Summary of PhD thesis

MOLECULAR EPIDEMIOLOGICAL INVESTIGATION OF UNICELLULAR PARASITES OF COMPANION ANIMALS

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1. Introduction

Companion animals have been playing an increasingly important role in people's lives, and attention has been focused on them more in recent decades. At the same time, the diagnostic tools in veterinary parasitology also developed. Protozoa can be considered common parasites, since the most frequently used anthelmintic drugs in pets are not effective against them, thus creating the opportunity for easier spread. Furthermore, various routes have been developed for their efficient transmission, such as vector-borne and non vector-borne.

Vector-borne protozoa are transmitted by different arthropods vectors e.g., ticks or mosquitoes, and mainly develop intracellularly in red blood cells or white blood cells. Among them in Europe, *Hepatozoon* spp. is worth mentioning of which tick vector needs to be consumed by the host to be infected. Additionally, the piroplasms are also very important protozoa of companion animals, e.g., *Babesia canis* which can manifest in fatal disease in dogs, while *Cytauxzoon europaeus* occurs in Europe has been considered less pathogenic compared to *Cytauxzoon felis* in the USA. Most piroplasms are primarily transmitted by

ticks, but there are species that have found other routes, such as *Babesia gibsoni* through dog bites and transplacentally, while *Hepatozoon felis*, *C. europaeus* also can use the latter way, in addition, carnivorism may take a role in their transmission.

Among non vector-borne protozoa *Giardia duodenalis* and *Acanthamoeba* spp. are the most common waterborne protozoa. Both are zoonotic, thus their importance to human health is also significant. *Giardia duodenalis* has nine Assemblages among which A and B can also be found in humans causing diarrhea, abdominal discharge and rarely skin lesions. The cysts of *Giardia* spp. are resistant to different undesirable environmental conditions; thus, animals' reinfestation occurs frequently. Acanthamoebae are opportunistic unicellular parasites and are primarily known for causing keratitis in humans, however, they have also been reported in animals, i.e., in dogs, cats, reptiles and horses.

Trichomonads are also significant homoxenous non-vector borne protozoa. They can be found in mucosal surfaces, such as *Trichomonas gallinae* in the upper gastrointestinal tract of pigeons, or *Tritrichomonas foetus* and *Pentatrichomonas hominis* in large intestine of dogs,

cats, and the latter also in humans. They are transmitted directly, though, it is assumed that slugs may have a paratenic host role in the life cycle of *T. foetus.*

Among heteroxenous protozoa *Toxoplasma gondii*, *Neospora caninum and Sarcocystis* spp. can have significant role in companion animals. All need intermediate host to complete their life cycle. *Toxoplasma gondii* is considered highly important in veterinarian and human health, since one of the most widespread zoonotic protozoa in the word causing serious problems in pregnant women, while *N. caninum* in pregnant cows.

Hungary is one of the few European countries where a low number of protozoan parasites have been discovered in companion animals in the past decades or at least analyzed with molecular biological tools. Thus, many unicellular parasites have long been waiting to be found and their occurrence, genetic and phylogenetic characteristics revealed. Additionally, their genetic diversity depending on the transmission route has also not yet been investigated. Based on these, we consider it very important and promising to conduct this gap-filling study.

2. Aims of the studies

Ad 1. to screen *Giardia*-infection in rodents and rabbits

Ad 2. to investigate avian *Trichomonas* species

Ad 3. to examine trichomonads infecting cats and dogs

Ad 4. to screen the feces of reptiles for different protozoa

Ad 5. to reveal the presence of *Acanthamoeba* spp. in canine and feline patients with ocular illnesses

Ad 6. to identify protozoa found in the feces of dogs and cats fed with raw meat

Ad 7. to examine *Sarcocystis* sporocysts from dog feces

Ad 8. to screen for the presence of *Hepatozoon felis* and *Cytauxzoon europaeus* in outdoor domestic cats

Ad 9. to investigate the prevalence of *Babesia gibsoni* and its molecular characteristics in “fighting dogs” in Hungary

Ad 10. to reveal whether the mode of transmission influences the genetic diversity of protozoa found in this study

3. Materials and methods

Altogether 1039 samples of feces, intestinal content, blood, oral cavity, oropharynx, conjunctiva and tissue were collected from companion animals and examined for the presence of unicellular parasites with traditional parasitological methods and/or molecular biological methods including phylogenetic analysis. Most of the samples originated from Hungary.

