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Investigatious on the blood fluke Schistosoma turkestanicum in game animals and its geographical distribution in Hungary

Doctoral Theses

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1. Introduction

The subject of our research is a medically and veterinary important parasite that is believed to have been living unnoticed for millions of years in Hungary while causing serious nuisance to people in agricultural work in Asia. Schistosoma turkestanicum Skrjabin, 1913, found in Central Asia, is predominantly an endoparasite in the vascular system of ruminants of the Bovidae family, the larvae that develop in the snail host cause skin inflammation by penetrating the skin (Sahba and Malek, 1979, Skrjabin, 1951). Research has shown that an isolated population of this blood fluke causes infection in red deer in the Gemenc floodplain and has been present in the area since at least the ice age (Lawton and Majoros, 2013; Majoros et al., 2010). This parasite can be found exclusively in deer in this habitat although it occurs sporadically in many other ruminants and other species in Asia.

A disease caused by human blood flukes, known as bilharziasis or schistosomiasis, is the most common parasitic disease in the world after malaria (Bush et al., 2001). At least 200 million people are estimated to be infected. The number of infections is rising, for example due to the increasing human mobility. In countries with a warm climate anyone can get infected by drinking or bathing in water. The number of infections can't be decreased despite regular treatments due to the presence of several rat species that carry the blood-fluke, even in the absence of humans, in deserted or in heavily populated areas. Altough in Europe and North America no human infection occurs because the source of infection, the intermediate hosts of human blood-fluke, does not live here. Therefore attention has turned toward those intervertebrates which would spread due to the global warming. For example it is thus possible that the blood-fluke spreading snails could appear far away from their original habitat. This fear is not unfounded as our research group had the opportunity to recognize the first occurrence of Biomphalaria tenagophila in the temperate zone (Europe), the species that spreads the South American blood-fluke disease (Majoros et al., 2008).

In cinsequence of the limited success of the defense against it schistosomiasis, this disease has been intensively investigated in recent years worldwide. Extensive literature is available on documented epidemiological analysis, cases of human and animal infection, as well as on factors influencing the risk of infection.

The more detailed local study of S. turkestanicum is justified in view of the above because the deeper knowledge of European the species is

primarily relevant to international research. The parasite that otherwise does not endanger the hungarian deer population could be an important model for combating against human schistosomosis. We can get to know more about the serological changes caused by fluke-cercariae, their role in dermatitis, and find out more about the nature of the organism that cause the "swimmers itch" occurs along the Danube.

Maintaining a population of flukes under laboratory conditions would be a great advance in studying schistosomosis safely.

The aim of the PhD program is to get more information about the natural conditions in which S.turkestanicum occur and studying it in laboratory.

During our 3-year survey, our objectives were as follows:

1. Is it possible to detect the eggs of S. turkestanicum or its larvae in deer faeces? The significance of this is that contamination of an area can also be investigated even if there is no way of detecting the specimens in the organs of shot animals.

2. Where does Radix auricularia, the intermediate host snail for S. turkestanicum, can be found in deer-populated areas of Hungary? Recognizing such areas would help find such habitats of S. turkestanicum that has not been discovered yet.

3. Are there other habitats in Hungary where S. turkestanicum is present outside of the Gemenc Danube Basin? The discovery of a natural foci of S.turkestanicum in a temperate zone suggests that this parasite potentially has a wide geographic range therefore it is possibe that this parasite could be present in other regions of Hungary or even other parts of Europe.

4. Is it possible to bredthis fluke in laboratory? For a more detailed study of this parasite it would be desirable to investigate wthether it could be maintained not only in ruminants but also in rodents. The European *Radix auricularia* snail has not yet been bred in laboratory that’s the reason why maintenance of a complete life cycle looks difficult.

5. Can we observe any serological changes and any symptoms in case of human contact with cercariae when they penetrate into the skin? Presumably the reactions caused by S. turkestanicum should be differentiated from symptoms and immune reactions caused by infection of human schistosomes.

2. MATERIALS AND METHODS

Adult S. turkestanicumt worms were collected mainly from the livers of red deer that live the Gemenc forest. Faecal samples of deer were collected near feeding platforms. In addition to these samples we also received liver samples from the following locations: Szigetköz, Zala: Kis-Balaton, Mátra: Sirok region and Parádsasvár, Nyírség, Pilis: Pilisszentlászló, Gödöllői Hegy, Bakony, Zselic. Droppings were also collected from Pilis, Szigetköz, Kis -Balaton, the Mátra and the Bükk Mountains.

