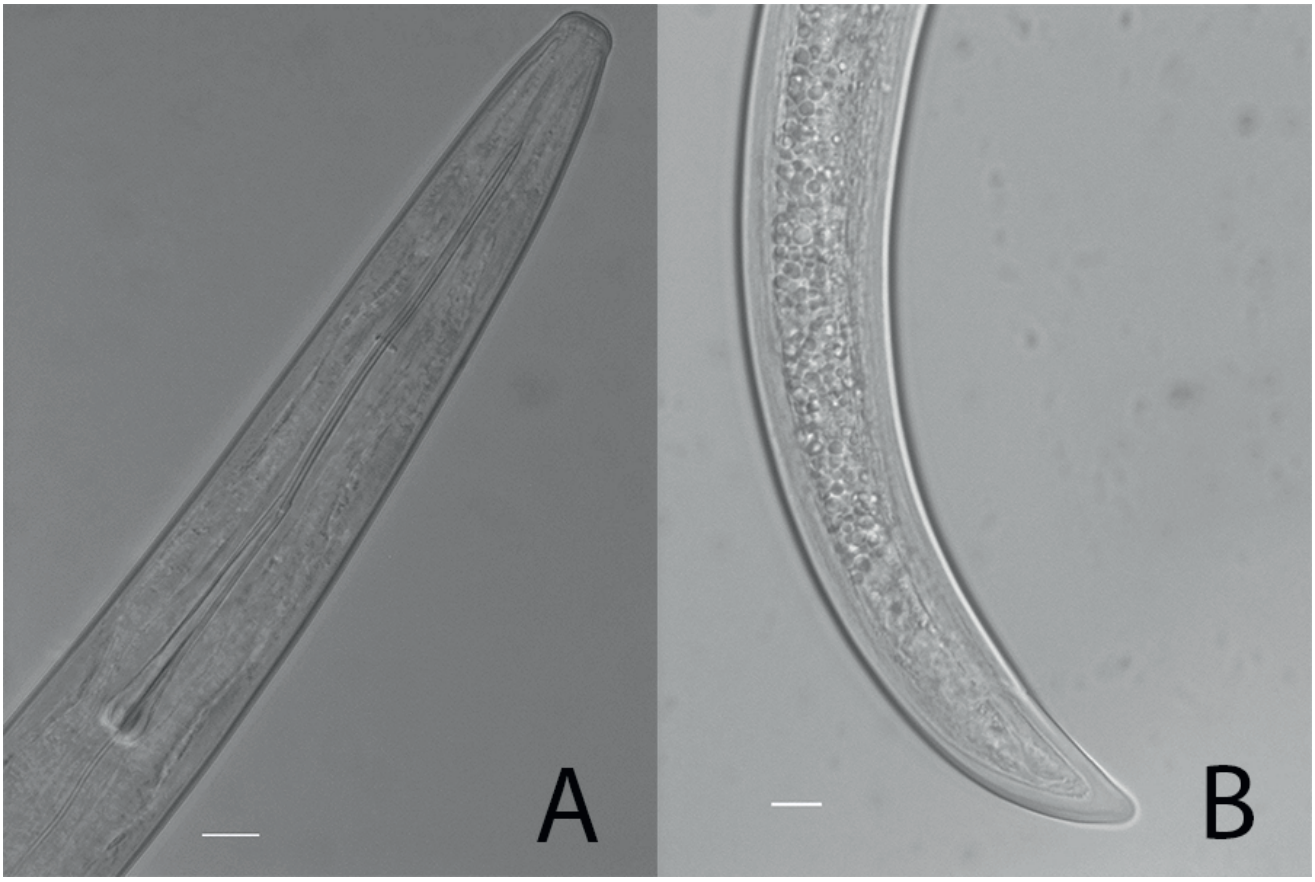

Fig. 1. Light micrographs of *Xiphinema santos* from Brazil. Female anterior (A) and tail regions (B). Scale bars=10 µm.

slide and added to tube with the sectioned nematode. Samples were submitted to PCR at 65 °C for 2 h, 99 °C for five minutes and stored at -20 °C (Consoli *et al*., 2012). The universal primers D2A (5'-CAAGTACCGTGAGGGAAAGTTG-3') and D3B (5'TCGGAAGGAACCAGCTACTA-3') were used to amplify the D2/D3 expansion segment of 28S rDNA by PCR (Al-Banna *et al*., 2004). A total of 12.5 µL of Gotaq Hot Start Green Master Mix (Promega, São Paulo State, Brazil), with the reagents necessary for reaction: 12.5 µL Go Taq®, 10 µL NFW (Nuclease Free Water), and 1 µL each primer [10 mM] and 0,5 µL of cDNA from the single female specimen, totaling 25 µL per reaction was submitted to PCR at 94 °C for seven minutes; followed by 35 cycles at 94 °C for 60 seconds, 55 °C for 60 seconds, 72 °C for 60 seconds; and 72 °C for 10 minutes (Mrácek *et al*., 2006). The internal transcribed spacer (ITS-1) region of the ribosomal DNA sequence (rDNA) was amplified using the forward primer BL58 (5'-CCCGTCGMTACTACCGATT-3') and reverse primer 5818 (5'-ACGARCCGAGTGATCCAC-3'). The PCR conditions were: denaturation at 94 °C for 5 minutes; followed by 40 cycles of 94 °C for 1 minute, 57 °C for 45 second, 72 °C for 2 min, and extension for 10 min at 72 °C. Five µL of PCR product were used for electrophoresis in TAE buffer on 1 % agarose gel, stained with ethidium bromide (0.02 mg/mL), visualized and photographed under UV light. The result of the amplification was compared to the molecular weight marker VIII (Roche Life Science).

The amplified fragments of D2/D3 expansion 28S rDNA and ITS-1 region were sequenced with the Big Dye Terminator kit (Applied Biosystems). A reagent mix containing 2 µL Big Dye, 3.2 pmol sense primer, 3.0 µL of amplified product containing 400 ng DNA and 2.0 mL of water was prepared for the product end of the PCR reaction. The reaction for sequencing was performed according to the manufacturer's protocol (Applied Biosystems) with further purification of the amplified product by precipitation with isopropanol. Samples were suspended in 10 uL of deionized formamide and denatured at 95 °C for three minutes. Electrophoresis was performed in a 3500xL Genetic Analyzer (Applied Biosystems).

The obtained sequences were aligned and compared to nucleotide polymorphism identification with the aid of BioEdit Aligment Sequence Editor Program. The consensus sequence was compared with other species of nematodes in the database (GenBank, http://www.ncbi.nlm.nih.gov) for identification based on genetic similarity.

Ethical Approval and/or Informed Consent

The conducted research is neither related to human nor animals use.

Results and Discussion

Morphometrics (Table 1) of the specimens obtained here generally agree with those of the paratype specimens of *X. santos* (Lamberti *et al*., 1993) except for a ratio and odontostyle length (36.5 – 46.8, 76 – 84 µm vs 54.0 – 59.0, 68 – 72 µm respectively). Nevertheless, these differences are within the range of intraspecific variation, as showed in different Portuguese, Egyptian and Spanish populations

Table 1. Morphometric data of female and four juveniles stages (J1, J2, J3 and J4) of *Xiphinema santos* from Brazil. Values are mean ± standard deviation (range).

Nematode	*Xiphinema santos*				
Stages Morphometrics*	female	J1	J2	J3	J4
n	24	1	1	1	14
L (mm)	1.7 ± 0.1 (1.6 – 1.9)	0.7	0.7	0.9	1.3 ± 0.1 (1.1 – 1.4)
Odontostyle (µm)	79.2 ± 2.4 (76 – 84)	32.0	42.0	51.0	64.9 ± 2.4 (61 – 69)
Replacement odontostyle (µm)	–	48.0	52.0	63.0	77.3 ± 2.9 (72 – 82)
Odontophore (µm)	51.3 ± 1.8 (47 – 55)	43.0	30.0	37.0	42.5 ± 1.7 (40 – 45)
Spear (µm)	130.5 ± 2.9 (126 – 136)	75.0	72.0	88.0	107.4 ± 2.7 (102 – 111)
Tail (µm)	34.4 ± 1.8 (30 – 37)	30.0	32.0	25.0	34.1 ± 2.2 (30 – 37)
Body diameter (µm)	40.1± 2.9 (36 – 45)	18.0	21.0	24.0	28.9 ± 2.6 (26 – 36)
V%	51.8 0.9 (50.0 – 53.5)	–	–	–	–
a	42.5 ± 2.8 (36.5 – 46.8)	41.4	33.8	39.0	43.4 ± 3.2 (37.2 – 47.2)
b	5.9 ± 0.7 (4.7 – 7.6)	5.5	4.9	5.5	5.4 ± 0.9 (4.0 – 7.5)
c	49.5 ± 3.0 (44 – 53)	24.8	22.2	37.4	36.6 ± 3.2 (32.5 – 44.3)
c`	1.6 ± 0.1 (1.4 – 1.8)	2.5	2.5	1.8	1.8 ± 0.2 (1.2 – 2.1)

* length of body (L), position of the vulva in relation to the anterior end of the body expressed as a percentage of the length of the body (V %), body length divided by largest body diameter (a), body length divided by oesophageal length (b), tail length divided by body diameter at the anus (c') and ratio of the length of the body and tail (c)].

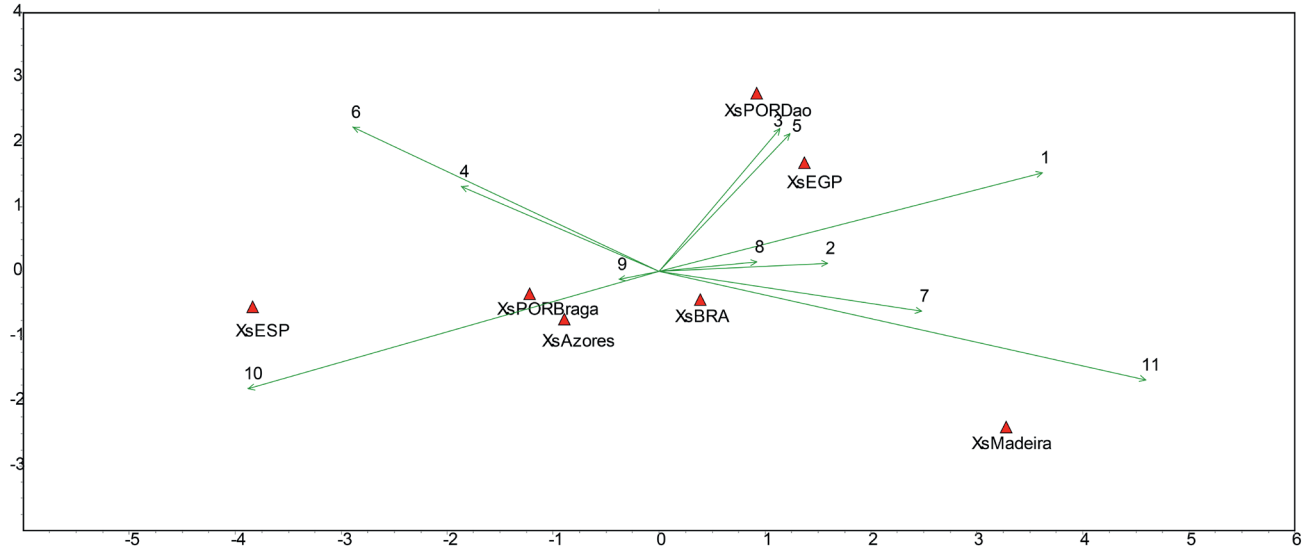

Fig. 2. Principal component analysis of seven *Xiphinema santos* populations based on mean values of 11 morphometric characters (Table 1). Brazilian (XsBRA), Portuguese from Dao Region (XsPORDao), Azores (XsAzores), Braga (XsPORBraga) and Madeira (XsMadeira); Egyptian (XsEGP) and Spanish (XsESP) populations.

of this species (Lamberti *et al*., 1994; 1996; Gutiérrez-Gutiérrez *et al*., 2012). The alpha-numeric codes for the Brazilian population of *X. santos* to be applied to the polytomic identification keys for the *X. americanum*-group species by Lamberti *et al*. (2000) are: (A 2/3, B 2, C 3, D 1, E 2, F 1, G 2, H 2, I 3) and by OEPP/EPPO (2017) are: (A 2, B 2, C 1, D 2/3, E 2, F 3, G 1, H 2, I 3, J 3).

Female: The Brazilian population of *X. santos* was characterised by a body forming a close C-shaped. Lip region rounded anteriorly, slightly expanded and separated from the body by a weak depression (Fig. 1 A). Reproductive system amphidelphic, both branches apparently equally developed, opposed and reflexed, without any uterine differentiation. Ovaries with symbiont bacteria. Tail short, conoid, weakly curved ventrally with conoid rounded terminus (Fig. 1 B).

Juveniles: All four juvenile stages were identified using morphological characters such as body length, length of replacement and functional odontostyle (Table 1). Specifically, J1 was characterised by the position of replacement odontostyle just posterior to functional odontostyle. Subsequent stages (J2, J3 and J4) were identified following Halbrendt & Brown (1992) by establishing the range of corresponding replacement and functional odontostyle length.

Male: No male was detected.

The PCA separated the seven *X. santos* populations into four distinct clusters (Fig. 2). The Brazilian population (XsBRA) was morphometrically closer to the Portuguese populations from Azores (XsAzores) and Braga (XsPORBraga) and formed a distinct cluster. Also, in agreement with Lamberti *et al*. (1996), the Egyptian population (XsEGP) was almost identical to the type population from Dao Region, Portugal (XsPORDao). The Spanish (XsESP) and Madeira (XsMadeira) populations were both isolated from all other populations.

Amplification of D2/D3 expansion 28S rDNA gene and ITS1 of the Brazilian population of *X. americanum*-group species (XsBRA) produced, respectively, 628 bp and 706 bp fragments and the sequences were deposited in the GenBank under the accession codes MN318337 and MN318338. The technique of DNA barcode sequence showed that the expansion D2/D3 28S rDNA gene of XsBRA was identical to three *X. santos* populations (GenBank JQ990029.1; JQ990030.1; AY601587) from Spain and Portugal, *Xiphinema* sp. (MH558570) from Spain and *X. citricolum* (DQ285668) from USA, with a similarity of 100 %. ITS1 showed 98 % similarity with *X. santos* (JQ990046; JQ990047) from Spain and 97 % with several *X. americanum*-group species isolated from USA (KF748440.1; KF748389.1; KF748451.1; KF748442.1; KF748438.1) and *X. georgianum* (DQ299521.1).

Xiphinema santos was described from specimens collected from the rhizosphere of vineyards in Dão region, Portugal (Lamberti *et al*., 1996), and has also been reported from vineyards in Nubaria, North Egypt (Lamberti *et al*., 1996), vineyards and stone pine in Rociana del Condado (Huelva, Spain) (Gutiérrez-Gutiérrez *et al*., 2012) and wild olive in Arcos de la Frontera (Cádiz, Spain) (Archidona-Yuste *et al*., 2016). In this study, *X. santos* occurred around roots of cultivated olive from Bom Sucesso de Itararé (SP). This is the first record of the species in Brazil.

Combining the results of this study and of other previous reports, a total of 31 *Xiphinema* species have now been recorded from Brazil. These include six *Xiphinema americanum*-group species: *X. brevicolle*, *X. diffusum*, *X. luci*, *X. oxycaudatum* and *X. peruvianum* (Oliveira *et al*., 2003; Silva *et al*., 2008) and *X. santos* (present study). As mentioned by Brown & Halbrendt (1997), the taxonomic status of *X. americanum*-group nematodes is controversial and the species identification based on morphological characters is problema-

tic because several species are only distinguished by minor morphometric or morphological differences. However, the use of the polytomous keys published by Lamberti *et al*. (2000) and OEPP/EPPO (2017) facilitated the identification of *X. santos*. Additionally, the sequences of the D2/D3 expansion 28S rDNA gene and ITS1 analysis by DNA barcode technique was useful for the diagnosis of *X. santos* Brazilian population.

Based on both morphological, morphometrical and molecular analysis we concluded that the longidorid nematodes associated with olive trees from state of Sao Paulo, Brazil, belong to the species *X. santos*. This is the first report of *X. santos* in Southern Hemisphere and outside the European and African continents, thus extending its geographic range.

Conflict of Interest

Authors state no conflict of interest.

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DOI 10.2478/helm-2020-0008

HELMINTHOLOGIA, 57, 1: 43 – 48, 2020

Case Report

Giant kidney worm: novel report of *Dioctophyma renale* in the kidney of a dog in Greece

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Article info

Received September 13, 2019
Accepted December 17, 2019

Summary

Dioctophyma renale is the largest nematode that infects domestic mammals and is the aetiologic agent of a serious renal disease, dioctophymatosis. It has an indirect life cycle with carnivores serving as final hosts and earthworms as intermediate hosts. The parasite can infect humans with significant zoonotic potential. The pathogenesis of dioctophymatosis is mainly associated with the extent of renal atrophy caused by the parasites, the risk of bilateral renal infestation and the location of the parasite (extrarenal cases). Clinical diagnosis is challenging, and the only treatment option is nephrectomy or nephrotomy to remove the adult nematode. A 6-year-old female crossbreed dog presented with tachypnea, tachycardia and severe hematuria, but died shortly after presentation. Postmortem examination found the right kidney was enlarged and two adult nematodes were found in the renal pelvis. The left kidney was normal. The nematodes were collected and submitted for identification. The two specimens were identified as *D. renale* using specific identification keys. Herein we present the first case of renal parasitosis by the nematode *D. renale* in a dog from Greece. This case highlights the need for investigation of the actual prevalence of the parasite and the use of measures for the control of its expansion aiming the protection of dogs and public health.
Keywords: *Dioctophyma renale;* kidney; nematode; dog; Greece

Introduction

Dioctophyma renale (Goeze, 1782), is a parasitic nematode that belongs to the order of Ascaridida and the family of Dioctophymatidae (Bowman, 2014). This parasite has a wide distribution and is also known as the giant kidney worm due to the "gigantic" dimensions of the adults; a female may measure more than 60 cm length and 1 cm diameter. It is the largest nematode that infects domestic mammals and is the aetiologic agent of a serious renal disease, dioctophymatosis. *D. renale* has been described in many different mammalian species as well as humans, highlighting the zoonotic importance of this parasite (Norouzi *et al*., 2017; Venkatrajaiah *et al*., 2014). The most frequent definitive hosts are mustelids (especially minks), although there are also other wild carnivores such as otters, martens and raccoons that may be infected. Among domestic mammalian hosts, dogs are most frequently affected, but it has been also reported in other domestic animals, such as felines, swine, cattle and horses (Bowman, 2014; Ribeiro *et al*., 2009; Verocai *et al*., 2009).