Within traditional parasitological methods, flotation using 1200 or 1300 g/l salt solution and blood smear analysis were performed. From all fecal samples examined in this study the DNA was extracted using the QIAamp® Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions or with slight modifications. While the DNA from oral, oropharyngeal, conjunctival, blood and tissue samples were extracted with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s blood or tissue protocol. As a next step, conventional PCRs were preformed to screen the presence of various protozoa (Table 1). All sequences retrieved from GenBank and included in the phylogenetic analysis had 97-100%

coverage with sequences from this study and were trimmed to the same length. The dataset was resampled 1,000 times to generate bootstrap values. Phylogenetic analysis was conducted by MEGA 7.0 and MEGA 11.0.

Finally, the genetic diversity of protozoan parasites was analyzed according to their different transmission routes, focusing on those species which were recently discovered or molecularly analyzed for the first time in Hungary or its geographical region.

Table 1. Protozoa and their target genes during PCRs.

Target group	**Target gene**
Giardia spp.	*bg*, *gdh*, *tpi*
Trichomonadida	18S rRNA, alpha-tubulin, ITS2
Acanthamoeba spp.	18S rRNA
Neospora - *Toxoplasma* - *Cystoisospora* spp.	COI
Neospora sp.	NC5
Toxoplasma sp.	repeat region
Piroplasms *Sarcocystis* spp.	18S rRNA
Hepatozoon spp.	18S rRNA
Cytauxzoon spp.	*cytb*
Babesia gibsoni	*cox*1, *cytb*

4. Results and discussion

Among protozoa tested in this study *Giardia* cysts were found with parasitological method in Norway rats, chinchillas and degus. Based on PCRs, *G. duodenalis* Assemblage B and G were detected in two rats, while Assemblage E in a beaver. In addition, Assemblage B occurred in one chinchilla. Our results confirm the significance role of rodents in the epidemiology of giardiosis. Most animals tested here were asymptomatic carriers, further increasing the necessity of awareness that clinically normal pet rodents may pose a risk of shedding cysts of even zoonotic *Giardia* genotypes.

Furthermore, *Trichomonas gallinae* was detected in Columbiformes with an overall prevalence of 73%. Based on the screening assay, racing feral pigeons had a significantly higher prevalence of *T. gallinae* infection than urban feral pigeons. Altogether, six *Trichomonas* subtypes were detected, among which *Trichomonas* sp. Hu-TG37 belonged to the phylogenetic group of *Trichomonas* spp. infecting domestic carnivores (*T. canistomae* and *T. tenax*) with moderate support, implying that this might be a new species. This calls for further epidemiological

studies on the possible contact between dogs and pigeons and its role in the transmission of these protozoan parasites. In addition, this is the first study on the genetic diversity of *T. gallinae* in Hungary, Romania and the whole southeastern European region.

As a result of investigation trichomonads in small animals, 13.8% of domestic cats were positive for *T. foetus* and 2.1% for *P. hominis*. In addition, one feline sample contained the DNA of a different *Tritrichomonas* species which was genetically most closely related to *Tritrichomonas casperi* isolated from mouse. The detection of DNA from the latter species could indicate a possible role of a predator-prey link in the evolution of this feline trichomonad, like what is known of avian trichomonosis. Regarding dogs, 16% proved to be *T. foetus* positive, and to the best of our knowledge, this is the first report of *T. foetus* in dogs in Europe north of the Mediterranean Basin.