Liver samples were cut into small pieces, worms were washed out of them with tap water and preserved in alcohol. Remaining liver pieces were suspended in water after soaking in alkaline. The suspension was then filtered through sieves and stained with fuschine to stain any eggs that may be present in tissues. The resulting solution was mixed with water and the supernatent was decanted. The sediment was searched for eggs before and after mixing with high-specific gravity saline solution. Eggs were detected int he watery sediment the surface of the saline solution. The eggs in the droppings collected from the ground, were detected similarly but using a variety of flotation solutions with different spec. gravity. The ITS regions were sequenced from adult S.turkestanicum worms and resulting sequences were deposited in GenBank.

Freshwater snails were collected from the Gemenc and identified by the morphology of their shell and inner organs. *Radix auricularia* was confirmed as the only intermediate host of S. turkestanicum in Hungary through cercarial shedding. Mice were placed in the water containing the cercariae to induce infection in a rodent host. One of the mice was killed after the infection, the next one after 1 month the other's 5 months after that, and the fourth was left alive to observe the egg laing.

The COX molecular marker was sequenced from *R. auricularia* snails from Hungary and from other european countries. We started keeping the snail in artificial laboratory conditions. Based on the museum samples we collected the data of the prevalence of snail in Hungary and created a map of the geographical distribution of this species.

We have tried various trapping and screening methods to detect the cercariae from the water. We tried ti find cercariae in he surfaces of floating traps were coated with linseed-oil-containing attractants, we also tried to collect cercariae in waer by floating screens and manual sieves

We have observed the consequences of human contact with S.turkesatnicum cercariae and have artificially induced infection of the cercariae to demonstrate their ability to cause cercarial dermatitis.

3. RESULTS

Out of the 176 deer livers collected in Gemenc and the 36 collected in Karapancsa, 50 and 3 contained blood flukes respectively.

No gross lesions caused by blood flukes were found in the livers. Co-infection between S. turkenstanicum and Fascioloides magna was often observed in the livers. Specimen of the latter could not bealways detected, but lesions characterestic of liver flukes were found in almost every liver. Hypertrophia of the bile duct caused by lancet fluke and cysticercus tenuicollis were found in 3 and 2 cases respectively.

The number of the blood flukes found in the liver samples were differing – sometimes 2-3, mostly 40-50 and rarely more than 100 flukes could be detected.

The flukes were almost exclusively adults. In most cases only male flukes were detectable, since the small females were damaged during the sample collection procedure and storage.

S. turkenstanicum infection could be detected only in livers collected in the forests of Gemenc and Karapancsa. We could not find S. turkenstanicum in the livers collected in the other 9 hunting areas of Hungary.

50% of the livers infected with S. turkenstanicum also contained eggs of the blood fluke. We separated the eggs from the tissue using thin alkali. Most of the eggs found in the liver did not contain viable larvae. Therefore we did not anticipate large amount of intact eggs in the faeces of the animals.

In order to find the S. turkenstanicum eggs in the droppings (collected from the ground), we suspended the faeces in water, filtered it through sieve and implamented a flotation method using a sequence of solutions with different special gravity.

Since the specific gravity of the excreted eggs were different, with aim of all of the solutions with a special gravity ranging from 1250g/l to 1400 g/l we could detect eggs.

By using fuchsin staining, we could detect red-stained parasite eggs in 24% of the faeces samples collected in Gemenc and Karapancsa. No blood fluke eggs were found by this method in the samples collected in the other 9 hunting areas of Hungary.

The intermediate host of the S. turkestanicum is the *Radix auricularia* which very often cohabits with the very similar Radix balthica snail. The two species can be differentiated only by anatomical examination of the mature specimens.

We have revealed a difference between the sculptures of shell surface of two species. The apical whorls of *R. auricularia* equipped with regular raws of fine linear depression while the apical whorls of *R. balthica* has only unequal striations developed by the graving of the shell. We could find schistosoma larvae only in *R. auricularia* snails, but not in *R. balthica*.