D. renale has a complex life cycle and requires two intermediate hosts. The prepatent period is approximately 155 days and the entire life cycle can take up to two years to complete (Dyer, 1998;

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Ferreira *et al*., 2010). The adult nematodes parasitize, mature and produce eggs in the renal pelvis of the final mammalian host. Eggs are passed through urine into the environment and they are ingested by the first intermediate host, an aquatic oligochaete annelid (e.g., *Lumbriculus variegatus*) (the common mud worm). The eggs hatch inside the intermediate host and start the development of the first and second larval stages. From this stage on, there are three potential routes of transmission. In the first case, the development and maturation to the third and fourth stage larva (the infective stage) takes place inside the same intermediate host, the aquatic oligochaete annelid and mammalian hosts become infected when they ingest the infected intermediate hosts. In the second case scenario, annelids containing the second stage larvae may be ingested by a fish or a frog, where the larvae develop into the final infective stage and encyst in the tissues of these hosts. In this case the mammalian hosts acquire the infection by consuming fish or frogs infected with the fourth-stage larvae. The third potential route of transmission is when the infected annelids parasitize on a cray fish (*Cambarus* spp.) and they are ingested by a fish or a frog. However, some investigators propose that fishes and frogs may act more likely as mechanical vectors or transporters rather than actual intermediate hosts (Dyer, 1998). In each of the above-mentioned scenarios, the definitive host becomes infected by ingesting the fourth-(infective) stage larvae. Inside the definitive host, the *Dioctophyma* larva penetrates the intestinal or duodenal mucosa, enters the peritoneal cavity and migrates to the kidney, where it develops and matures into the adult form (Bowman, 2014; Pedrassani *et al*., 2017). Adults are able to live up to 3 years in the definitive host and the fertilized eggs that are shed into the environment may survive for up to 5 years (Radman *et al*., 2017). *D. renale* nematodes are most commonly found inside the renal pelvis where they mature, however they can also be encysted within the abdominal cavity, the uterus, ovary, mammary gland, urethra, subcutaneous tissues of the inguinal region and in the mesenteric lymph nodes (Bowman, 2014; Nakagawa *et al*., 2007). Most cases have unilateral renal colonization, most frequently the right kidney, due to the anatomic proximity to the duodenum (Bowman, 2014).

Dioctophymatosis in dogs is a parasitic disease that results in compressive atrophy of the renal parenchyma of the infected kidney, dilation of the renal pelvis and ureteral obstruction due to the size of the parasite colonizing the renal pelvis space. However, the main pathologic finding is the progressive destruction and atrophy of the renal parenchyma, resulting in a cystic structure containing the adult worms in a hemorrhagic exudate with only a thin capsule remaining of the original renal parenchyma (Nakagawa *et al*., 2007; Soler *et al*., 2008).

Renal failure may not be evident when only the one kidney is affected, due to adequate compensation by the unaffected kidney, although gross or microscopic hematuria may be detectable. Rarely if bilateral infection occurs, clinical renal failure is inevitable, resulting in end stage kidney disease and death. If the parasite establishes an extrarenal location within the abdominal cavity, abdominal distension, pain and peritonitis may be develop. However, most of the reported canine dioctophymatosis cases were asymptomatic (Ferreira *et al*., 2010).

Several clinical diagnostic methods for the detection of *D. renale* infection are available. The analysis of urine sediment is most commonly used to detect *D. renale* eggs, which is a low-cost with high sensitivity and specificity technique. Other reported diagnostic techniques include advanced imaging techniques (Nakagawa *et al*., 2007; Soler *et al*., 2008) and enzyme-linked immunosorbent assay (ELISA) for the detection of anti-*Dioctophyma renale* antibodies (Pedrassani *et al*., 2015). However as many cases are asymptomatic, most of the reported cases rely on postmortem diagnosis of canine dioctophymatosis (Ferreira *et al*., 2010). Treatment of canine dioctophymatosis remains challenging for veterinary practitioners. Surgical removal of the nematodes is recommended with ureteronephrectomy for unilateral infection or bilateral nephrotomy for bilateral infections (Ferreira *et al*., 2010).

At the current time, there has been no report of canine dioctophymatosis in Greece, and only one confirmed case of human infection (Katafigiotis *et al*., 2013). The primary aim of this case report is to present the first confirmed case of renal parasitosis by the nematode *D. renale* in a dog from Greece and secondly to highlight the zoonotic potential of this infection in countries where it has not been previously reported and is unlikely to be detected ante-mortem.

Case report

A 6-year-old female mixed breed dog of 27 kg body weight was presented with a history of lethargy, muscle weakness and severe hematuria to a veterinary clinic in the city of Xanthi, northern Greece. The owners reported weight loss and hematuria. The dog lived in a farmhouse located in the sub-rural area of Xanthi and was allowed free range activity in the area around the farm but had not travelled outside of Greece. There was a river and water sources in the local vicinity.

At clinical examination the dog presented with tachypnea (60/min), tachycardia (140 – 170/min), and hyperemic oral mucosa (> 2 – 3 min capillary refill time). The dog was slightly pyrexic with a body temperature of 39.2 °C. Following clinical examination, urinalysis, complete blood count (CBC) and serum biochemistry were performed. Urinalysis revealed severe hematuria, but no evidence of bacteriuria or infection. Microscopy of the sediment failed to show any evidence of parasitic infection or ova. Significant serum biochemistry findings were azotemia with serum creatinine of 3.8 mg/dL (reference value: 0.8 – 1.8 mg/dL), serum urea of 49 mg/dL (reference value: 15 – 40 mg/dL), and all other parameters were within normal ranges. The hematologic profile of the patient revealed leukocytosis (> 50 x 10^9 WBC/L) with left shift neutrophilia, microcytosis and anisocytosis. Serological analysis for the detection of the antigen of the nematode *Dirofilaria immitis* and the specific antibodies against the pathogens *Anaplasma platys/*

phagocytophilum, Ehrlichia canis/ewingii and *Borrelia burgdorferi* were also performed using specific in-clinic rapid enzyme-linked immunosorbent assay (ELISA) kit (SNAP® 4Dx® from IDEXX® Laboratories, Westbrook, Maine, USA), according to the manufacturer instructions. The results of this examination revealed seropositivity only to *D. immitis.*

Emergency supportive treatment was initiated with intravenous fluid therapy Lactated Ringer's solution (Vioser S.A., Greece) at 10 ml/kg/hr however the dog suffered a cardiac arrest and was not resuscitated. The following day, a postmortem examination was performed. The pleural and the abdominal serosal surfaces appeared normal. In the thorax, the lungs appeared normal, however the heart appeared generally enlarged. Internal examination of the heart found about 45 whiteish nematodes later identified as adults of *D. immitis,* positioned in the right side of the heart from the right atrium, the caval opening and the apex of the right atrial appendage. On inspection of the abdominal cavity, all the visceral organs appeared normal. However, the right kidney was enlarged and soft on palpation, with a cystic appearance. (Fig. 1). The left kidney was normal. During examination and opening of the left kidney, two large nematodes were found (Fig. 2). The entire renal structure was destroyed and there was a thin outer capsule. The two nematodes were collected and placed in saline solution and sent to the Laboratory of Parasitology and Parasitic Diseases of the Aristotle University of Thessaloniki, Greece. At the laboratory, the two specimens were examined under the stereoscope and identified using specific identification keys (Bowman, 2014). The two nematodes were identified as a pair of female and male *D. renale* measuring 58 cm long and a maximum of 4.2 mm width and 23 cm long and 2.9 mm width maximum respectively.

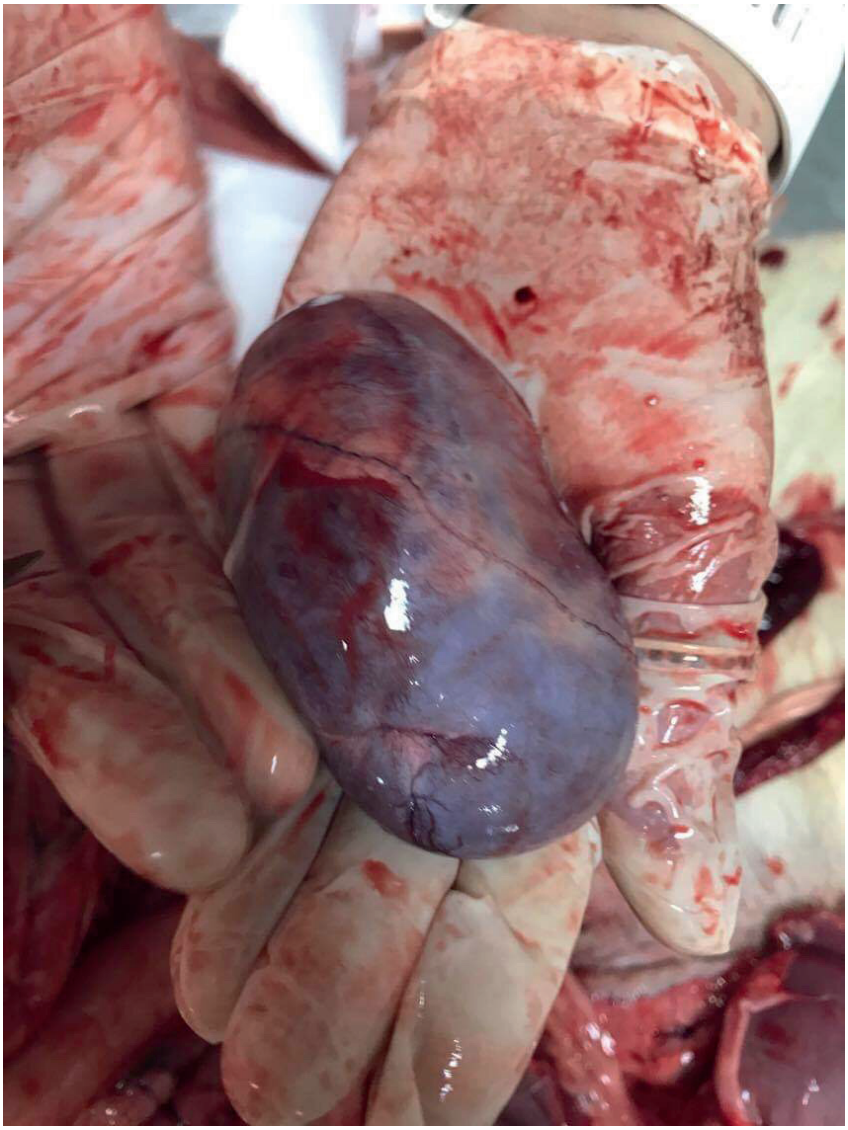

Fig. 1. Enlarged right kidney of the infected dog.

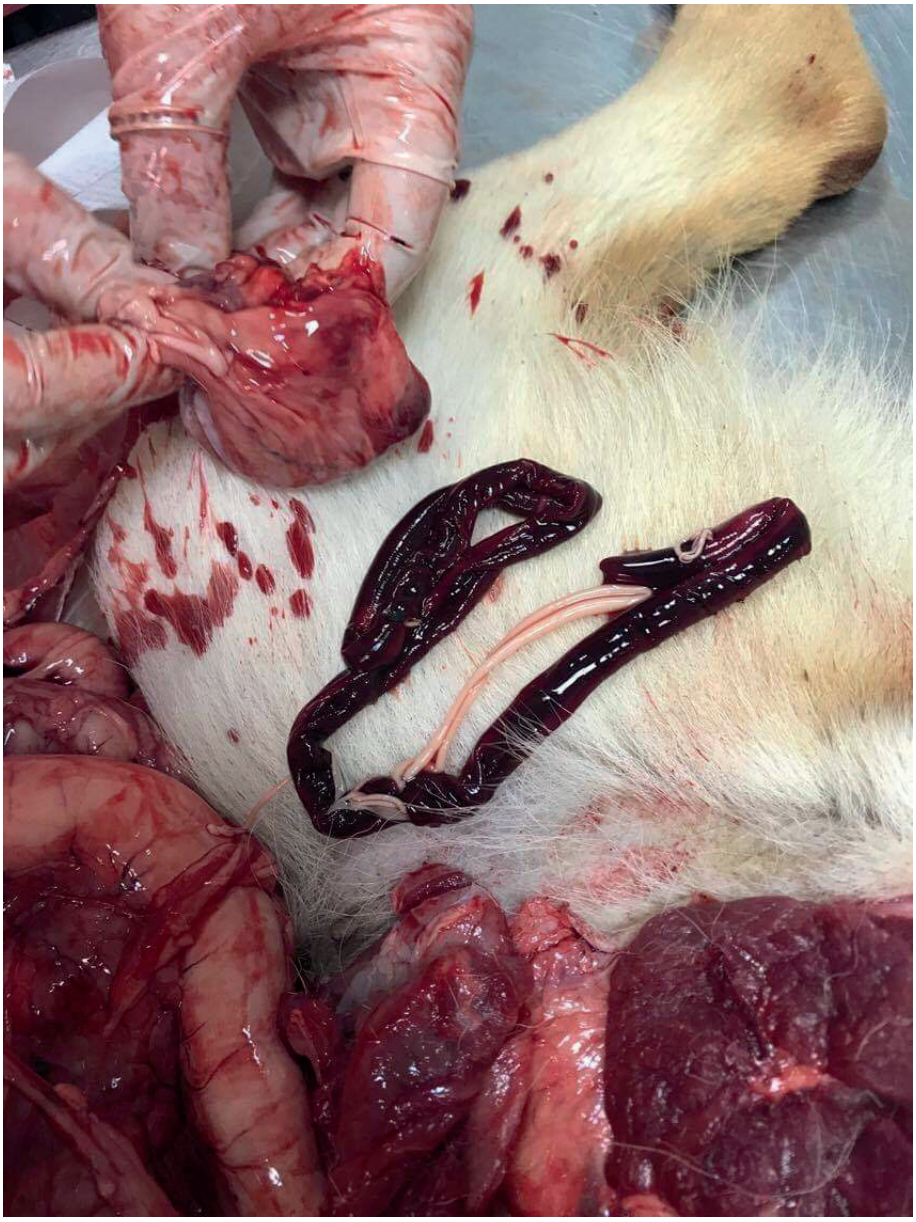

Fig. 2. *Dioctophyma renale* adult parasites recovered at the *post mortem* examination of the right kidney.

Discussion

Canine dioctophymatosis is a rare parasitic disease that poses serious threat for the canine health as well as for public health due to the zoonotic significance. The majority of reported cases come from the cold temperate regions where freshwater is available, as its life cycle requires an aquatic environment. Brazil is the country with the highest number of reported *D. renale* cases in domestic dogs with prevalence rates up to 14.2% (Ferreira *et al*., 2010; Nakagawa *et al*., 2007; Pedrassani *et al*., 2017; Rappeti *et al*., 2017). Canine dioctophymatosis has also been reported recently in other Latin America countries, such as Colombia (Florez *et al*., 2018) and Argentina (Radman *et al*., 2017). Recent studies have also reported *D. renale* in dogs in Iran (Vafae Eslahi *et al*., 2017; Zolhavarieh *et al*., 2016). In the European continent, the parasite has been reported sporadically in several countries, i.e. France, Holland, Germany, Bulgaria, Romania, Italy, Poland, Spain and former USSR (Smits *et al*., 1965; Measures, 2001; Soler *et al*., 2008).

Until recently, there was no officially reported case of canine *D. renale* infection in Greece. However, in 2013 a scientific team from the University hospital in Athens reported for the first time a rare and life-threating human case of *D. renale* infection (Katafigiotis *et al*., 2013). Human infection with *D. renale* is rare and is usually as-

sociated with the consumption of raw fish, however human cases have been reported in China (Yang *et al*., 2019; Yang *et al*., 2016), India (Chauhan *et al*., 2016; Venkatrajaiah *et al*., 2014), Iran (Norouzi *et al*., 2017), and the USA (Kuehn *et al*., 2016).

The present case of canine dioctophymatosis presented with severe hematuria and general lethargy, typical symptoms of a *D. renale* infection (Taylor *et al*., 2017). However, these symptoms are very nonspecific and parasitic infection would not be immediately suspected in countries where the disease is not prevalent. The clinical history was significant in that the animal lived in close vicinity and had free access to fresh water sources that facilitates the transmission route of the parasite. However, this patient also presented with severe tachypnea and tachycardia, clinical symptoms that were more likely to be secondary to the simultaneous infection with *D. immitis*. It is possible that the co-existence of these two pathogenic parasites in this host proved fatal for the animal. The pathogenesis and prognosis of untreated cardiopulmonary dirofilariasis is more severe and acutely life-threatening when compared to dioctophymatosis. At the post-mortem examination of the dog, the two giant worms, were retrieved from the right kidney, which was destroyed. As it was expected, the left kidney was normal due to the fact that the right kidney (close vicinity with the duodenum) is mostly infected by the nematodes (Bowman, 2014). At the time of presentation of this dog to the emergency clinic, *D. renale* had not previously been reported in the veterinary literature regarding Greece and therefore treatment efforts were focused on the most likely infection which was dirofilariosis and the renal infection was only identified at postmortem examination. Canine dioctophymatosis is a serious disease with poor knowledge of any effective anthelmintic treatment. Surgical excision is the treatment of choice, however the prognosis is very poor, and the majority of animals, if symptoms appear, die soon after the etiological diagnosis. The only known prophylaxis consists of avoiding consumption of freshwater fish or the other hosts (Beugnet *et al*., 2018).

Finally, it is of concern that, according to the owner, the dog had no history of travelling outside the country or living in close vicinity with dogs imported from other countries, confirming the autochthonous character of this infection. Similarly, the human case that was previously reported in Greece (Katafigiotis *et al*., 2013) was also described as autochthonous. Consequently, these cases have a significant importance in terms of epidemiology as they both support the hypothesis that this parasite circulates within the Greek canine or wildlife population, which is extremely important with respect to public health.

Conclusion

Dioctophymatosis is a life-threatening disease with severe clinical manifestations and guarded prognosis in dogs as well as in humans. The presence of this nematode infection both in dogs and humans in Greece stresses its zoonotic importance in terms of public health. Veterinary and medical practitioners should be aware of this disease and include it in the differential diagnosis in cases presenting with hematuria. A large-scale epidemiological study must be conducted in the country in order to estimate the accurate prevalence of this parasite and address possible risk factors that are associated with it.

Ethical Approval and/or Informed Consent

For this study formal consent is not required.

Conflict of Interest

Authors state no conflict of interest.

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DOI 10.2478/helm-2020-0009

HELMINTHOLOGIA, 57, 1: 49 – 56, 2020

Case Report

First report on clinical feline heartworm (*Dirofilaria immitis*) infection in Romania

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Article info

Received October 9, 2019
Accepted December 16, 2019

Summary

Dirofilaria immitis (Nematoda: Filarioidea) is the causative agent of heartworm disease (HWD), a severe and potentially fatal condition in dogs. Although cats are considered more resistant to infection than dogs, they are also susceptible to heartworm infection. Moreover, the clinical importance of feline dirofilariosis has increased in recent years, especially in heartworm endemic areas. In contrast to dog, definitive antemortem diagnosis of heartworm infection in cat is difficult to achieve and a combination of testing methods must be used for clinical confirmation. Here we describe a clinical case of heartworm infection in a 12-year-old male mixed breed cat, originated from Southern Romania, which was referred to a veterinary clinic with a history of vomiting and clinical signs of respiratory distress. The thoracic radiograph showed a diffuse bronchointerstitial pattern of the pulmonary parenchyma. The serological test for *D. immitis* circulating antibody was positive and heartworms were visualized by echocardiography in the main pulmonary artery. In conclusion, the present study clearly shows that cats are at risk for *D. immitis* infection in heartworm-endemic areas in Romania. Additionally, the findings highlight the urgent need for increased awareness among veterinary practitioners of the existence of feline heartworm diseases and for adequate prophylactic measures to be applied. To our knowledge, this is the first report on clinical evolution and radiographic and echocardiographic features of a naturally heartworm-infected cat in Romania.