From fecal sample of a leopard gecko a not yet reported *Monocercomonas* species was found which had the closest match with *Monocercomonas colubrorum*. In addition, fecal samples of six reptile species showed positivity in the PCR targeting genus *Acanthamoeba*. In

two samples, one from a yellow anaconda and the other from a Gila monster/beaded lizard kept together, *Acanthamoeba hatchetti* was identified which clustered with the strains of T11 genotype. From a bosc monitor *Acanthamoeba castellanii*, while from a frilled dragon *Acanthamoeba lugdunensis* was detected. Furthermore, the sample of a green iguana proved to be identical to the *Acanthamoeba* T13 genotype. These findings of opportunistic pathogens highlight the importance of monitoring protozoa in the feces of pet reptiles as a source of infections for other animals and humans living nearby.

Investigating the presence of *Acanthamoeba* species in the eyes of dogs and cats with ocular signs, no positive results were acquired. Even though *Acanthamoeba* is a well-known cause of eye disease in humans, the risk of small animals presenting to veterinarians with ocular lesions due to the presence of *Acanthamoeba* might be negligible.

Moreover, dogs and cats kept on BARF diet were examined for the presence of various protozoa. Although the molecular tests became negative for the presence of *Neospora*, *Toxoplasma* and *Sarcocystis* spp., in the fecal

samples of two dogs, oocysts of *Cystoisospora canis*, *Cystoisospora ohioensis*-like sp. and *Eimeria stiedai* were detected. In addition, in one sample sporocysts of a *Sarcocystis* sp. were seen. While the latter is certainly associated with raw meat consumption, the occurrence of *Cystoisospora* spp. may not have been due to BARF-diet, because their prevalence was higher in the era preceding the widespread application of raw meat feeding. In adddition, *E. stiedai* was detected as pseudoparasites in this study. Freezing the meat/viscera for 2-3 days is strongly advised to avoid living protozoa that would risk the health status of BARF-fed pets or others living nearby.

In a dog's fecal sample, the DNA of *Sarcocystis morae* was present, in addition, its sporocysts were also observed with oval shape and size of 14.95 × 9.75 µm. *S. morae* has recently been described as a new species, with red deer and fallow deer as typical intermediate hosts. Although canids have been suspected to be the final hosts based on phylogenetic properties, this is the first molecular evidence of the final host role of domestic dogs in the life cycle of *S. morae*. The *Sarcocystis*-infected dog presumably ingested meat from cervids as part of the raw meat-based diet.

During investigation of piroplasms and *Hepatozoon* spp. in cats, only 1 of 127 domestic cats tested positive for *Hepatozoon felis*, while *Cytauxzoon europaeus* was not detected. The positive cat was kept in a region where *H. felis* is endemic in wild cats. Furthermore, the *H. felis* genotype II found here is the first to be identified in a domestic cat in Europe. The infection most likely happened by ingesting infected ticks in the habitat shared with wildcats. Overall, feline hepatozoonosis and cytauxzoonosis are emerging infections in the southern part of Central Europe. Hitherto *H. felis* and *C. europaeus* have only been found in wildcats in this endemic area, but according to the present results at least *H. felis* also emerged in domestic cat.

Further investigating piroplasms in dogs, 40.5% and 10.1% of the 79 dogs were positive for *B. gibsoni* and *Babesia vulpes* with PCR, respectively. In addition, 62.45% of *Babesia*-infected dogs harbored hemotropic mycoplasmas. The *cox*1 gene sequence of *B. gibsoni* isolated here showed the closest identity with *B. gibsoni* reported from Japan but had a nonsynonymous mutation (M33I). Furthermore, the 11 *B. gibsoni*-positive samples analyzed for sequence variants of the *cytb* gene showed

the presence of a common mutation (P310S). Most importantly, *B. gibsoni* had two further nonsynonymous mutations, M121I and F258L, in a dog with severe and relapsing anemia following atovaquone treatment. Phylogenetically, both *cytb* sequence variants clustered together showing the closest relationship of both haplotypes identified in Hungary with those from China and Japan. To the best of our knowledge, this is the first *cox*1 and *cytb* characterization of *B. gibsoni* in Europe, as well as the first report on the emergence of this piroplasm and hemoplasmas with high prevalence among “fighting dogs” in Hungary and its region.