We realized, that even if there are some fossilic specimens prove the endemism of the R. auricularia, it could not have been very common speciesduring the ice ages. We examined the cox genes of the *R. auricularia* specimens collected in Hungary and other countries and determined the haplotypes. Based on the sequence analysis of the 170 *R. auricularia* specimens collected in 14 countries, we prepared the phylogenetic tree of the species, indicating the evolutionary relationships between the european and asian populations of the species. The biggest genetic diversity can be observed in the European region.

Based on malacological collections and own observations, we detected 81 localitiesof the *R. auricularia* in Hungary. Using the data of the habitats we constructed a map where we indicated a map separately the natural and the artificial habitats of the snail. We observed that, despite being endemic, this snail prefer to live in artificial habitats.

We also observed the swarming from the intermediate host of the S. turkestanicum cercariae as well as their lifepsan outside the host and their virulence. The larvae leave the intermediate host in the morning hours and they swim near the water surface for about a day before they die. The cercariae are capable of penetrating the skin of a mouse or a human, causing dermatitis in the latter accidental host. We showed, that in the liver of an infected mouse, it takes one month for the S. turkestanicum to reach a length of 200-300µm, and even after 5 months, it do not reach maturity.

In the natural habitat of the snails, we implemented various methods in order to detect the cercariae. We could collect cercariae swimming in the water by using a filter *towed* by remotely controlled ship model or by hand. By staining the filtered debris with neutral red dye, the moving and stained cercariae could be detected

4. DISCUSSION

Altough S. turkestanicum is a close relative to human schistosomes, despite its larvae being capable of penetrating human skin the fluke itself is not able to develop to maturity in humans. This feature makes it interesting for scientific research, as it can be assumed that better understanding of the behavior of tropical schistosomes can provide more knowledge and more efficient protection against them. Therefore the study of S. turkestanicum in Hungary does not primarily have significance to animal health but deserves attention as a zoonotic agent and as a safely studied model of human parasitic infections. According also our investigations, it seems in Europe it lives only in red deer but in Asia it has multiple mammalian hosts.

The infection can be recognizable from few dkg liver of shooted deer, if the part of the organ had been extracted from the hilus of the liver. This fact isimportant because usually there is no option to examine whole amount of intact organs which acould be infected by the blood flukes (for example the wall of the gut, mesenterium). The eggs and adults of S. turkestanicum can be also found together with the infection with *F. hepatica* or *D. dendriticum,* although in contrast with these flukes the bllod fluke never cause any kind of lesions.

Although human and animal blood flukes have been detected in Europe several times, a native species of the mammalian schistosomes could not be. Ont he course of our investigations, not only the S. turkestanicum which was discovered in 2001 have proved to be a native species but also its intermediate host, therefore according our current knowledge this parasite is the unique, non introduced mammalian blood-fluke of Hungary and Europe.

The national examination of the visceral organs of the deer all over Hungary has proven to be unsolvable. That is why the distribution of these flukes in the country is only possible by faecal examination.

FIn the shed faecal samples we could detect in any seasonsof the eggs. Together the sedimentation we developed a staining procedure. With the help of thestaining, during the microscopic examination, we could easly separate the blood fluke eggs from the other components of the feacal sample. The importance of this method it that the eggs of blood flukes can be find only in small amount of the fecal sample.

The possible spread of the fluke in Hungary depends on zheintermediate host snails. Therefore we examined the distribution of *Radix auricularia* in Hungary.

Inspite of the snail is native to Hungary, it prefersthe artificial habitats and lives mostly in warm waters than in natural waters. This suggests that this freshwater snail could have been spread in Central Europeafter the ice age. In Hungary S. turkestanicum can develop only in *R. auricularia* but not in its closest relative, *R. balthica*. The cercariae of the fluke swarm out from the intermediate host in the second part of the summer and survive only one day next to the surface of the water. Traps, which contains attractant material, probably can not be detected but with a screening tool the cercariae can be detected in the water which has some importance regardingto people who have a bath in public water bodies.

We established that ~~the~~ S. turkestanicum, which lives in deer, can ~~be~~ enter an inadequate host for example mice and humans and can cause “swimming itches” in humans known as dermatitis.

These findings, on the one hand, help to find the occurrence of S. turkestanicum in places where the red deer and the *R. auricularia* snails are present, or detect the cercariae directly from the water, if no any hosts can be examined directly. Hopefully in this way the cercariae of other flukes may be detected from water which can cause dermatitis in humans, such as larvae of some worms of waterbirds.