Keywords: *Dirofilaria immitis* infection; respiratory distress; thoracic imaging; cat; Romania

Introduction

Heartworm disease (HWD) is a severe and potentially fatal cardio-pulmonary disease caused by the mosquito-borne filariod *Dirofilaria immitis*, primarily infecting dogs, but also other carnivores, including domestic and wild felids. Adult worms are located in the pulmonary arteries and occasionally in the right heart. The dog is considered the main reservoir, although *D. immitis* can also affect cats and other mammal species, and in rare cases, humans (McCall *et al.,* 2008). HWD is endemic in Mediterranean countries but currently an increase in the geographical range and its spreading towards central and eastern European areas are reported (Genchi *et al.,* 2011).

Cats are also susceptible to heartworm infection, although they are considered more resistant to infection than are dogs. There are some indications based on which the cat is considered an imperfect or not ideal host for heartworms, such as: low adult worm burden (1 – 6 worms) (Dillon *et al.,* 2007), their short life span (2 – 3 years) (Genchi *et al.,* 2008), lack or short duration of microfilaremia, and a prolonged pre-patent period (8 months) (Dillon *et*

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al., 2007; Atkins *et al.*, 2008). Therefore, most cats seem to tolerate heartworm infection for prolonged period without or only with transient clinical signs (González-Miguel *et al.,* 2010).
Moreover, clinical signs associated with HWD in cats are usually nonspecific, comprising predominantly respiratory sings (intermittent coughing, dyspnea, tachypnea, ataxia) but also gastrointestinal disorders (e.g. vomiting, diarrhea, weight loss, anorexia), as well as the frequent asymptomatic course of feline dirofilariosis in which the sudden death of the animal is sometimes the only clinical sign shown (Dillon, 1984; McCall *et al.,* 1994; Lee & Atkins 2010). Some spontaneously self-cure as a result of natural death of parasites in infected cats without showing clinical signs or a suddenly acute respiratory syndrome may arise without warning (Atkins *et al.,* 2000; McCall *et al.,* 2008). All these facts make the diagnosis of HWD in cats more difficult and underestimated (Lee & Atkins 2010).
Dirofilariosis is usually diagnosed by detection of the circulating microfilariae in the peripheral blood, immunological tests (ESDA, 2017) and molecular approaches (Liu *et al.,* 2005; Nuchprayoon *et al.,* 2006). Because of the specific features that characterize HWD in cats, reflected by the relatively low adult worm burden, low intensity and transitory microfilaraemia, low circulating antigenemia, the diagnostic approach to feline heartworm infection must incorporate a combination of testing methods (Lee and Atkins, 2010). Of these, heartworm serology (antigen test, antibody test), thoracic radiography and echocardiography are considered the most useful tools of clinical confirmation (Atkins *et al.,* 2000; ESDA, 2017).
Though microfilaraemia is seldom present in infected cats, because patency is short, and performing a modified Knott test for detecting circulating microfilariae is rarely successful, when present, microfilaraemia is considered a definitive diagnosis (ESDA, 2017). However, serological tests are typically the initial screening tool for feline dirofilariosis and have been used for large-scale studies (Kramer & Genchi, 2002) and also to assess clinical infections (Atkins *et al.,* 2000). Currently, commercially available antigen tests, that target a protein in the reproductive tract of adult females, are considered highly specific and sensitive (100 % and 98.9 %, respectively) methods for infection screening or for confirming a clinical suspicion (Genchi *et al.,* 2018). However, sensitivity can be lower in case of low burden or single worm/sex infection (ESDA, 2017). Antibody tests are able to detect infection by both male and female worms and are the only tests that can be positive as early as 2 months post infection (Morchon *et al.,* 2004) but do not confirm a current infection or the presence of adult worms (Snyder *et al.,* 2000).
In cats with either immature or adult heartworms, significant parenchymal and airway disease is manifested radiographically and histologically (Atkins *et al.,* 2000; Dillon *et al.,* 2007). Therefore, additionally, and independent of serologic test results, thoracic radiography may provide strong evidence of feline HWD and is valuable for assessing the severity of disease and its monitoring (ESDA, 2017). Also, echocardiography is a useful adjunctive test in cats with clinical suspicion of HWD and, in almost all cases, is the only definitive way to diagnose infection antemortem (Atkins *et al.,* 2008).
During the last years, several different Polymerase-Chain-Reaction (PCR)-based protocols have been developed for single species or simultaneous detection of different *Dirofilaria* species in cats using nested and multiplex PCR and PCR-RFLP (Restriction Fragment Length Polymorphism), respectively assays, as useful diagnostic tools to detect the *D. immitis* low infection in feline HWD (Liu *et al.,* 2005; Nuchprayoon *et al.,* 2006).
The clinical importance of HWD in cats has increased in recent years, especially in heartworm endemic areas, mainly do to increased awareness of this disease, as even a small number of worms are potentially life-threatening (McCall *et al.,* 1994).
In the last decade the dynamics of canine HWD in Romania has been changed from sporadic to emerging (Ionita *et al.,* 2012) and currently an alarming increased prevalence is reported, with endemic foci in South-Eastern areas (Mircean *et al.,* 2012; Girdan *et al.,* 2015; Pana *et al.,* 2018). However, to our knowledge, there are no reports about feline heartworm disease in Romania. Here we describe a clinical case of natural heartworm infection in a cat in Southern Romania.

Material and Methods

Case presentation
A twelve year-old male mixed breed cat was referred, in October 2018, to a veterinary clinic in Bucharest with a history of vomiting and difficult breathing. The cat originated from a rural area from Giurgiu county (44°24'54"N 25°49'28"E) (Southern Romania), at about 30 km far by Bucharest. The cat, with a mixed indoor and outdoor life style, lived with other 2 cats and 24 dogs in the same household and she had not received heartworm prevention prior to the visit, as the owner stated. The history of cat, as recalled by the owner, did not include any travel outside the home city limits. Cat spent most of the time outdoors.
Laboratory investigations
Cat blood samples were collected and submitted for routine hematological and biochemical investigations and for parasitological testing.
In order to asses the cause and the severity of the disease, the cat was also subjected for imagistic investigations consisting of thoracic radiography (by using a Roentgen Examion Maxivet DR apparatus) and cardiac echocardiography (by using a General Electric Logiq 9 ultrasound system).
Parasitological evaluation included a modified Knott test (Knott, 1939), for detecting circulating microfilariae of *D. immitis* and two commercial immuno-chromatographic rapid tests for detecting circulating *D. immitis* antigen (Ag) (SNAP®4DX® Plus, Idexx) and antibody (Ab) (FeliCheck-3, VetExpert), respectively.
The modified Knott test was applied for the concentration, detec-

tion and identification of microfilariae, as briefly described here: 1.0 ml of EDTA venous blood was mixed in a 15 ml conical centrifuge tube with 9.0 ml of 2 % buffered formalin and centrifuged for 5 minutes at 1500 rotation per minute (rpm). The supernatant was decanted, and a drop of 0.1 % methylene blue was added to the sediment and stained for 2 minutes. A drop of the sediment was transferred to a glass slide and covered with a cover slip. The slide was examined under the microscope at 10x to detect the presence of microfilariae and then at 40x to observe the morphological features. The whole sediment was analyzed.

The SNAP®4DX® Plus test is a point-of-care ELISA-based immunochromatographic commercial kit for the detection of *D. immitis* antigen; additionally, it is able to simultaneous detect antibodies against *Anaplasma phagocytophilum/Anaplasma platys*, *Ehrlichia canis, Ehrlichia ewingii*, and *Borrelia burgdorferi* (IDEXX Laboratories, Westbrook, ME).
The FeliCheck-3 Ab test used is a chromatographic immunoassay for the simultaneously qualitative detection of feline heartworm Ab (FHW Ab), feline immunodeficiency virus antibody (FIV Ab), and feline leukemia virus antigen (FeLV Ag).

Ethical Approval and/or Informed Consent

All applicable national and institutional guidelines for the care and use of animals were followed.

Results

Clinical and laboratory findings
On physical and clinical examinations, the cat was in a good body condition but showed, intermittent coughing, cyanotic mucous membranes and dyspnea, without murmur and arrhythmia.
The hematological examination revealed increased number of the white blood cells (WBC 25.99 K/µL) characterized by lymphocytosis (13.6 K/µL), mild neutrophilia (NEU 11.09 K/µL) and monocytosis (0.54 K/µL). The biochemical analysis showed increased hepatic enzymes (alaninaminotranspherase 67 UI/L; alkaline phosphatase 118 UI/L; total protein 8.7 g/dl) (Table 1).

Table 1. Hematological and serum biochemical parameters of a 12-year old mixed breed cat with heartworm infection.

Parameter	Measure unit	Reference value	Results (interpretation*)
White blood cells (WBC)	K/µL	5.1 – 16.2	25.99 (↑)
Hematocrit (HCT)	%	27.7 – 46.8	38.9
Hemoglobin (HGB)	g/dL	8.1 – 14.2	13.1
Neutrophils (NEU)	K/µL	2.3 – 10.7	11.09 (↑)
Lymphocytes (LYM)	K/µL	1.2 – 6.8	13.6 (↑)
Monocytes (MON)	K/µL	0.1 – 0.4	0.54 (↑)
Platels (PLT)	K/µL	156 – 626	216
Blood Uruine Nitrogen (BUN)	mg/dL	15 – 35	27
Glucose (GLU)	mg/dL	61 – 120	162 (↑)
Alkaline phosphatase (ALKP)	UI/L	12 – 65	118 (↑)
Total protein (TP)	g/dL	5.4 – 7.9	8.7 (↑)
Alaninaminotranspherase (ALT)	UI/L	8 – 53	67 (↑)
Creatinine (CRE)	mg/dL	0.5 – 2	1.5
Amylase (AMYL)	UI/L	370 – 1200	894
Total bilirubin (TBIL)	µmol/L/dL	0.15 – 0.5	0.3

* ↑: increased

Subsequently to the parasitological testing, circulating *D. immitis* microfilariae and Ag were not detected, but the serology was positive for feline heartworm Ab, as well as for FIV Ab (Fig. 1).
The thoracic radiograph showed a diffuse bronchointerstitial pattern of the pulmonary parenchyma (more evident in the caudal lung lobes) (Fig. 2) and enlarged caudal lobar pulmonary arteries (Fig. 3).
During of cardiac ultrasonographic examination, adult heartworms were detected in the main pulmonary artery. The heartworm appeared as short, segmented strongly echogenic parallel lines (Fig. 4).

Treatment and follow-up
After diagnostic, a treatment protocol was designed for the infected

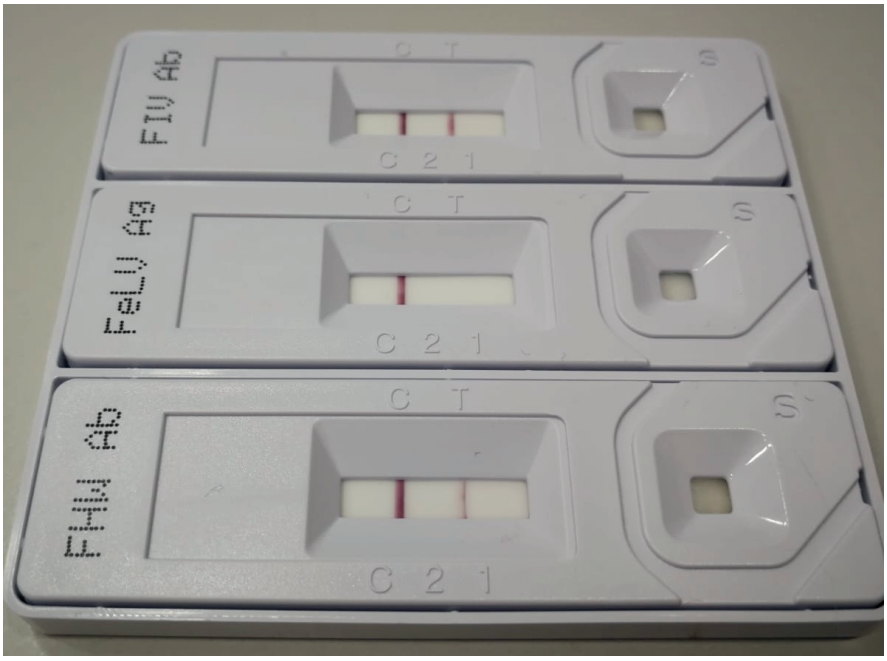

Fig. 1. Serologic (FeliCheck-3) test showing positive reaction for feline immunodeficiency virus (FIV) and feline heartworm (FHW) infection

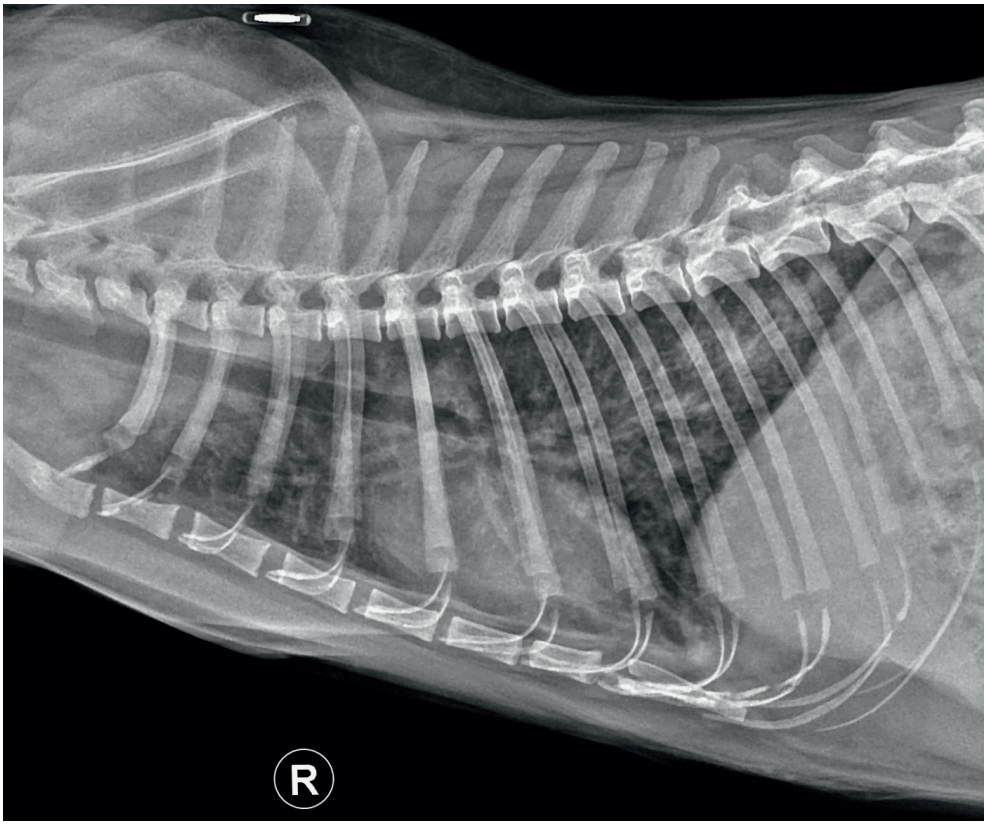

Fig. 2. Thoracic radiograph showing a diffuse bronchointerstitial pattern in a 12-year old cat with heartworm infection (lateral view)

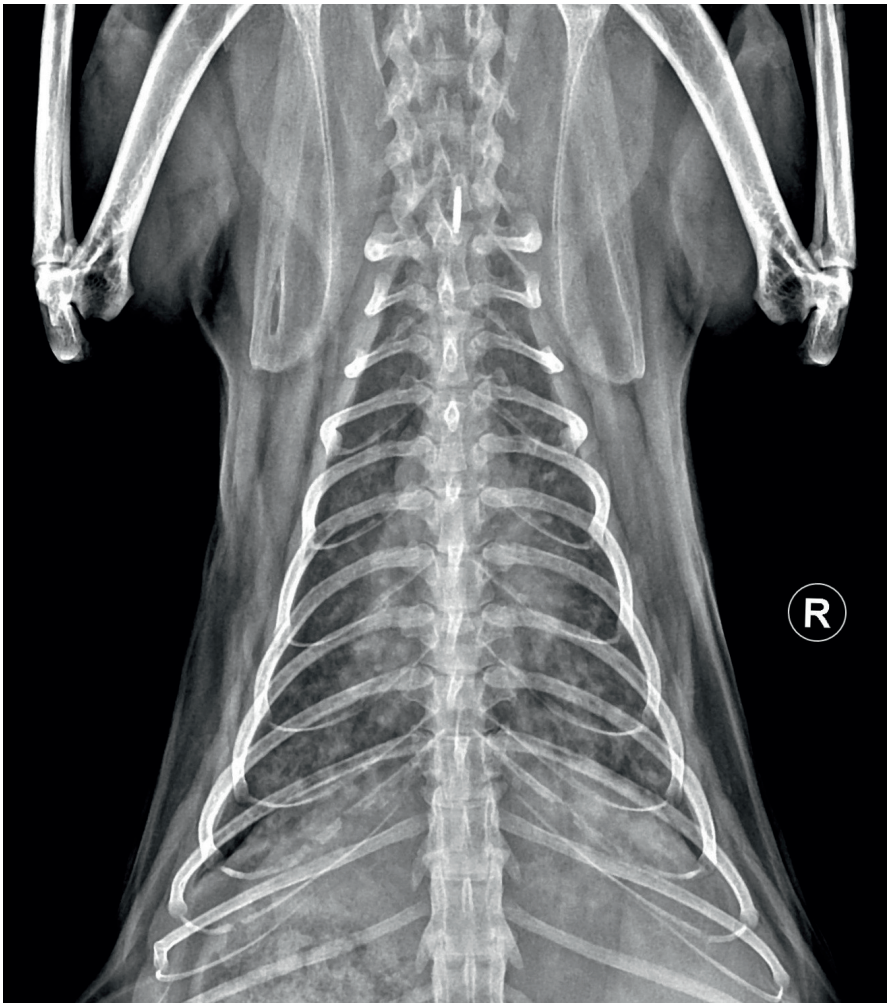

Fig. 3. Thoracic radiograph showing enlarged caudal lobar pulmonary arteries in a 12-year old cat with heartworm infection (dorsoventral view)

cat aiming firstly to stabilize the respiratory signs and to stop the progression of cardiac disease. A symptomatic treatment for the pulmonary hypertension (sildenafil, 1.0 mg/kg, every 12 hours, 30 days) was commenced. Additionally, supportive hepatic and immuno-stimulating therapy was recommended, for 30 and 60 days, respectively. After few days, the cat stopped vomiting, showing normal breathing and displayed no other suspicious clinical signs. Further heartworm chemoprophylaxis with monthly doses of either milbemycin oxime orally, or topical selamectin was recommended for the cat to avoid repeat infection (Litster & Atwell 2008), and serologic retesting at 6 – 12-month intervals for the purpose of monitoring infection status (ESDA, 2017).

As the positive cat lived in a house with other at risks' animals, 2 cats and 24 dogs, we recommended to the owner to test all the co-habiting animals. The 2 cats were tested by serologic (Felicheck 3, VetExpert) for and by Knot tests, for circulating *D. immitis* Ab and microfilariae, respectively, and both were negative.

The 24 co-habiting dogs were tested for circulating *D. immitis* Ag, by using the Ag test (Snap 4Dx) and microfilariae, by the modified Knott test. Of them, five dogs (5/24; 20.8 %) were positive for the both, circulating *D. immitis* Ag and microfilariae.