Based on the results obtained during the comparison of the genetic diversity of the selected protozoa, among four apicomplexan parasites (*B. gibsoni*, *C. europaeus*, *S. morae* and *H. felis*) the latter had the highest genetic diversity. While, among non-apicomplexan protozoa (*T. gallinae*, *P. hominis*, *T. foetus* and *A. castellanii*) the latter proved to have the highest genetic diversity. Transmission mode had a significant impact on the genetic diversity among protozoan parasites, depending on life cycle strategies and consequent frequency/chance of sexual reproduction vs binary fission. In particular, the absence

of direct transmission between hosts is a common trait of *H. felis* and *A. castellanii*, contributing to their high genetic diversity.

5. Overview of the new scientific results

Ad 1. First report on the *Giardia*-carrier status of pet degus, and on Assemblage E of *G. duodenalis* in beavers

Ad 2. First report on the genetic diversity of *T. gallinae* in the southern central and southeastern European region

Ad 3. First evidence of *T. foetus* in dogs of our region, and a hitherto unknown large intestinal *Tritrichomonas* sp. was shown to be present in a cat

Ad 4. Not yet reported species of the genus *Monocercomonas* and *Acanthamoeba* spp. with possible clinicopathological significance in reptiles, and including zoonotic species

Ad 5. No correlation between the presence of *Acanthamoeba* and ocular illnesses in canine and feline patients

Ad 6. BARF-feeding may contribute to the contamination of the environment with *E. stiedai* oocysts

Ad 7. First molecular evidence in support of the final host role of domestic dogs in the life cycle of *S. morae*

Ad 8. First report in Europe on the presence of *H. felis* from genogroup II in free-roaming domestic cats

Ad 9. The first *cox*1 and *cytb* characterization of *B. gibsoni* in Europe, as well as the first report on the emergence of this piroplasm and hemoplasmas with high prevalence among “fighting dogs” north of the Mediterranean Basin

Ad 10. The absence of direct transmission between hosts may play a role in the high genetic diversity of certain protozoan parasites, as exemplified by *Hepatozoon* and *Acanthamoeba* spp.

6. Publications in the topic of the dissertation

1. Hornok S, Keve G, **Tuska-Szalay B** (2025) **Transmission route-dependent genetic diversity of selected protozoan parasites as reflected by the phylogenetic analysis of the 18S rRNA gene.** Acta Vet Hung. https://doi.org/10.1556/004.2025.01128

2. **Tuska-Szalay B**, Sipos D, Czabán D, Kalmár Z, Keve G, Szekeres S, Kelemen BS, Sándor AD, Hornok S (2025**) Pet and wild rodents as hosts of *Giardia duodenalis* in Central Europe, Hungary.** Acta Vet Hung. https://doi: 10.1556/004.2024.01115.

3. **Tuska-Szalay B**, Jerzsele Á, Hornok S (2024) **Antiprotozoal agents used in veterinary medicine: synopsis** [In Hungarian]. MAGYAR ÁLLATORVOSOK LAPJA, 146 (8). pp. 487-500. ISSN 0025-004X.

https://doi.org/10.56385/magyallorv.2024.08.487-500

4. **Tuska-Szalay B**, Gilbert J, Takács N. Boldogh SA, Fáy J, Sterczer Á, Psáder Á, Kontschán J, Izsó Á, Hornok S (2024) **Molecular-phylogenetic investigation of trichomonads in dogs and cats reveals a novel *Tritrichomonas* species.** Parasites Vectors 17, 271. https://doi.org/10.1186/s13071-024-06343-0

5. **Tuska-Szalay B**, Papdeák V, Vizi Z, Takács N, Hornok S (2024) **Parasitological and molecular investigation of consequences of raw meat feeding (BARF) in dogs and cats: implications for other pets living nearby.** Parasitol Res. 123(2):114. https://doi: 10.1007/s00436-024-08124-1.