Invesigation of the human cercarial dermatitis revealed that the larval invasion of S. turkestanicum, causes sok ind of seroconversion with is detected by the serological method use in rutine diagnostics as if it was an nfection with tropical blood-flukes

That´s why that we need to separate the type of seroconversion caused by S. turkestanicum from the type of seroconversion caused by human schistosomes.

The laboratory white mouse may be successfully infected with the S. turkestanicum but eggs do not develop in it within at least 5 months period. As the infected animal does not suffer from any symptoms there is promise for future infection which would be a great progress in maintaining the blood fluke amongartificial environments.

5. NEW SCIENTIFIC RESULTS

1. I developed a test method for the detection of blood-fluke eggs in the liver, which method is useful in that case if there is no worms found in the organ sample to be examined.

2. I developed a combined process for the detection of S. turkestanicum eggs in the deer faeces, which combines the benefits of sedimentation method flotation method and contrasting with a stain. This way the eggs in the sample can be found even when they are only present in very small concentration..

3. I put together a distribution map of the *Radix auricularia* snail, separating this species from the very similar Radix balthica and Radix labiata species.

4. I described those morphological criteria that make the difference between the *R. auricularia* and *R. balthica* species. This way not only the living adult speciments but the juveniles and empty shells of them can be distinguised from each other.

5. In Hungary I proved first the occurrence of cercarial dermatitis caused by Schistosoma turkestanicum in humans.

6. I have found that the special symptom recognized by the Gemenc fishermen since the beginning of the last century the so called „water-scabbiness" is caused by Schistosoma turkestanicum, the ordinary host of this is the red deer.

7. In Hungary, for the first time, I could detect cercariae directly from natural waterin the fields.

5. PUBLICATIONS RELATED TO THE TOPIC OF THE PRESENT THESIS

Full text papers in peer-reviewed journals

Juhász A., Majoros G. (2018) **Investigations on the distribution Schistosoma turkestanicum Skrjabin, 1913 (Trematoda: Schistosomatidae) infection of red deer in Hungary and a combined method for detection of its eggs in droppings**, Acta Parasitologica, in press. IF.: 1,16

Majoros G., Juhász A., (2018) **Temporary puddles on forest roads as wallow sites of red deer may have important role to sustain Fascioloides magna infection in flood area of Danube River**, Biologia, in press. IF.: 0.759

Juhász A., Dán Á., Dénes B., Kucsera I., Danka J., Majoros G (2016) **A rare zoonosis in Hungary: cercarial dermatitis caused by Schistosoma turkestanicum blood-fluke**. Orvosi Hetilap 157. évfolyam 40. szám. IF.: 0,291

Impact factors in total: 2,21

Oral presentations on national conferences

Juhász A (2018) A *Schistosoma turkestanicum* vérmétely magyarországi köztigazdájának vizsgálata; Akadémiai Beszámolók, Budapest, 2018. január 24.

Juhász A (2017) Detection of eggs of *Schistosoma turkestanicum* in droppings of deer p.: 31. 3rd International Congress on Parasites of Wildlife, Kruger National Park, South Africa

Juhász A (2017) A *Schistosoma turkestanicum* fertőzöttség kimutatása a végleges gazdában; Akadémiai Beszámolók, Budapest, 2017. január 25

Juhasz A (2016) Search for snail-vectors of an endemic *Schistosoma* species in Hungary The 19th International Congress of Unitas Malacologica, The World Congress of Malacology (WCM) Malajzia, Georgetown, 2016. július 18-24.

Juhász A (2016) A magyarországi Schistosoma fertőzöttség elterjedésének vizsgálati lehetőségei; Akadémiai Beszámolók, Budapest, 2016. január 27

Juhász A (2015) Vérmétely cerkáriák kimutatása vízből; MPT Jubileumi Ülés, Budapest, 2015. június 3.

Juhász A (2015) *Radix auricularia,* mint a *Schistosoma turkestanicum* köztigazdája; 39. Malakológiai Kongresszus, Budapest, 2015. szeptember 26.

Juhász A, Majoros G (2015) A felszíndúsítás során identifikált peték DNS kivonáshoz történő koncentrálása; Akadémiai Beszámolók Budapest, 2015. január 28.