For all the owned-cats and dogs, heartworm chemoprophylaxis was also recommended.

Discussion

Over the recent decades feline dirofilariosis, caused by *D. immitis* heartworm, has gained the attention of the scientific community which recognized significant differences between feline and the classical, better known, canine heartworm disease, in all aspects: host response, patho-physiology, and clinical presentation (Lee & Atkins 2010; ESDA, 2017). Therefore, a conscious awareness of clinicians of its existence is critical.

Here we describe the first survey carried out on the clinical evolution and radiographic and echocardiographic features of a naturally heartworm-infected cat in Southeastern Romania. To the authors'

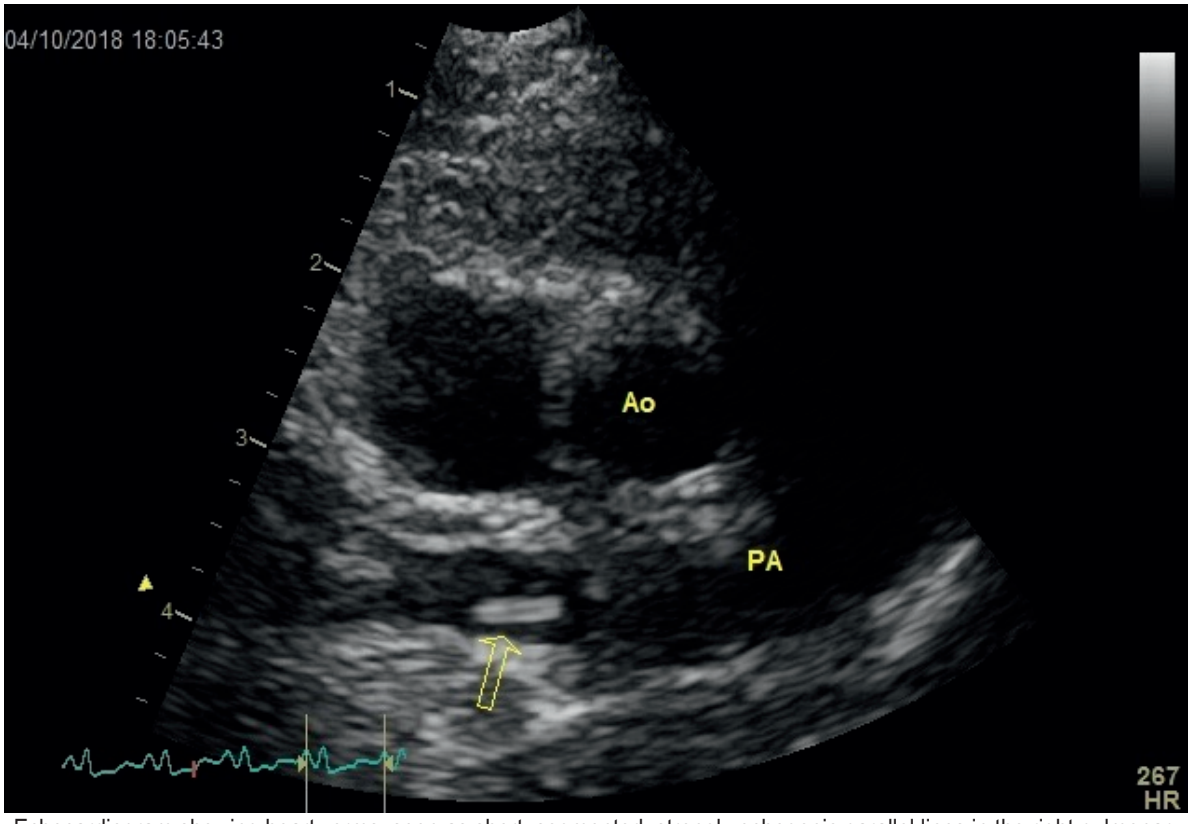

Fig. 4. Echocardiogram showing heartworms, seen as short, segmented, strongly echogenic parallel lines in the right pulmonary artery (arrow) of a 12-year naturally infected cat (Ao: aorta; PA: pulmonary artery) (right parasternal short axis view at the heart's base)

knowledge, no epidemiological surveys were carried out to investigate the exposure of cats to *D. immitis* infection in Romania.

In contrast to heartworm infection in dogs, the prevalence of feline heartworm infection is less well defined because definitive ante-mortem diagnosis is difficult to achieve. Additionally, according to the current opinion, most feline HW infections are asymptomatic, which support the idea that the true prevalence of HW infection in cats in HW-endemic areas is underestimated (Venco *et al.,* 2008). However, it is generally estimated to be 5 % to 20 % that of the canine population in a particular area (Hermesmeyer *et al.,* 2000). This estimation is supported by recent field surveys, such as epidemiological surveys in Italian endemic areas and the metropolitan area of Barcelona (Spain), that reported a prevalence of feline HW infection of approximately 10 % and 10.4 %, respectively of that of dogs in the same area (Venco *et al.,* 2011; Montoya-Alonso *et al.,* 2014).

For Romania, recently, several studies have been reported on existing endemic foci for canine HWD in Southeastern Romania, the infection rate values varying from 13.4 % to 23.0 % (Ionita *et al.,* 2012; Girdan *et al.,* 2015; Anghel *et. al.,* 2016; Pană *et al.,* 2018). The infected cat in the present study originated from such heartworm-endemic area in Southeastern Romania, Moreover, the cat was living with dogs of which 20.8 % were confirmed to be heartworm infected. These demonstrate that cats from that area are at high risk for HW infection and it is very likely that many clinicians are not aware about the real risks of cats for HWD. Further epidemiological surveys will elucidate the current status of feline dirofilariosis in Romania.

Moreover, it is suggested that cats that are living in the urban areas are also at risk to be infected with heartworm. Building construction and human activity increase the density of potential hosts and develop suitable environments for the proliferation of mosquitoe populations due to an increase in the provision of water sources and vegetation (Harrus & Baneth, 2005; Petríc *et al*., 2014).

The outdoor lifestyle of the infected cats in the present study appears as a risk factor for HW infection. It is states that outdoor lifestyle increases cat's exposure to mosquito vectors in HW-endemic areas (Genchi *et al.,* 2008). However, different studies showed that cats living strictly indoors are not fully protect against HW infection, as between 19 % and 27 % of confirmed HW-infected cats were indoor only (Atkins *et al.,* 2000).

The observed patho-physiology and clinical picture including history of vomiting, intermittent coughing, and increased respiratory efforts, are of the most common clinical signs heartworm-infection associated in cats (Venco *et al.,* 2015). The echocardiography has proved the clinical suspicion of heartworm disease in the cat with positive serology (Ab test), but the Ag test for circulating *D. immitis* microfilariae was negative. These findings clearly support the re-

commendation that multiple test diagnosis must be used for clinical confirmation of HWD in cats, some of which may need to be repeated, especially in endemic areas characterized by high index of suspicion (ESDA, 2017).

Recent evidence-based knowledge acquired from various field studies on the detrimental effects of the both larval and adult stages of *D. immitis* that can potentially have of any exposed cat, clearly demonstrate that prevention against infection in cats is the best approach to feline heartworm disease (Venco *et al.,* 2015). For this, there are several highly effective heartworm preventive products commercially available, all from the macrocyclic lactone group. Therefore, a monthly feline heartworm chemoprophylaxis with either ivermectin (24 µg/kg) or milbemycin oxime (2.0 mg/kg) orally, or topical selamectin (6 – 12 mg/kg), moxidectin (1.0 mg/kg), or eprinomectin (0.48 mg/kg) is recommended at least during of the heartworm transmission season (Lee & Atkins, 2010; ESDA, 2017).

Conclusion

The present study shows that cats are at risk for *D. immitis* infection in heartworm-endemic areas in Romania. Additionally, this study highlights the urgent need for increased awareness among veterinary practitioners of the existence of feline heartworm disease and for adequate prophylactic measures to be applied.

Conflict of Interest

Authors state no conflict of interest.

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DOI 10.2478/helm-2020-0004

HELMINTHOLOGIA, 57, 1: 57 – 62, 2020

Research Note

Drug efficacy of ivermectin against primary nematodes parasitizing captive Przewalski's horse (*Equus ferus przewalskii*) after ten years of annually treatment

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Article info

Received July 23, 2019
Accepted December 4, 2019

Summary

Reintroduction of endangered species to natural habitat is considered as an important tool for conservation. The effect of drug management on captive population of reintroduced species is largely neglected. Decreased drug efficacy could pose a substantial threat to health of animals. More importantly, captive population without proper drug administration could act as transmission medium of resistance nematodes to wild population, making it important to delay the occurrence of drug resistance in captive population. Ivermectin have been used in captive Przewalski's horse (*Equus ferus przewalskii*) to eradicate intestinal parasitic nematodes annually, while no available studies describing the drug efficacy in the recent ten years. Here, fecal egg counts pre- and post-treatment were performed with ivermectin through individual trace. Both large and small strongyles were identified by larval culture. The fecal egg count reduction was almost 100% based on egg counting data of 448 samples from 13 Przewalski's horses. Feces of two Przewalski's horses were sampled for successive 20 days. Eggs per gram feces usually increased dramatically at the period of 1 – 2 post-treatment days and declined persistently to 0.0 within 15 days. A sustained high ivermectin efficacy against neither *Parascaris equorum* nor strongyles was indicated, which can be partly explained by the low deworm frequency.

Keywords: drug efficacy; Captive Przewalski's horses; fecal egg count reduction test; Ivermectin; parasitic nematodes

Introduction

The Przewalski's Horse (*Equus ferus przewalskii*; PH for short), once considered as the only extant true wild horse that has never been domesticated, is classified as endangered by the IUCN Red List (King, *et al*., 2015) and as a class I protected species by the Chinese government at the same time. PH was extinct in the wild since the 1960s, and reintroduced to China in captivity about twenty five years later (Xia *et al*., 2014). Latest study has shown PH is the feral descendant of the earliest-known domesticated Botai herds and form a monophyletic group independent from the modern domestic horses (Gaunitz *et al*., 2018). However, PH is still important in culture, genetic diversity and conservation since it is the last species live in the wild and have a unique adaptive evolutionary history (Oakenfull *et al*., 2000). Now, the primary threats to captive populations are inbreeding depression and diseases (Wakefield *et al*., 2002). With respect to the latter, debilitating infections pose a substantial threat to the reestablishment of PH populations. Captive populations repeatedly infected with parasitic nematodes, including roundworms (*Parascaris equorum*) and

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strongyles, which typically parasitize the alimentary canal of PH and trigger many clinical signs (Zhang, 2006). In 2000, *P. equorum* infections caused the death of several PH at the Xinjiang Uygur Autonomous Region Wild Horse Breeding Research Center (Ente & Zhang, 2003).

Since 2005, ivermectin (IVM) has been used as the single equine anthelmintic once a year in winter to combat parasitic diseases on all of the horses kept in captivity without preexamination. Long-term use of single deworming drug may cause anthelmintic resistance (AR) in highly adaptable parasitic nematodes (Leathwick, 2013). Decreased IVM efficacy against important intestinal parasites of horses, especially *P. equorum*, has been widely recognized in many countries (Boersema *et al*., 2002; Schougaard & Nielsen, 2007; Lyons *et al*., 2008; Milillo *et al*., 2009). Captive PHs were chosen to be released to wild periodically, which may act as transmission channel of anthelmintic-resistant nematodes between domestic and wild animals (Chintoan-Uta *et al*., 2014). However, there was only one study described performance of IVM in PHs (Zhang, 2006). A better understanding of gastrointestinal nematode resistance against anthelmintic is urgently required for PHs breeding. A range of methods can be utilized for detecting AR in animals, including in vivo and in vitro tests (Coles *et al*., 1992; von Samson-Himmelstjerna, 2012). The Fecal Egg Count Reduction Test (FECRT) (Coles *et al*., 1992) is the most widely used in vivo test, and considered as the only viable method for declaring the presence of IVM-AR in equine gastrointestinal nematodes (Kaplan, 2002; Nielsen *et al*., 2014).

Given that the efficacy of anti-parasitic drugs against gastrointestinal nematode is key to control parasite that prevalent in captive population and prevent the spread of resistant strains to released population, the present study evaluated the efficacy of IVM against gastrointestinal nematode parasitizing captive PHs based on FECRT method and could serve for the deworming work in species reintroduction program.

Materials and Methods

Study area

All PHs were maintained at the Xinjiang Uygur Autonomous Region wild horse Breeding Research Center located in Jimsar County, Changji City, Xinjiang Uygur Autonomous Region, China (44°12'12"N, 88°44'26"E). Environmental conditions of temperature were -14 °C ±6 °C during sampling period in November and December. The horses were bred outdoor in different size of irony stalls all day long. Captive population were divided into breed, female or bachelor groups. There is no vegetation in the stalls. Alfalfa and carrots were offered daily. No water were provided as the water freeze, and horses could replenish water by eating snow.

Study design

Sixteen horses were randomly selected for an infection survey, of which two horses with typical histories of parasitic nematode disease were selected as representatives to explore the change pattern of egg counts number in 20 successive days during November and December 2015. One representative horse (#105) is an adult female, born in 1998; the other (#345) is a young male foal, born in 2013. Furthermore, the EPG were compared in the same animals both before and after treatment in 3 foals (<1 year of old) and 8 sub-adults (<2 years of old) in 2017 January. The larvae culture was applied to verify the composition of strongyles. Only small group of animals were enrolled (11 individuals including 3 foals and 8 sub-adult) because EPG before treatment in most horses was too low to take into account. Another practical justification for this small group experimental design is that drug treatment was only allowed on certain days in winter due to restrictions of management measures. The limited time and cold weather made it hard to obtain enough samples by individual track in deworm process or use unpaired design which need extra control group. Although control groups were not adopted, its influence can be eliminated since pre-treatment counts can be used as the compared baseline (McKenna, 1990).

Experimental Method

An oral paste of IVM (Beijing Wanfeng Pharmaceutical Laser Target Designator, Beijing 100193, China; 2 mg/g i.m) was mixed with corn meal and fed to all captive PHs at a dosage of 100 μg/kg following the IVM deworming instructions reported before (Zhang, 2006). Records of body weight of the horses was offered by the Xinjiang Uygur Autonomous Region wild horse Breeding Research Center. Deworming was carried out by a qualified veterinary surgeon. No dose errors were reported.

The surface and interior of each fecal pellet feces were randomly mixed. For fecal egg counts (FEC), a total of 1.0 g feces was ultimately sampled and fixed in 10 mL centrifuge tube with 2.0 g 10 % formalin solution. Each sample was replicated three times at least. For larval culture, about 5.0 g feces were collected from each horse before treatment. All feces samples were collected from ground immediately or within two hours after they were naturally excreted, without disturbing PHs. All PHs lived in their assigned paddocks during and after collection and all acted normally. Samples were stored at 4 °C in order to preserve the morphological characteristics of the nematode eggs and maintain accurate egg counts (Nielsen *et al*., 2010).

The FEC were performed based on a Modified Wisconsin centrifugal-flotation technique (Cox & Todd, 1962) with analytical sensitivity of 1.0. 6 mL saturated $NaNO_3$ were added to each sample tube. Pellet was vortexed vigorously using Vortex Genie 2 mixer (Scientific Industry, Vortex Genie 2) for 3 minutes, and then filtered with a 60-mesh sieve. The filtrate was poured back into the centrifuge tube and centrifuged for 5 minutes at 950 r/min. Saturated $NaNO_3$ solution was added until a meniscus was formed on the top of the tube. The samples were stand for 10 minutes and then placed a 24 mm × 24 mm coverslip on the top. After an additional 5 minutes, the coverslip was removed and placed on a microscope slide. The

numbers of eggs of each parasite type were counted under a light microscope (OLYMPUS CX22). Then the process was repeated with new coverslips until no more eggs were found on the coverslip. Larval cultures were performed to determine the composition of strongyles. The fecal samples collected from each horse were pooled and humidified with sawdust and water. The cultures were incubated in 28 °C for 14 days. The L3 larval were recovered using Baermann apparatus and identified using morphological keys by Bevilaqua *et al*. (1993).

Data analysis

The FEC reduction (FECR) of individual PH was calculated by using arithmetic means according the following formula: $FECR(\%)=[(FEC_1–FEC_2)/FEC_1] \times 100$ (Francisco *et al*., 2011), where FEC_1 is the mean of FEC before treatment and FEC_2 is the FEC at the 15th post-treatment day. Since only small groups of horses were available and FEC_1 in the current study were low, a reliable FECR-result can hardly been calculated by regular formula based on the arithmetic mean of pre- and post-treatment FEC (Levecke *et al*., 2018). An alternative Bayesian based methodologies was applied in a user friendly web interface, egg Counts (Torgerson *et al*., 2014), to assess the FECR and coverage probability of 95 % highest posterior density (HPD) intervals of group. We adopted the paired design (fecal samples obtained before and after the treatment from each horse). Resistance is identified if both the FECR is less than 90 % and the 95 % lower confidence level is less than 90 %, following the typical criteria recommended by the World Association for the Advancement of Veterinary Parasitology (Coles *et al*., 1992).

Ethical Approval

The authors declared that all sample procedures in this study were approved by the Xinjiang Uygur Autonomous Region Wild Horse Breeding Research Center (Under the jurisdiction of Forestry Department of Xinjiang Uyghur Autonomous Region) and Beijing Forestry University. Sample collection were performed in accordance with the IUCN Policy Statement on Research Involving Species at Risk of Extinction (Approved by the 27th Meeting of IUCN Council, Gland Switzerland, 14 June 1989). Non-lethal and responsible collections were applied in the case of species listed as endangered under criterion C.

Results

Before the administration of IVM, 75 % of the 16 selected PHs tested positive for *P. equorum* and 81.3 % for strongyles. The results were consistent with previous reports (Zhang, 2006). Fertilized *P. equorum* FEC per horse (1 – 2862) was much higher than fertilized strongyles FEC per horse (1 – 70). Both small strongyles (Cyathostomins) and large strongyles (*Strongylus* spp.) were identified from larval culture.

As is shown in Figure 1, before fed with IVM, the *P. equorum* mean FEC of PHs #105 and #345 rose briefly on the 1st and 2nd post-treatment days, higher than any pre-treatment FEC, and then fell sharply in the next 2 days and keep in 0 EPG until the end. The maximum of strongyles FEC in both PH #105 and #345 did not exceed 10 EPG at any time, so the data were not shown. Apart from the two representative horses, examination of PHs before (screening FEC > 20 EPG) and after treatment (FEC = 0 EPG) show a 100 % FECR for both *P. equorum* and strongyles, with the lower confidence limit (99.2 %) above the designated 90 % cut-off.

Discussion

A common pattern is observed in *P. equorum* FEC among the different captive PH individuals: the number of FEC usually rises dramatically at the period of 1 – 2 days after deworming (Fig. 1). Consistent with the FEC, 22 and 32 of adult *P. equorum* have been collected from the foal #345 feces on 1st and 2nd post-treatment days respectively. The number of *P. equorum* FEC in the PHs #345 and #105 rose 57.1 % and 33.7 % respectively, but level of parasitic infection in PH #105 peaks earlier than in PH #345. No true positive correlation between EPG and worm burden have been verified in *P. equorum* (Reinemeyer, 2009). A hypothesize that parasite eggs might act as a proxy for female parasites in horses could be considered as sharply decline time interval of EPG comes in line with duration of adult parasite excretion. It is clear that nematode parasites of PHs #105 and #345 are wiped out after drug treatment, but the number of strongyles FEC before treatment at single digit level is not sufficient to declare truly anthelmintic efficacy.