6. **Tuska-Szalay B**, Boldogh SA, Farkas R, Rompos L, Takács N, Beresnyák V, Izsó Á, Kontschán J,

Lanszki J, Hornok S (2023) **Screening of domestic cats from north-eastern Hungary for *Hepatozoon felis* and *Cytauxzoon europaeus* that cause infections in local wildcat populations.** Pathogens. 12(5):656. https://doi: 10.3390/pathogens12050656.

7. **Tuska-Szalay B**, Sipos G, Takács N, Kontschán J, Sándor AD, Péter Á, Berta K, Kerek Á, Jerzsele Á, Votýpka J, Hornok S (2022) **Molecular epidemiological study of *Trichomonas gallinae* focusing on central and southeastern Europe.** Front Vet Sci. 9:1050561. https://doi: 10.3389/fvets.2022.1050561.

8. **Tuska-Szalay B**, Kelly H, Takács N, Kontschán J, Votýpka J, Hornok S (2022) **Molecular evidence of *Monocercomonas* and *Acanthamoeba* in the feces of captive reptiles.** Parasitol Res. 121(12):3681-3687. https://doi: 10.1007/s00436-022-07677-3.

9. **Tuska-Szalay B**, Vizi Z, Hofmann-Lehmann R, Vajdovich P, Takács N, Meli ML, Farkas R, Stummer-Knyihár V, Jerzsele Á, Kontschán J, Szekeres S, Hornok S (2021) ***Babesia gibsoni*** **emerging with high prevalence and co-infections in "fighting dogs" in Hungary.** Curr Res Parasitol Vector Borne Dis. 1:100048. https://doi: 10.1016/j.crpvbd.2021.100048.

10. **Tuska-Szalay B**, Takács N, Kontschán J, Vizi Z, Hornok S (2021) **Dogs are final hosts of *Sarcocystis morae* (Apicomplexa: Sarcocystidae): First report of this species in Hungary and its region** - Short communication. Acta Vet Hung. 69(2):157-160. https://doi: 10.1556/004.2021.00017.

7. Publications not related to the dissertation

1. Hornok S, Boldogh SA, Takács N, Sándor AD, **Tuska-Szalay B** (2022) **Zoonotic ecotype-I of *Anaplasma phagocytophilum* in sympatric wildcat, pine marten and red squirrel** - Short communication. Acta Vet Hung. https://doi: 10.1556/004.2022.00021.

2. Hornok S, Boldogh SA, Takács N, Kontschán J, Szekeres S, Sós E, Sándor AD, Wang Y, **Tuska-Szalay B** (2022) **Molecular epidemiological study on ticks and tick-borne protozoan parasites (Apicomplexa: *Cytauxzoon* and *Hepatozoon* spp.) from wild cats (*Felis silvestris*), Mustelidae and red squirrels (*Sciurus vulgaris*) in central Europe, Hungary.** Parasit Vectors. 15(1):174. https://doi: 10.1186/s13071-022-05271-1.

3. Hornok S, Daccord J, Takács N, Kontschán J, **Tuska-Szalay B**, Sándor AD, Szekeres S, Meli

ML, Hofmann-Lehmann R (2022**) Investigation on haplotypes of ixodid ticks and retrospective finding of *Borrelia miyamotoi* in bank vole (*Myodes glareolus*) in Switzerland.** Ticks Tick Borne Dis. ;13(1):101865. doi: 10.1016/j.ttbdis.2021.101865.

4. Kerek Á, Csanády P, **Tuska-Szalay B**, Kovács L, Jerzsele Á (2023) **In Vitro Efficacy of Hungarian Propolis against Bacteria, Yeast, and *Trichomonas gallinae* Isolated from Pigeons—A Possible Antibiotic Alternative?** Resources. 12(9):101. https://doi.org/10.3390/resources12090101