Strongyles FEC was generally low among horses. The infection level of strongyles in both the adult and the foal was lower than 200 EPG, which caused the experiment of inadequate sample with low pre-FEC and led to a reduction of credibility (Levecke *et al*., 2018). A low analytic sensitivity method and “egg Counts” package, which allowed for a much smaller pre-FEC threshold, were employed to compensate the negative impact and hence provide a more reliable result. This high efficacy still needs to be validated in the further study if a higher number of pre-FEC was observed.

Feces of all tested PHs were negative for both larval and adult nematode parasites by the 15th post-treatment day, which means the efficacy of IVM is 100 %. It is indicated that IVM, a broad-spectrum antiparasitic agent used against both nematode and arthropod domestic animal parasites since 1979 (Chabala *et al*., 1980), remains highly effecacy against both *P. equorum* and strongyles in PH. However, since the drug were fed by oral and relative small group of animals were enroll in the study, a more deliberate design must be applied in future if possible.

Given failures of IVM treatment to decrease *P. equorum* EPG reported after ten years use of IVM as predominant anthelmintic (Boersema *et al*., 2002), the high efficacy of IVM against *P. equorum* reported here is instructive since PH in Xinjiang have traditionally been dewormed at least for a decade. Various drivers could influ-

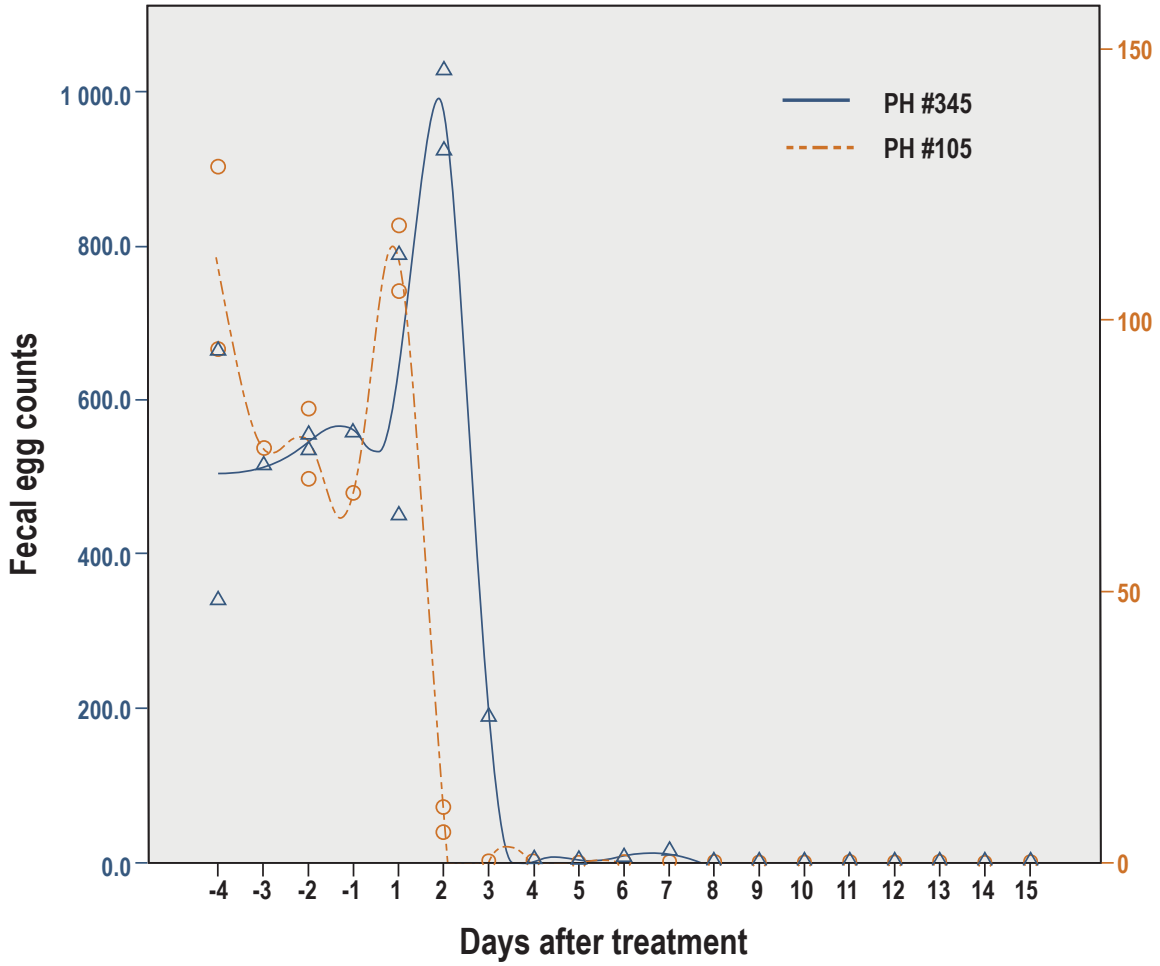

Fig. 1. Changes in *Parascaris equorum* egg counts per gram of feces in PH #105 and PH #345 from November 27th to December 15th, 2015.

ence the occurrence of AR, including various factors associated with breeding and veterinary management (Traversa *et al*., 2012). A research in the Netherlands noted that IVM resistance occurred in *P. equorum* on account of frequently treatment of foals (van Doorn *et al*., 2007). The current research provides an example of successful parasitic nematodes control program of reintroduced animals for a decade using single deworming drug in a low treatment frequency. It is reasonable to assume that low frequency treatment and inadequate feces management slow down the development of AR, because there are large number of susceptible genotype eggs and free-living larvae exist on pasture, which also known as refugia population (van Wyk, 2001). Raise of refugia population even could restore the deceased anthelmintic efficacy (Sissay *et al*., 2006).

The potential role of captive population of reintroduced species serve as vectors of resistant parasites to wild animals, make it important to keep a high anthelmintic efficacy. The managers should consider the importance of slowing down the occurrence of drug resistance instead of wiping out all nematodes. Leaving foals untreated should be avoided as they might be infected with *P. equorum*. Deworming sub-group horses (which have an EPG exceeding average level) at a low frequency which can help maintain refugia, which allow populations of nematodes unexposed to treatment to survive, could be undertook to reduce the selection for resistance in captive populations of reintroduced species. Fecal monitoring is required to set a baseline for screening horses enrolled in the drug treatment. The current study provids a pilot exploration of IVM-efficacy against nematodes parasitize PHs. The findings could be instructive to deworming program of domestic and wild animals, especially reintroduced endangered species that need to recover population in capacity before released to wild.

Statement of interest

Authors state no conflict of interest.

Acknowledgments

The authors are grateful to Jie Cao, Zhenshan Wang, Chen Wang, Zhenbiao Wang, Shouqin Gao and other professionals at the Xin-

jiang Uygur Autonomous Region Wild Horse Breeding Research Center, for their support in animal individual identification and sample collection. This work was supported by the Fundamental Research Funds for the Central Universities (No. JC2015-04).

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DOI 10.2478/helm-2020-0010

HELMINTHOLOGIA, 57, 1: 63 – 70, 2020

Research Note

Comparative study among lactophenol blue, lactophenol solution and proteinase-K lytic solution for rostellar hooks morphometry of *Echinococcus granulosus* protoscolices

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Article info

Received November 15, 2019
Accepted December 13, 2019

Summary

Echinococcus granulosus is a tapeworm whose life cycle includes dogs and other canines as final hosts, while domestic and wild ungulates act as intermediate hosts for the tissue-invading larval stage (metacestode). *E. granulosus* has a worldwide geographical distribution. Protoscolices and rostellar hooks of *E. granulosus* are useful for diagnosis and rostellar hook morphometric features may be useful to discriminate *E. granulosus* and related species. The present study was aimed to determine a more suitable lytic solution and to obtain a clearest vision for performing morphometric studies on the rostellar hooks of *E. granulosus* protoscolices. Five fertile hydatid cyst samples were collected from sheep in Kirkuk slaughterhouse, Iraq, during June of 2015. According to the results of the present study, proteinase-K lytic solution is the best approach in morphometric analysis to get a clear vision of rostellar hooks and a safer usage in comparison with solutions containing lactophenol (lactophenol, lactophenol blue).
Keywords: *Echinococcus granulosus*; protoscolices; rostellar hook; morphometric study

Introduction

Echinococcus granulosus is a tapeworm whose life cycle includes dogs and other canines as final hosts for the intestinal tapeworm, while domestic and wild ungulates act as intermediate hosts for the tissue-invading larval stage, metacestode (Moro & Schantz, 2009). *E. granulosus* has a worldwide geographical distribution (Eckert & Deplazes, 2004). Hydatid disease (hydatidosis) is the larval infection characterized by long-term growth of hydatid cysts in the intermediate host. In internal organs, mainly liver and lungs of humans and other intermediate hosts, hydatid cysts of *E. granulosus* develop as unilocular fluid-filled bladders (McManus *et al*., 2003). Hydatid cyst consists of external acellular laminated layer and internal nucleated germinal layer; the last one may give rise by asexual budding to brood capsules. Protoscolices originate from the internal layer of the brood capsules (Thompson & McManus, 2001).

For morphometric study of rostellar hooks from *E. granulosus* protoscolices, a lytic solution must be used for digesting protoscolices and preparing the microscopic slides. Lactophenol blue solution is a common reagent used in mycology, but it is also very useful in evidencing protozoal cysts, trophozoites and ova of parasitic helminths, in addition to studying the internal structure of the parasitic pathogen (Jada *et al*., 2016). Lactophenol blue solution contains cotton blue, which stains internal structures. Lactophenol blue and lactophenol solution contain phenol and lactic acid, which kill viable trophozoites, and may also kill protozoal cysts and helminthic eggs. Finally, glycerol in lactophenol blue and lactophenol solution

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provide semi-permanent preparations (Parija & Prabhakar, 1995). Proteinase-K is a main proteolytic enzyme, obtained upon purification from *Tritirachium album*. This enzyme was named "proteinase-K" with respect to its keratin hydrolyzing activity. Proteinase-K has a strong proteolytic activity- about six times more than pronase and about three times more than bovine trypsin- on denatured proteins, for example, hemoglobin and casein (Ebeling *et al*., 1974). Protoscolices and rostellar hooks of *E. granulosus* are useful for diagnosis, also in relation to cysts vitality. In addition, rostellar hook morphometric features may be useful to discriminate the isolates of *E. granulosus* from the related species *E. canadensis* (Harandi et al., 2012), and the morphometric features of rostellar hooks of protoscoleces are useful in differentiation between strains of *E. granulosus* of different intermediate hosts (Mowlavi *et al*., 2012; Arbabi *et al*., 2017). The present study is aimed to compare different suitable lytic solutions in order to obtain a clearest vision for performing morphometric studies on the rostellar hooks of *E. granulosus* protoscolices.

Materials and Methods

Collection of samples

Out of 34 fertile hydatid cysts collected from 18 infected sheep in Kirkuk slaughterhouse, Iraq during June of 2015, five fertile hydatid cyst samples were selected from liver to perform the present study. According to Perez-Serrano *et al.* (1995), protoscolices were isolated from each hydatid cyst and washed with phosphate buffer saline (PBS), then stored in ethanol 70% tubes until used.

Preparation of slides for microscope

The study was designed for the first time for comparing among three lytic solutions (Lactophenol blue, lactophenol and proteinase-K lytic) for preparation of microscopic slides. Lactophenol blue solution of Central Drug House company (India) was used. Lactophenol solution was prepared by mixing 50 ml of phenol (CDH, India), 10 ml of lactic acid (Solvo Chem, UK), 20 ml of glycerol (Solvo Chem, UK) with 900 ml of distilled water (Humason, 1979). Proteinase-K lytic solution was prepared according to Wu *et al*. (2007) with modification, by mixing 300 µl of TE9 buffer with pH 7.5 and 200 µl of 10 mg/ml of proteinase-K stock solution. TE9 buffer was prepared according to Wu *et al*. (2007) and Blasco-Costa *et al*. (2012) with modification on the volume of components. TE9 buffer contained 10 mM of tris-HCl (Scharlau, Spain), 125 mM of sodium chloride (SCRC, China) and 10 mM of EDTA (Panreac, Spain) with pH 8. Proteinase-K stock solution with 10 mg/ml (w/v)% was prepared according to Harvey (2000) by dissolving 50 mg of proteinase-K (Bio Basic, Canada) in 5 ml of distilled water (Alkem laboratories, India). Lactophenol blue, lactophenol and proteinase-K lytic solutions were used on all samples (for comparison) as in the following steps: i) one drop of protoscolices with ethanol 70 % was taken by 3 ml Pasteur pipette and laid on the middle of microscope slide for each solution and was examined under microscope before adding the lytic solutions to act time zero; ii) each slide was putted in separated Petri dish, and then a drop from each lytic solution was added on the specimens separately. All slides were incubated for 4 hours with repetitive microscopic examinations under 100x and 400x magnification every 30 minutes (time 30, time 60, time 90, time 120, time 150 and time 240 minutes) with continued repetitive additions of the all lytic solutions to prevent specimen drying. Slide with proteinase-K lytic solution was incubated at 56 °C, while, lactophenol blue and lactophenol solution slides were incubated at a room temperature; iii) at time 240, the incubation was stopped, and a drop of aqueous gelatin-glycerol was added to all specimens to preserve it for a long time. Aqueous gelatin-glycerol consisting

Table 1. Clustering behavior of protoscolices and visibility of components at different times after treated with lactophenol-based solutions and Proteinase-K lytic solution.

Time of exposure	Lactophenol blue or lactophenol solution	Proteinase-K lytic solution
Time 30 minutes	Protoscolices aggregated/ Components and rostellar hooks not visible (Fig. 2a-b)	Protoscolices separated/ clearer vision of components (Fig. 2c-d)
Time 60 minutes	Protoscolices aggregated/ Components and rostellar hooks not visible (Fig. 3a-b)	Protoscolices separated/ clearer vision of components (Fig. 3c-d)
Time 90 minutes	Protoscolices aggregated/ clearer vision of components (Fig. 4a-b)	Protoscolices separated/ vision of components same as time 60 (Fig. 4c-d)
Time 120 minutes	Protoscolices partially separated/ components visible but with no lysis of membrane (Fig. 5a-b)	Protoscolices well separated/ starting of membrane lysis (Fig. 5c-d)
Time 150 minutes	Protoscolices partially separated/ components visible but with no lysis of membrane (Fig. 6a-b)	Protoscolices well separated/ increasing of membrane lysis (Fig. 6c-d)
Time 240 minutes	Protoscolices partially separated/ components visible (better with lactophenol) but with no lysis of membrane (Fig. 7a-b)	Protoscolices well separated/ membrane lysis semi-completed (Fig. 7c-d). Rostellar hooks free and visible (Fig. 8)

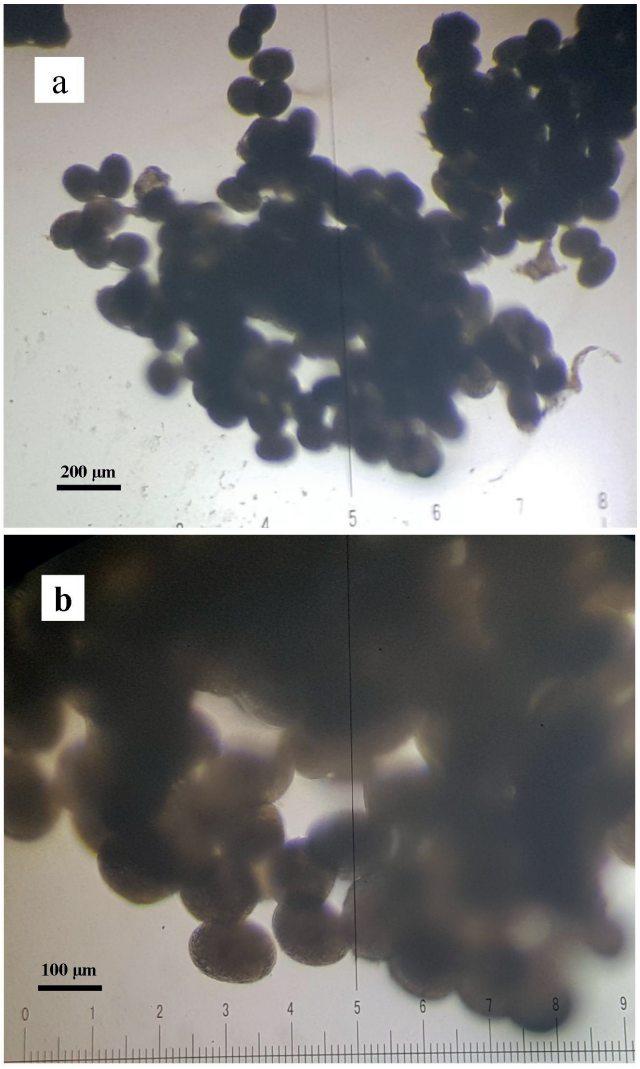

Fig.1. Microscopic photography of protoscolices at different magnification (a, b) before use of lytic solutions (zero time), showing aggregation of protoscolices within the preservation solution (ethanol 70%).

of 5 g of gelatin (Panreac, Spain), 50 ml of glycerol (Solvo Chem, UK), 5 ml of phenol (CDH, India) and 50 ml of distilled water (Humason, 1979). In conclusion, a microscopic examination was carried out under 1000x with oil immersion and fine pressure on the coverslip was done to identifying the rostellar hooks.

Ethical Approval and/or Informed Consent

This article does not contain any studies with human participants or animals by any of the authors therefor the present study formal consent is not required.

Results

During the first inspection, before adding the lytic solutions, the protoscolices resulted aggregated after examination using a microscope with 400x, thus not allowing a clear definition of the internal structures and rostellar hooks (Fig. 1a, b). Whereas, at the last step, after 240 min from the addition of lytic solutions, lactophenol blue provided still little clear vision and no lysis of protoscolices membranes even after putting a cover-slide on sample; lactophenol solution gave a more clear vision, the cellular components were noticed but also un-differentially and no lysis of cellular membrane even after putting a cover-slide on sample was observed; proteinase-K lytic solution determined the clearest vision with semi-complete lysis of cellular membrane and with complete lysis after putting a cover-slide on sample. After using proteinase-K lytic solution, the hooks appeared distributed in two separated rings alternatively between large and small hooks, and each one had three regions (blade, guard and handle). Also, after using proteinase-K lytic solution, it became easier to count the number of hooks (mean= 34, SD= 4) and to distinguish between large hooks (larger, less robust, had more pointed blade) and small hooks.
Clustering behavior of protoscolices and visibility of internal components at each time after time zero are summarized in Table (1).

Discussion

In the present study, treatments of rostellar hooks of *E. granulosus* protoscolices with lactophenol blue and proteinase-K lytic solution were compared for the first time for morphometric analysis. Lactophenol solution or polyvinyl lactophenol have been used in several studies for morphometric characters on rostellar hooks of *E. granulosus* protoscolices, but no information about comparison with lytic solutions are so far available (Hobbs *et al*., 1990; Constantine *et al*., 1993; Almeida *et al*., 2007; Karimi & Dianatpour, 2008; Almeida *et al*., 2009; Yildiz & Gurcan, 2009; Calderini *et al*., 2012; Harandi *et al*., 2012; Soriano *et al*., 2013; Fadakar *et al*., 2015; Mustafa *et al*., 2015).
According to Central Drug House (CDH), the manufacture company, lactophenol either in lactophenol blue or in lactophenol solution is harmful if swallowed or inhaled by humans, provoking severe skin burns and eye damage, and is suspected of causing genetic defects due to germ cell mutagenicity and may cause damages to organs following prolonged or repeated exposure.
Proteinase-K lytic solution is also frequently used in molecular studies for DNA extraction on various living cells and tissues. Also, proteinase-K lytic solution was used as enzymatic digestion technique to obtain and study the sclerotized structures of monogenean parasites as *Ligophorus* and *Solostamenides*, in addition to using it in molecular studies for the identification of these monogenean parasites (Hernández-Orts *et al*., 2010; Blasco-Costa *et al*., 2012; Rodríguez-González *et al*., 2015, Al-Nasiri & Balbuena, 2018). So far, proteinase-K lytic solution was not used previously

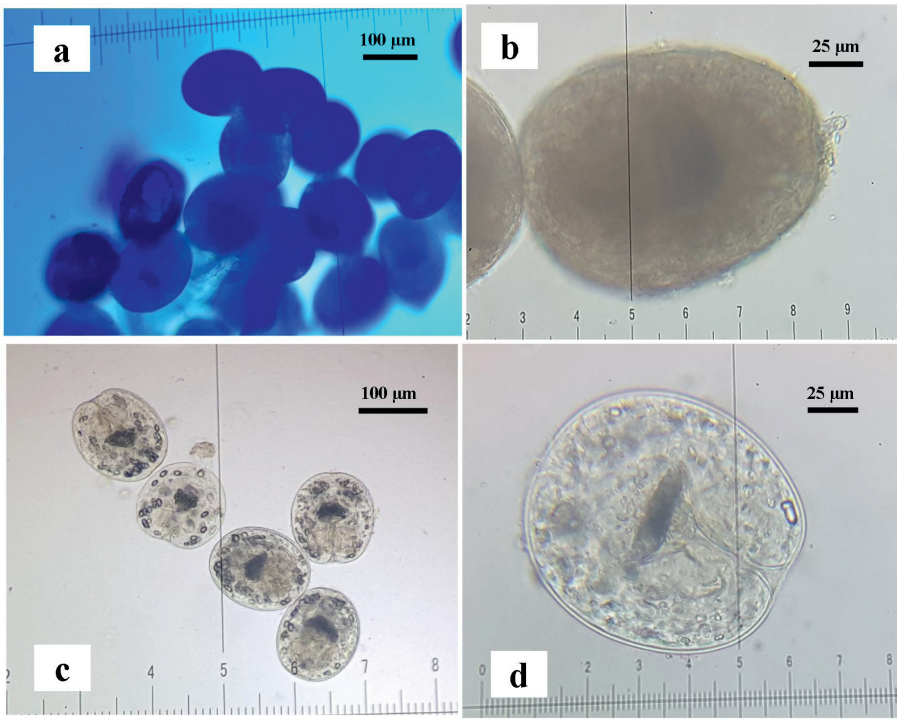

Fig. 2. Microscopic photography of protoscolices after 30 min from zero time of addition of lactophenol blue (a), lactophenol solution (b), proteinase-K lytic solution (c, d).

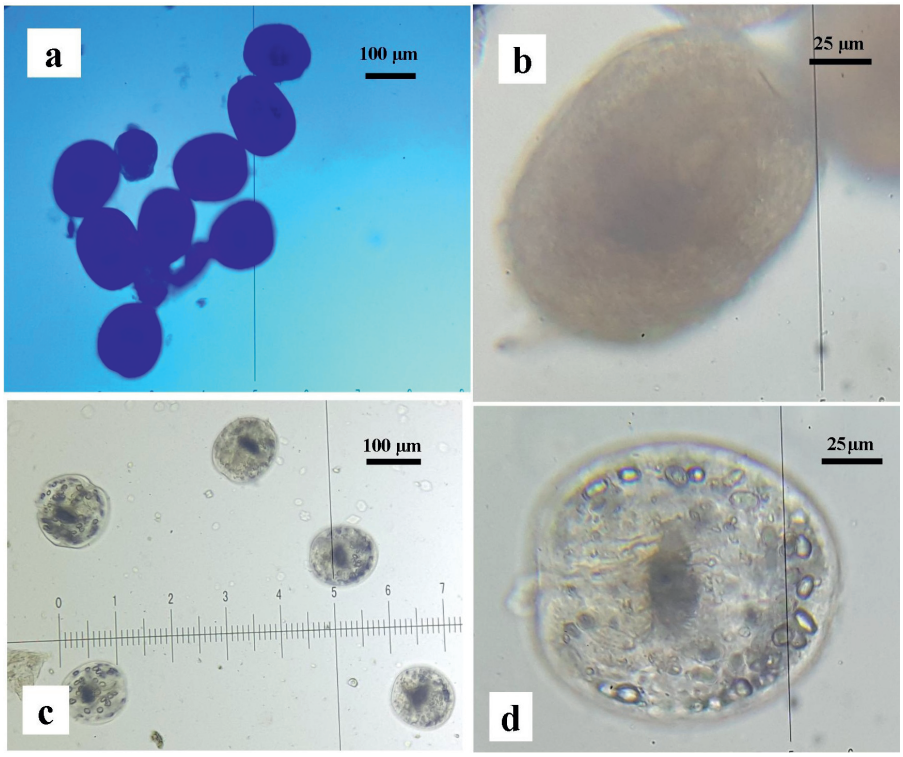

Fig. 3. Microscopic photography of protoscolices after 60 min from zero time of addition of lactophenol blue (a), lactophenol solution (b), proteinase-K lytic solution (c, d).

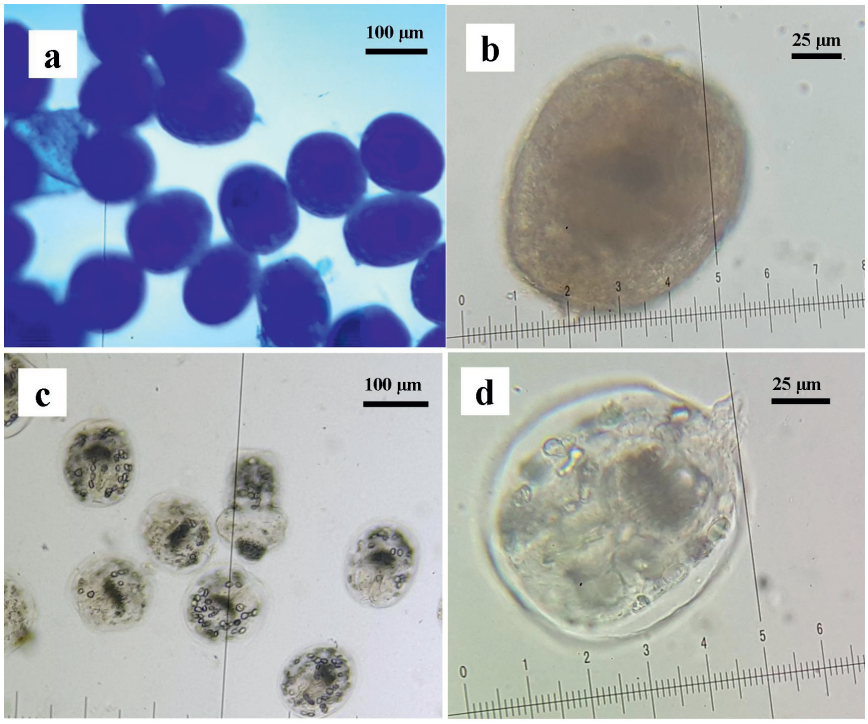

Fig. 4. Microscopic photography of protoscolices after 90 min from zero time of addition of lactophenol blue (a), lactophenol solution (b), proteinase-K lytic solution (c, d).

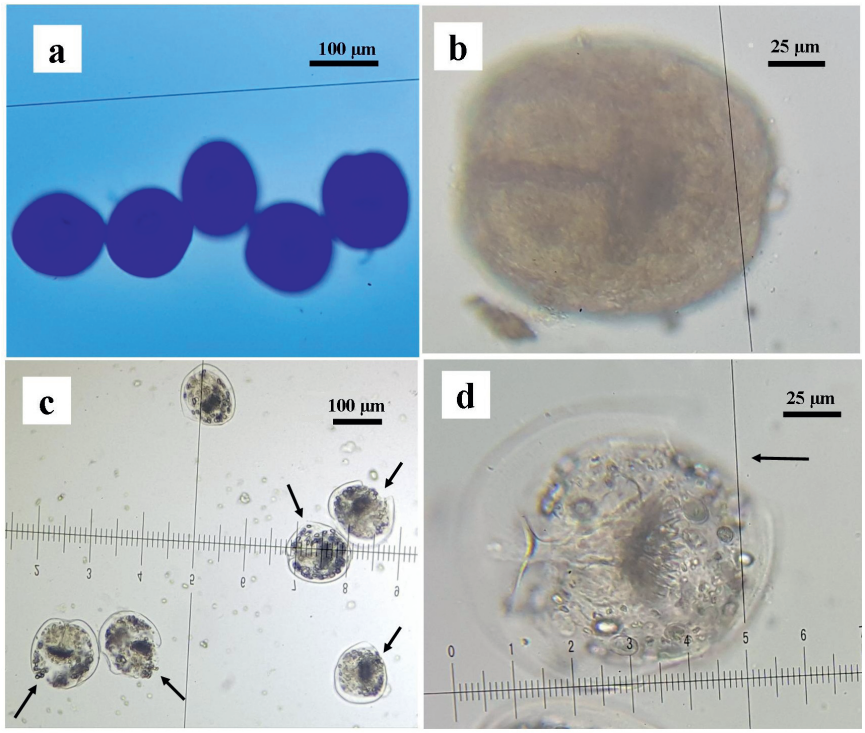

Fig. 5. Microscopic photography of protoscolices after 120 min from zero time of addition of lactophenol blue (a), lactophenol solution (b), proteinase-K lytic solution (c, d); arrows indicated the sites of lysis in cellular membrane.

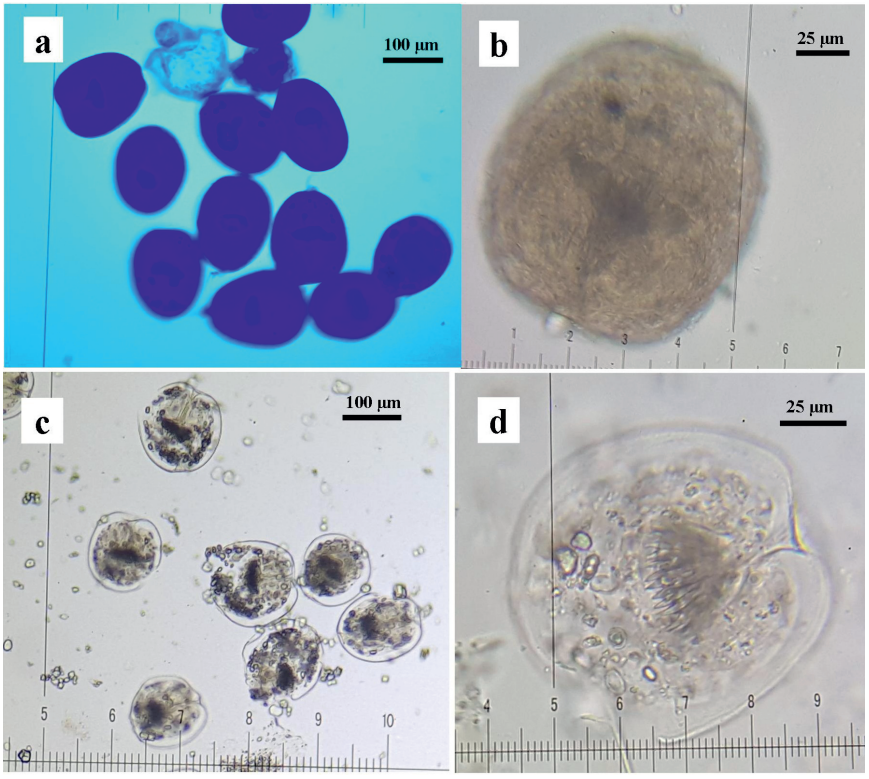

Fig. 6. Microscopic photography of protoscolices after 150 min from zero time of addition of lactophenol blue (a), lactophenol solution (b), proteinase-K lytic solution (c, d).

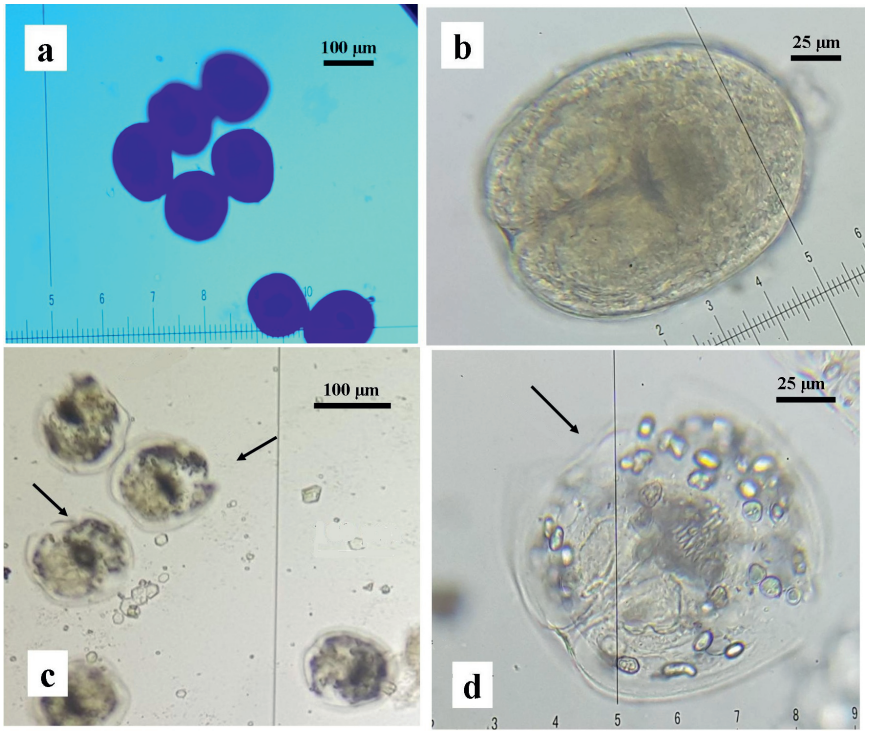

Fig. 7. Microscopic photography of protoscolices after 240 min from zero time of addition of lactophenol blue (a), lactophenol solution (b), proteinase-K lytic solution (c, d), arrows indicated the sites of lysis in cellular membrane.

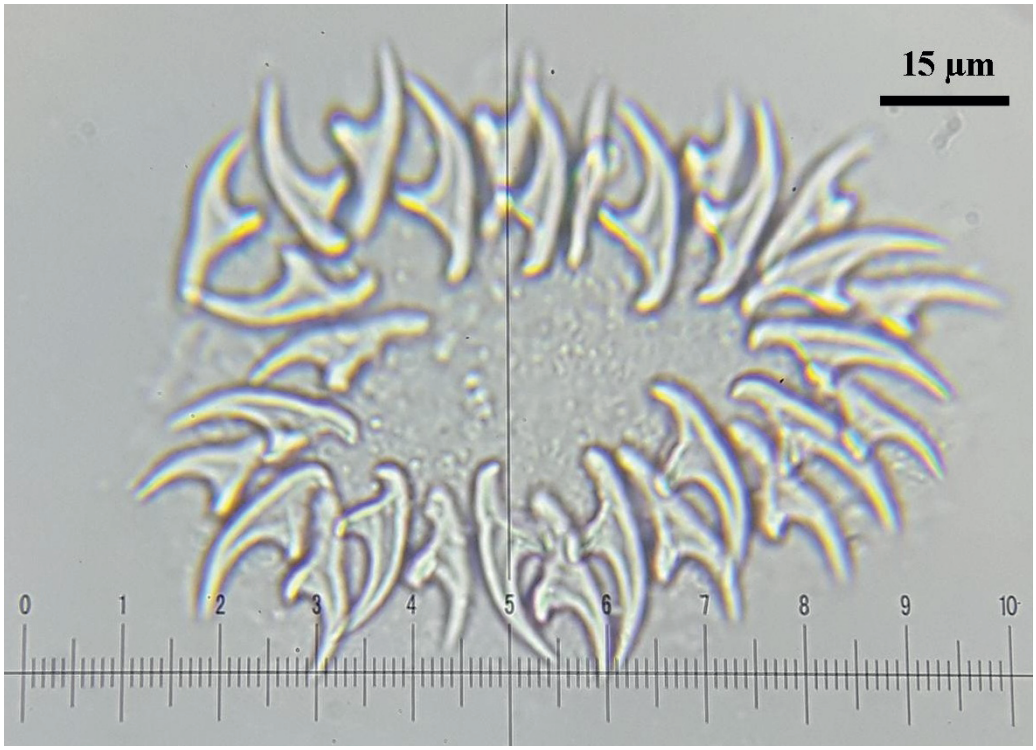

Fig. 8. Microscopic photography of hooks after lysis the membrane of protoscolices after 240 min from zero time of addition of proteinase-K lytic solution to the protoscolices.

for morphometric study on rostellar hooks of *E. granulosus*. Additionally, in the present study, proteinase-K lytic solution made well available to the observer those morphometric features of high taxonomic values, such as large and small hook length and blade length, as described in previous papers (Kumaratilake & Thompson, 1984; Hobbs *et al.,* 1990).

The present study is the first study that evaluates lactophenol blue and proteinase-K lytic solution in digestion of *E. granulosus* protoscolices for morphometric study of rostellar hooks.

Based on the obtained results, the present study recommends the use of proteinase-K lytic solution in morphometric of rostellar hooks, as it provides a clearer vision of hooks if compared with lactophenol-based solutions and may guarantee safer conditions for operators, although a protective equipment must be used in any case.

Conflict of Interest

Authors state no conflict of interest.

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sciendo

DOI 10.2478/helm-2020-0006

HELMINTHOLOGIA, 57, 1: 71 – 77, 2020

Research Note

Phylogenetic position of *Pleurogenoides* species (Plagiorchiida: Pleurogenidae) from the duodenum of Indian skipper frog, *Euphlyctis cyanophlyctis* (Amphibia: Dicroglossidae) inhabiting the Western Ghats, India

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Article info

Received September 10, 2019
Accepted October 30, 2019

Summary

Two species of digenetic trematodes of the genus *Pleurogenoides viz*., *P. cyanophlycti* Shinad & Prasadan (2018a) and *P. euphlycti* Shinad & Prasadan (2018b) have been described from India. Information regarding the molecular data of various species of the genus *Pleurogenoides* Travassos, 1921 is virtually lacking. This study addresses the application of molecular markers to validate the phylogenetic position of *P. cyanophlycti* and *P. euphlycti*. In the present study, two species *P. cyanophlycti* and *P. euphlycti* were collected between January 2016 to October 2017, infecting the freshwater frogs inhabiting the Western Ghats, India. In the present study, the two species were identified morphologically and by PCR amplification of the 28S ribosomal RNA gene. Phylogenetic tree results clearly demonstrate that both *P. cyanophlycti* and *P. euphlycti* belongs to the family Pleurogenidae Looss, 1899. Based on these results, we presented and discussed the phylogenetic relationships of *P. cyanophlycti* and *P. euphlycti* within family Pleurogenidae from India. Phylogenetic analyses showed that *P. cyanophlycti* and *P. euphlycti* cluster according to their vertebrate host and revealed an important congruence between the phylogenetic trees of *Pleurogenoides* and of their vertebrate hosts. *P. cyanophlycti* and *P. euphlycti* clearly constitute a separate, sister branch with other species of the genera, *Pleurogenoides, Pleurogenes* (=*Candidotrema*), *Prosotocus* and *Brandesia*. The present study firstly provides important information about the molecular study and phylogenetic analysis of *P. cyanophlycti* and *P. euphlycti*. This study will also serve as a baseline for *Pleurogenoides* species identification for further studies.
Keywords: *Pleurogenoides*; *P. cyanophlycti*; *P. euphlycti*; 28S; frogs; India

Introduction

Studies on the metazoan parasites of amphibians in the Indian subcontinent are fragmentary. Darrel (2013) suggested that like other vertebrates a considerable range of metazoan parasites harbors frogs as preferred vertebrate hosts. The genus *Pleurogenoides* was proposed by Travassos, 1921 to accommodate type-species *P. tener* that was described by Looss, 1898 as *Prosotocus tener*. The genus comprises species described from frogs worldwide. Brinesh & Janardanan (2014) documented nine species reported from Indian frogs. Recently, three new species of *Pleurogenoides* were described including *P. cyanophlycti* Shinad & Prasadan (2018a), *P. euphlycti* Shinad & Prasadan (2018b) and *P. wayanadensis* Shinad & Prasadan (2018b) from *Euphlyctis cyanophlyctis* of the Wayanad region of the Western Ghats.

During a parasitological survey of trematode parasites of frogs from

* – corresponding author

the Wayanad region of the Western Ghats, India, *Pleurogenoides cyanophlycti* and *P. euphlycti* were recovered from the gastrointestinal tracts of *Euphlyctis cyanophlyctis* Schneider, 1799. Both the species of *Pleurogenoides* collected in this study were characterized on the basis of their morphology and molecular characteristics. The systematic position of *Pleurogenoides* species within the family Pleurogenidae has also been worked out. The main aim of this study was the molecular identification of *Pleurogenoide*s species collected from Indian frog and a comparison of the resulting data with that of the available species in the GenBank database.

Material and Methods

The hosts were collected from the Wayanad region of the Western Ghats, which stands second only to the Eastern Himalayas as a treasure trove of biological diversity in India. The map of the study area (Fig. 1) was prepared using QGIS2.16.1 software. Information on the collections of specimens of *Pleurogenoides cyanophlycti* and *P. euphlycti* is presented in Table 1.

Specimens of *Euphlyctis cyanophlyctis* collected during the period from January 2016 to October 2017 from various water bodies using sweep hand net were brought to the Laboratory, maintained in cement cisterns and fed occasionally with insects. The hosts were narcotized with chloroform, dissected and their body parts were examined for digenetic trematodes under a stereozoom dissecting microscope (Labomed Luxeo 4Z, USA). Internal organs were also dissected out from each frog, placed in separate Petri dishes containing 0.75 % saline, macerated and examined under the stereo zoom microscope. Adults trematodes were carefully removed from the duodenum, transferred to 0.75 % saline in separate watch glasses and studied under Nikon ECLIPSE Ni-U phase contrast research microscope (Nikon Ni-SS 935179, Japan) without supra vital staining or after staining with neutral red. Permanent slides of adult parasites were prepared after fixing them in 5 % formalin under slight cover glass pressure and staining with acetocarmine, following the procedure outlined by Cantwell (1981). Prevalence of infection was measured following Bush *et al*., (1997). Prevalence is the number (%) of hosts infected with one or more individuals of

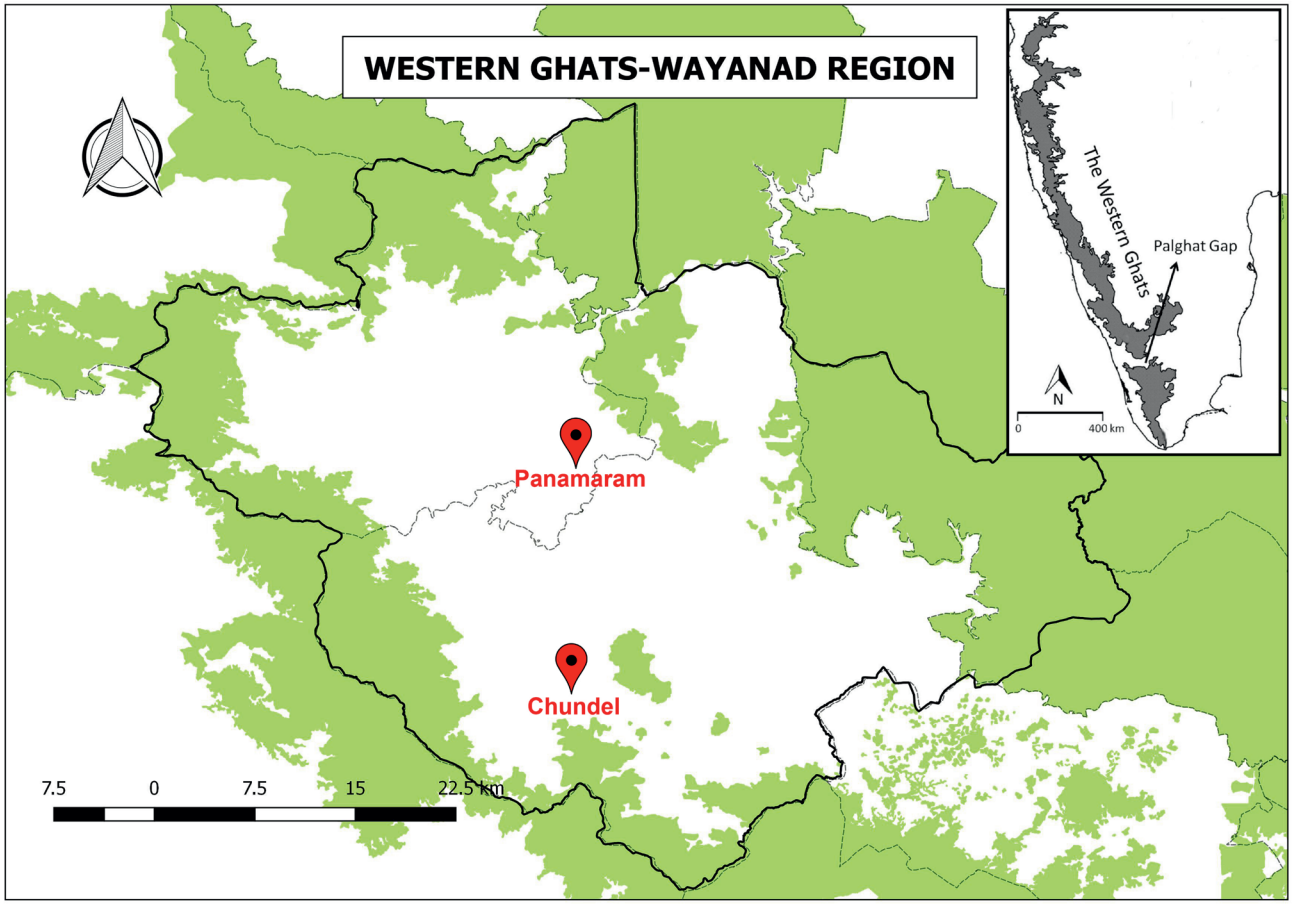

Fig. 1. Study area – Western Ghats – Wayanad region, Localities – Chundel (11°34'26.2"N 76°03'32.5"E) and Panamaram (11°45'03.6"N 76°04'01.3"E).

Table 1. *Pleurogenoides* species collected from January 2016 to October 2017 from duodenum of host from Wayanad District, India.

Species	*P. cyanophlycti*	*P. euphlycti*
Sample no.	PC1 and PC2	PE1 and PE2
Host	*Euphlyctis cyanophlyctis*	*Euphlyctis cyanophlyctis*
Infected organ	Duodenum	Duodenum
Location	Chundel	Panamaram
Coordinates	11°34'26.2"N 76°03'32.5"E	11°45'03.6"N 76°04'01.3"E
Prevalence (Parasite no. per host) (%)	16 out of 100 frogs (16%)	10 out of 100 frogs (10%)
Voucher no.	Z-P/H-F 101*	Z-P/H-F 109*
GenBank accession nos.	MN218390 and MN218388	MN218391 and MN218389

*Voucher specimens deposited in the Helminth parasite collections, Ecological Parasitology and Tropical Biodiversity Laboratory, Department of Zoology, Kannur University, Mananthavady Campus, Wayanad-670645, Kerala, India.

a particular parasite species (or taxonomic group) divided by the number of hosts examined for that parasite species. Information about the collections of specimens of *Pleurogenoides* species is presented in Table 1.

DNA from *Pleurogenoide*s species was extracted from two different individuals of both species using the DNeasy™ Tissue Kit (Qiagen, Germany), according to the manufacturer's instructions. 28S gene region of the rDNA was amplified using primers, Ancy55F (5'-GAGATTAGCCCATCACCGAAG-3') (Plaisance *et al*., 2005); LSU1200R (5'-GCATAGTTCACCATCTTTCGG-3') (Littlewood *et al*., 2000) and L300F (5'-CAAGTACCGTGAGGGAAAGTTG-3'); ECD2 (5'-CCTTGGTCCGTGTTTCAAGACGGG-3') (Littlewood *et al*., 2000) respectively. Polymerase chain reaction (PCR) was carried out in a total volume of 25 µl consisting of 2.5 µL 10× PCR buffer, 4 µL 1 mM deoxyribonucleotide triphosphates (dNTPs) mix, 0.8 µL of each primer, 1 U Taq polymerase (1U; Biotools) and 4 µl genomic DNA. The cycling conditions were as follows: one cycle of initial denaturation at 94 °C for 3 min; 35 cycles at 94 °C for 40 s, 55 °C for 1 min, and 72 °C for 1 min; with a final extension at 72 °C for 7 min. Negative sample with no DNA was used in per amplification run to exclude contamination. Amplified PCR products were analyzed by electrophoresis in agarose gel stained with ethidium bromide, purified using the Purelink™ Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen) and sequenced with the above primers using Big Dye Terminator vr. 3.1 cycle sequencing kit in ABI 3130 Genetic Analyzer, Applied Biosystems.

Obtained contigs were assembled and compared for similarity by searching the GenBank database using the BLAST search (www.ncbi.nlm.nih.gov/blast). Sequences obtained for 28S were retrieved and aligned with sequences from other related species downloaded from GenBank using ClustalW with default parameters implemented in MEGA version 7.0 (Kumar *et al*., 2016). For 28S gene, GTR + G + I were estimated as the best-fitting nucleotide substitution model using the Akaike Information Criterion (AIC). For phylogenetic analyses, maximum likelihood (ML) and Bayesian inference (BI) analyses were performed using MEGA v. 7.0 (Kumar *et al*., 2016) and TOPALI 2.5 (Milne *et al*., 2009) respectively. BI analysis was run for 1,000,000 generations, sampling every 100th tree and discarding as 'burn in' the first 25 % of the sampled trees. For the ML analysis, the bootstrap values based on 1,000 resampled datasets were generated. The genetic divergence among taxa was estimated using uncorrected 'p' distances in MEGA version 7.0. In addition, the sequence of *Fasciola hepatica* (AY222244) was used as the out group. The obtained sequences of the 28S gene for both species were submitted to GenBank for accession numbers (Table 1).

Ethical Approval and/or Informed Consent

The research related to the experiments and handling of frog in the present study has been conducted with all the relevant national regulations and institutional policies for the care and use of animals.

Results and Discussion

During the study, *Pleurogenoides* Travassos, 1921 species were found from the duodenum of freshwater frog *E. cyanophlyctis*. On the basis of morphological characteristics, the two species were identified as *P. cyanophlycti* Shinad & Prasadan (2018a) and *P. euphlycti* Shinad & Prasadan (2018a) respectively.

We have generated partial 28S sequences of ribosomal RNA for the two species of *Pleurogenoides* recovered from the Indian skipper freshwater frog of the Western Ghats, India. These sequences were analyzed together with other sequences of order Plagiorchiida under which the present studied species fall. ML and BI analyses produced similar topological tree, with somewhat different support values at some nodes, therefore, only the ML tree was presented here. The resulting tree branch topologies from both, ML and BI analyses, were in consensus and representing species genetic lineages (Fig. 2). Moreover, the newly generated sequences of *Pleurogenoides* isolate clustered within family Pleurogenidae with well-supported clade A that representing parasite infecting frogs (Fig. 2). Both the two *Pleurogenoides* species (*P. cyan-*

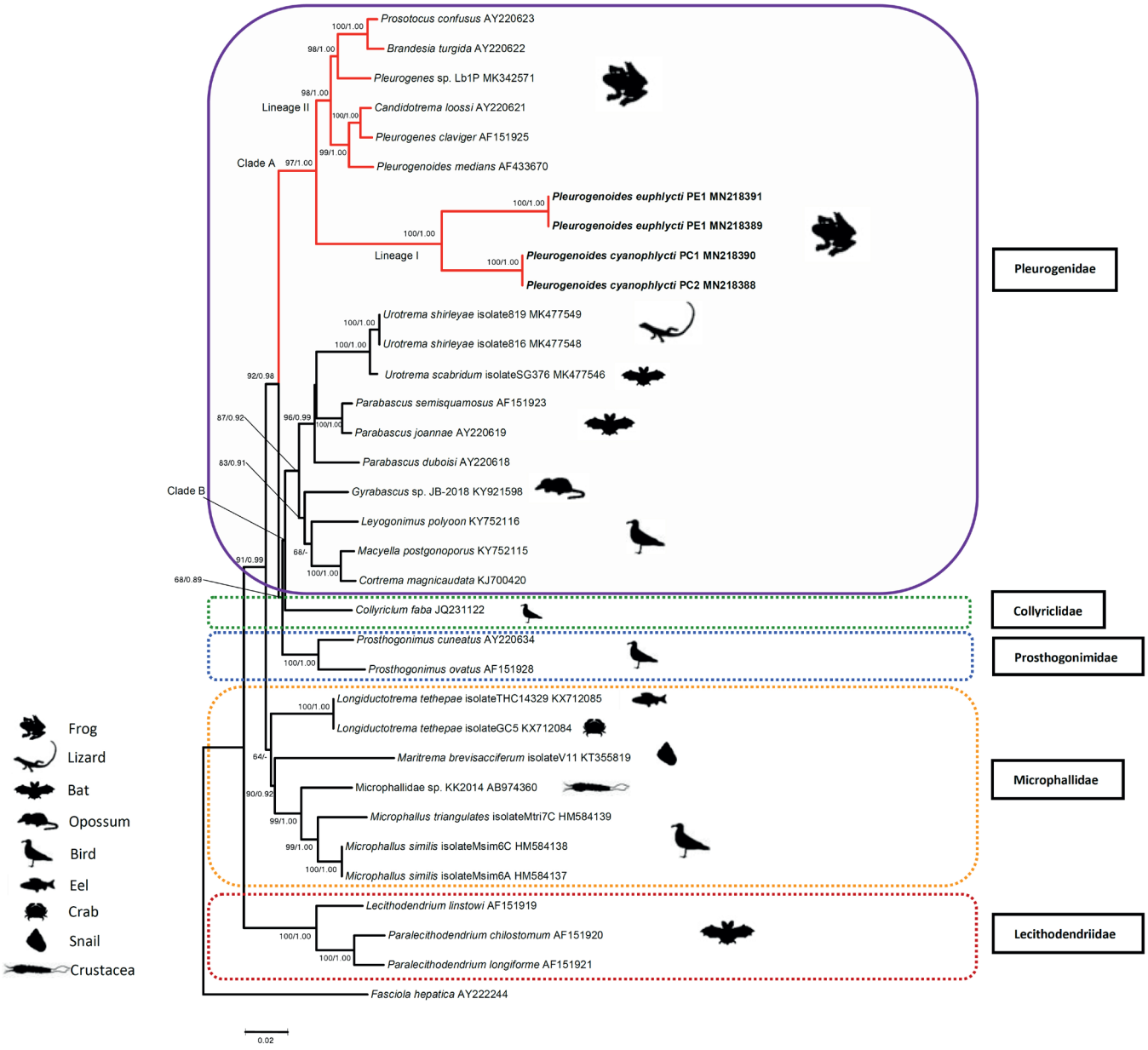

Fig. 2. Phylogenetic tree based on partial sequences of 28S rDNA gene. Nodal support from maximum likelihood (ML) and Bayesian Inference (BI) analyses is indicated as ML/BI. Hyphen indicates node unsupported by BI. GenBank accession numbers are provided alongside the species names. The scale-bar indicates the expected number of substitutions per site.

Table 2. Genetic distances (below diagonal) and sequence similarities (above diagonal) of Pleurogenidae species infecting frogs. Taxa are listed as they appear in Fig. 2.

	1	2	3	4	5	6	7	8	9	10
1. *Pleurogenoides cyanophlicti* PC1 MN218390		100	75.0	75.3	69.6	69.2	69.5	67.6	69.8	70.4
2. *Pleurogenoides cyanophlicti* PC2 MN218388	0.00		75.0	75.3	69.6	69.2	69.5	67.9	69.8	70.4
3. *Pleurogenoides euphlicti* PE1 MN218391	0.23	0.23		100	78.4	78.2	78.4	77.9	79.4	79.7
4. *Pleurogenoides euphlicti* PE1 MN218389	0.23	0.23	0.00		78.7	78.5	78.7	78.1	79.4	80.0
5. *Pleurogenoides medians* AF433670	0.28	0.28	0.21	0.21		97.8	97.8	95.8	95.4	96.0
6. *Pleurogenes claviger* AF151925	0.28	0.28	0.21	0.21	0.02		99.0	95.9	94.9	95.5
7. *Candidotrema loossi* AY220621	0.28	0.28	0.21	0.21	0.02	0.01		95.8	95.0	95.4
8. *Pleurogenes* sp. Lb1P K342571	0.28	0.28	0.21	0.21	0.04	0.04	0.04		95.7	96.6
9. *Brandesia turgida* AY220622	0.28	0.28	0.21	0.21	0.04	0.05	0.05	0.04		98.7
10. *Prosotocus confusus* AY220623	0.29	0.29	0.21	0.21	0.04	0.04	0.04	0.03	0.01	

ophlycti and *P. euphlycti*) were resolved as the 'Lineage II' representing them with well supported bootstrap values and formed a sister relationship with other species of Pleurogenidae belongs to different genera in 'Lineage II': *Pleurogenes* Looss, 1986 (Synm. *Candidotrema* Dollfus, 1951); *Prostocus* Looss 1899; *Brandesia* Stossich, 1899 and all parasites of amphibians (Fig. 2). Table 2 represents the pairwise distance (uncorrected p-distance range) and identity values of both *Pleurogenoides* species with closely related species. The intraspecific divergence observed within the 28S sequences of isolates of *P. cyanophlycti* and *P. euphlycti* shows no differences while interspecific divergence between *P. cyanophlycti* and *P. euphlycti*, was found 0.23 %, and with other species of 'Lineage II' ranged between 0.21 – 0.29 % (Table 2).

Travassos, 1921 erected the genus *Pleurogenoides* with type-species *P. tener* that was earlier described by Looss, 1898 as *Prosotocus tener*. This genus comprises more than 26 species worldwide that infect frogs (Brinesh & Janardanan, 2014). In India, about 12 species of genus *Pleurogenoides* have been reported from frogs to date *viz*., *P. gastroporus* (Luhe, 1901) Travassos, 1921; *P. sphaericus* Klein, 1905; *P. sitapurii* Srivastava, 1934; *P. orientalis* Srivastava, 1934; *P. bufonis* Kaw, 1943; *P. ovatus* Rao, 1977; *P. jamshedpurensis* Hasnain & Sahay, 1987; *P. ranchiensis* Dan & Hasnain, 1991; *P. malampuzhensis* Brinesh & Janardanan, 2014; *P. cyanophlycti* Shinad & Prasadan, 2018a; *P. euphlycti* Shinad & Prasadan, 2018b & *P. wayanadensis* Shinad and Prasadan, 2018b. Description and characterization of all the above species were made on the basis of morphological characteristics; however, additional molecular work is needed to support their taxonomic validity that is also important in the case where several species of the same genus are reported from the same host.

A nuclear gene (28S) was used as molecular marker for *Pleurogenoides cyanophlycti* and *P. euphlycti* for the present study. The only member of Pleurogenoides with a DNA sequence available is *P. medians* (AF433670) from *Rana lessonae* which demonstrates the scarcity of molecular data of this genus. The results of the present study demonstrate the need for DNA sequence data of other congeneric species distributed worldwide to understand the evolution and taxonomy of this group of parasites. Remarkably, *Pleurogenoides* Travassos, 1921; *Pleurogenes* Looss, 1896 (Synm. *Candidotrema* Dollfus, 1951); *Prostocus* Looss, 1899 and *Brandesia* Stossich, 1899 are morphologically differentiated with each other by the variability of the position of the genital atrium (Sharpilo & Iskova, 1989; Lotz & Font, 2008). In *Pleurogenoides* it is antero-lateral and situated distantly from the ventral sucker; in *Pleurogenes* (=*Candidotrema*) it is antero-lateral in position and located close to the oral sucker; in *Brandesia* it is placed in a lateral position and situated close to the posterior part of the body; while in *Prostocus* it is positioned laterally and distantly placed from oral sucker. Tkach *et al*., 2003 in a study on molecular phylogeny and morphological data of the Microphalloidea Ward, 1901 synonymized *Candidotrema* Dollfus, 1951 with *Pleurogenes* Looss, 1896.

Our study has revealed the phylogenetic similarity of *P. cyanophlycti* and *P. euphlycti* and allocated their correct systematic position that they belongs to the family Pleurogenidae within the superfamily Microphalloidea, that corresponds to the trees generated in other studies (Kanarek *et al*., 2014, 2015, 2017; Bella *et al*., 2018; Tkach *et al*., 2019). Though, for molecular study of *Pleurogenoides* we have only one representative species, *P. medians* and more data are required for a more congruent phylogeny. In contrast, *P. medians* seem to occur in 'Lineage II' that results a key question is the genus *Pleurogenoides* is not monophyletic, but it is very early to predict anything regarding paraphyly without addition and analyzing of other congeneric species data of this genus should be revised. Although, molecular sequences of the various genera included in the Pleurogenidae in future studies will clarify their phylogenetic affinities and systematic positions.

In the phylogenetic tree, *Pleurogenoides cyanophlycti* and *P. euphlycti* were nested in a 100 % supported clade formed for Indian species and suggested that the status of *Pleurogenoides* species needs to be re-evaluated in light of more molecular sequences and included in future phylogenetic analyses.

Acknowledgements

We would like to acknowledge the Head, Department of Zoology, Chaudhary Charan Singh University, Meerut, for providing laboratory facilities. The authors are grateful to the Kerala State Council for Science, Technology and Environment (KSCSTE), Government of Kerala for providing financial assistance in the form of a major research project (SRS/220/2015/ KSCSTE) to carry out this study. The permission accorded by the Department of Forest and Wildlife, Government of Kerala (Order No WL10-63909/2016) for collecting frogs from the Wayanad forest region is also gratefully acknowledged.

Conflict of interest

The authors declare that they have no conflict of interest.

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sciendo

DOI 10.2478/helm-2020-0003

HELMINTHOLOGIA, 57, 1: 78 – 82, 2020

Research Note

Evaluation of a marketed polyherbal dewormer against intestinal strongyles in naturally infected donkeys

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Article info

Received July 15, 2019
Accepted September 23, 2019

Summary

The study evaluated the effectiveness of a commercially available polyherbal dewormer to control intestinal strongyles in naturally infected donkeys. The animals were allotted to two groups: treated with the herbal dewormer (n=8) according to manufacturer recommendations and untreated control group (n=6). Fecal samples were taken from each animal on days 0 (day of treatment), 14, 21 (day of second additional treatment), 35, and 42. Faecal egg count reduction tests showed very negligible or no reduction in number of strongyle eggs for donkeys in the phytotherapeutic treatment group compared to those in the control group. Thus, the herbal dewormer was mostly ineffective in reducing fecal egg counts in donkeys infected with intestinal strongyles. These findings can make equine practitioners aware of possible disadvantages of herbal dewormers.
Keywords: Donkeys; intestinal strongyles; herbal dewormer; phytotherapy

Introduction

Donkeys play a key role as working animals in developing countries, and they are also used for meat and milk productions, social activities, tourism and leisure (Camillo *et al*., 2018). Consequently, the interest in the welfare and diseases of this species is constantly increasing (Bonelli *et al*., 2016; Sgorbini *et al*., 2017, 2018), including parasitic diseases and strategies for their control.

The high occurrence of large and small strongyles in donkeys has been widely reported (Matthee *et al*., 2000; Getachew *et al*., 2010; Matthews *et al*., 2013; Ismail *et al*., 2016; Jajere *et al*., 2016; Dibaba *et al*., 2017). Intestinal strongyles may negatively affect body conditions, live weight and haematological parameters of donkeys (Matthee *et al*., 2002; Yoseph *et al*., 2005) and their control is typically performed by the administration of anti-helminthic drugs licensed for use in horses (Matthee *et al*., 2002). However, the development of drug-resistant populations of small strongyles have been reported in donkeys (Matthee *et al*., 2002; Lawson *et al*., 2015). This factor and the growing consumer trend to ask for food products free of chemical residues, including milk from donkeys raised according to organic farming methods (Camillo *et al*., 2018), prompt researchers, practising veterinarians, and organic donkey farmers to search for sustainable alternatives to control gastrointestinal parasites in this host species. Among the sustainable alternatives, there are various medicinal plants that are reported to have anthelmintic properties in donkeys (Scantlebury *et al*., 2013). However, to the best of the authors' knowledge, the effectiveness of herbal formulations marketed for parasite control in donkeys under field conditions has not been yet investigated so far. Hence, the aim of the present study was to determine the effectiveness of a commercially available polyherbal dewormer, marketed for use in equines, to reduce the faecal output of strongyle eggs in donkeys.

* – corresponding author

Materials and Methods

Study animals, sample collection, and laboratory procedures

The study was carried out at the facilities of the veterinary teaching hospital "Mario Modenato" (University of Pisa, Italy) between January and February 2019. The study period was chosen for practical reasons. Indeed, female donkeys selected for the study were going to start the reproductive season in the following months (late winter/early spring). So, we wanted to avoid using the animals for the study purposes during the heat period, mating, and pregnancy. Fourteen asymptomatic Amiata donkeys (*Equus asinus*) of both sexes (10 unpregnant females and 4 intact males) were enrolled in the study irrespective of the parasite burden. They were aged 7 – 13 years and none of them had received any anthelmintic treatment for at least 12 months before the beginning of the study. The donkeys were naturally infected by intestinal strongyles. Infections were confirmed prior to the beginning of the study by the Mini-FLOTAC technique in combination with Fill-FLOTAC. A commercial sodium nitrate solution with specific gravity of 1.200 (Coprosol®, Candioli Farmaceutici S.p.A., Beinasco (TO), Italy) was used as flotation solution. The Mini-FLOTAC technique in combination with Fill-FLOTAC has a reported analytic sensitivity of five eggs per gram (EPG) of faeces and it was performed according to the detailed instructions provided at the web site of the supplier (https://www.parassitologia.unina.it/wp-content/uploads/2015/07/001-Erbivori-fresh_Layout-1-1_cut.pdf). This tool was chosen for copromicroscopic diagnosis and EPG counts because of the excellent performance previously shown in various studies to assess gastrointestinal helminth infections and anthelmintic drug efficacy in veterinary medicine (Rinaldi *et al*., 2014; Bellaw *et al*., 2018).

Study design

Each of the 14 donkeys was allotted to one of two groups: treated group (n=8) and untreated control group (n=6). Because there is no indication in previous studies that the response to anthelmintic treatment is different between sexes in donkeys, animals were allotted as follows. Females were randomly assigned to either treated or control group and males were assigned to control group. Thus, the treated group contained 8 females whilst the control group contained 2 females and 4 males. After allotments, the two groups were kept in separate paddocks as follows. Eight unpregnant females in the treatment group were kept all together in the same paddock. Due to management reasons, the 6 donkeys in the control group were separated by sex into five paddocks, that is 2 unpregnant females were kept together in the same paddock whilst the 4 intact males were kept separately in individual paddocks. All the donkeys allotted to each group were fed with hay from mixed-grass meadows and water ad libitum during the entire study period. On day 0, faecal samples were individually collected from the rectal ampulla of each donkey into plastic containers. Samples were appropriately labelled and brought to the laboratory where individual EPG counts were performed by using the Mini-FLOTAC technique in combination with Fill-FLOTAC, as above reported. The same day the bodyweight of each donkey in the treated group (n=8) was estimated by a weight tape, ranging from 280 to 393 kg. Thereafter, these animals were treated by oral administration of a marketed polyherbal dewormer at a dose rate of 8.3 gr per 100 kg bodyweight. The product is commercialized in the form of paste and is licensed for use in equines in Italy. It contains extracts of *Mallotus philippinensis*, *Carduus marianus*, *Urtica urens*, *Genziana lutea*, and *Eucalyptus globulus*. It is considered atoxic, can be used in pregnant and young animals, and has no withdrawal time. According to the dose suggested in the drug package, a 50 gr syringe is useful for treatment of horses of weight up to 600 kg. Thus, the dose used for treatment of donkeys in the present study was extrapolated from the dose recommended for horses by the manufacturer. During the oral administration of the herbal dewormer, all donkeys in the treated group showed that they enjoyed the taste very much. The remaining 6 animals, assigned to the control group, were kept untreated. Subsequently, faecal sample collections and EPG counts were individually performed as above reported in both groups on days 14, 21, 35, and 42. Furthermore, on day 21 all the donkeys in the treated group were administered again an additional administration of a 50 gr syringe of the paste used for the initial treatment, as adjunct treatment irrespective of bodyweight. After treatment, they were observed for possible adverse reactions for about 1 hour.

Data analysis

The faecal egg count reduction (FECR) test was used to determine the anthelmintic efficacy of the tested polyherbal dewormer (Kaplan & Nielsen, 2010). Arithmetic means (AMs) of the EPG counts were calculated to determine the mean percentage reductions on day 14 and on day 35, according to the following formula:

$$\text{FECR } \% = \frac{\text{Pre-treatment EPG - Post-treatment EPG}}{\text{Pre-treatment EPG}} \times 100$$

Egg shedding reduction can vary based on the drug tested and the number of horses investigated (Kaplan and Nielsen, 2010) but there are currently no available indications for phytotherapeutic products in literature. Thus results were interpreted following the classification of Kaplan and Nielsen (2010) and we arbitrarily assumed that the cut-off value for appropriate efficacy of the tested polyherbal dewormer against intestinal strongyles in donkeys was FECR at least ≥90 %.

AMs ± standard error of the mean (SEM) of the EPG counts were determined on each sampling day after treatment. AMs of the EPG counts were compared between treated and untreated groups by Student's t test. *P* values ≤0.05 and ≤0.01 were considered statistically significant or highly significant, respectively.

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 and Discussion

On day 0, EPG counts ranged from 180 to 700 and from 100 to 770 in faeces of treated and untreated donkeys, respectively. No side effects were observed after treatment. FECR test showed that the polyherbal dewormer produced a very negligible reduction (5 %) of strongyle eggs on day 0 onwards and no reduction or lower reduction (2 %) on day 21 till the end of the study, even though donkeys were treated again with a much higher dose, as adjunct treatment, on day 21. The values of AM±SEM of the EPG counts on day 0 to day 42 in treated and untreated donkeys are presented in Table 1. Unexpectedly, the AMs of the EPG counts were higher in the treated group both on day 14 and on day 21, reaching highly significant differences (P=0.0033 and P=0.0029, respectively). The values of the AMs in the treated donkeys became lower in comparison with their untreated counterpart on day 35 and on day 42 but differences did not reach statistically significant values (P=0.6530 and P=0.1796, respectively).

Results of the FECR test in the present study were compared to cut-off limits proposed by Kaplan and Nielsen (2010) as general guidelines to assess the occurrence of anthelmintic resistance after treatment with drugs currently used in horses, when a recommended range of at least 5 – 10 horses on each farm is included. Reductions >90 % for benzimidazoles and pyrantel or >95 % for ivermectin and moxidectin suggest that the strongyle population is susceptible to the drug tested. Lack of effectiveness is suspected when reductions <80 % for benzimidazoles and pyrantel or <90 % for macrocyclic lactones are found. In this study, only a very negligible reduction (5 %) was seen on day 14. In general, the anthelmintic activity of plants has been reported to be lower than that found for synthetic anthelmintic drugs (Macedo *et al*., 2010). In addition, Iqbal *et al*. (2004) reported that an increase in EPG reduction was noted with an increase in the dose of *Artemisia brevifolia* administered as crude powder, crude aqueous extract, and crude methanol extract against mixed infection of gastrointestinal nematodes in sheep. Similarly, Zajac and Gipson (2000) reported that a single treatment with fenbendazole was able to reduce EPG counts by only 50 % but 2 doses administered in a 12 h interval increased efficacy to 92 % in a goat herd. Thus, it is possible that the dose rate (8 gr of paste/100 kg bodyweight) recommended by the manufacturer for the polyherbal dewormer tested in our study may not be high enough to produce anthelmintic effects in donkeys. For this reason, a second treatment with a much higher dose of the product (50 gr of paste irrespective of the bodyweight) was additionally administered as adjunct treatment on day 21. Despite of this, no egg reduction could be demonstrated on day 35. Moreover, no important differences in individual FECR tests were observed among donkeys treated with the herbal dewormer. Finally, the occurrence of anthelmintic resistance can be excluded since the phytotherapeutic product tested had never been used before in the donkey population of the study. Therefore, our results show that the polyherbal dewormer administered in the present study failed to produce any suitable anthelmintic efficacy against intestinal strongyle infections in donkeys after both the first and mostly the second administration.

Our findings are in agreement with those of other authors. Lugin-

Table 1. Individual counts and arithmetic means ± standard error of the mean (AM±SEM) of eggs per gram (EPG) of faeces from day 0 to day 42 in donkeys treated with a commercial polyherbal dewormer on day 0 and on day 21 (I to VIII), and in donkeys of the control group kept untreated (IX to XIV).

Groups	Donkeys	EPG counts				
		Day 0	Day 14	Day 21	Day 35	Day 42
Treated	I	380	610	325	1000	230
	II	180	120	230	315	180
	III	240	440	570	305	495
	IV	580	480	480	745	160
	V	700	325	230	490	395
	VI	630	600	555	865	440
	VII	200	190	175	275	140
	VIII	370	350	325	510	260
	AM ± SEM	410 ± 71.9	389.4 ± 62.9[a]	361.2 ± 54.6[b]	563.1 ± 97.7	287.5 ± 48.4
Control	IX	770	215	185	285	215
	X	410	115	100	1115	400
	XI	280	170	150	835	585
	XII	110	45	225	1005	640
	XIII	100	60	150	195	285
	XIV	200	15	95	435	305
	AM ± SEM	311.6 ± 103	103.3 ± 31.7[a]	150.8 ± 20.3[b]	645 ± 159.5	405 ± 70.3

[a, b]highly significant differences

buhl *et al*. (2006) reported the lack of anthelminthic effects of a commercially available herbal dewormer to reduce EPG counts in meat goats. Burke *et al*. (2009a) showed that another marketed herbal dewormer, involving the use of two different formulations, was ineffective to control gastrointestinal nematodes in dairy goats. The two herbal mixtures contained *Artemisia absinthium*, *Allium sativum*, *Foeniculum vulgare*, *Juglans nigra*, and *Stevia rebaudiana* as well as *Cucurbita pepo*, *Hyssopus officinalis*, and *Thymus vulgaris*. Similarly, a commercially available certified organic garlic product, fresh garlic juice, and garlic bulbs or papaya seeds failed to control gastrointestinal strongyles in goats and lambs (Burke *et al*., 2009b). In a field trial to assess the usefulness of a mineral lick containing herbal extracts with anti-parasitic properties for the control of gastrointestinal helminths in grazing sheep, there were no significant differences in the prevalence and intensity of helminth infections between the treated and control groups (Nosal *et al*., 2016). The commercially available product consisted of the extracts of *A. absinthium*, *Artemisia cina*, *Tanacetum vulgare*, *T. vulgaris*, *A. sativum*, *Alsidium helminthochorton*, *Dryopteris* filix-mas, *Daucus carota*, *Chenopodium*, and *Punica granatum*. However, contrary to the aforementioned studies, other in vivo investigations have reported the efficacy of some medicinal plants against gastrointestinal strongyles. Iqbal *et al*. (2004), Masamha *et al*. (2010), Jabbar *et al*. (2007), and Tariq *et al*. (2009) reported the anthelmintic activity of *A. brevifolia*, *A. sativum*, *Chenopodium album* and *Caesalpinia crista*, or *A. absintium* in sheep, respectively.

To conclude, the phytotherapeutic potential and pattern of anthelmintic effectiveness of plant extracts vary widely from study to study. Consequently, though phytotherapy has become more and more popular with both donkey breeders and veterinarians, phytotherapic compounds marketed as herbal anthelmintics need evidence-based validation. For this reason, our trial tested the effectiveness of an herbal dewormer against intestinal strongyles in naturally infected donkeys. Based on the FECR test and current criteria used to evaluate the efficacy of anthelmintic drugs, the marketed polyherbal product tested in this study cannot be recommended as an effective alternative or complementary treatment for the control of intestinal strongylosis in donkeys. The findings of our study can help make practitioners aware of the possible disadvantages of natural herbal medicines for the treatment of parasitoses. In our opinion, monitoring FECR test results after treatment with herbal medicines is strongly recommended to provide the equine practitioner with the information necessary to administer extracts of anthelmintic plants consciously. Further in vivo trials focusing on suitable plants with promising potential anthelmintic properties are advisable for the development and the release of commercially available alternative and complementary anthelmintics to be used in donkeys.

Conflict of Interest

Authors state no conflict of interest.

Acknowledgements

This study was funded by University of Pisa. We also would like to thank Ente Terre Regionali Toscane for allowing us to use of the animals for the purpose of the study.

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