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**Examinations of canine leishmaniosis
and the parasites vectors in Hungary**

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1. Introduction

Leishmania (L.) infantum is a unicellular parasite causing visceral leishmaniosis, a systemic multiorgan disease of dogs and humans. Endemicity of the parasite is restricted to the regions of Europe where vector sandfly species occur. Latency of the disease in dogs can reach months, sometimes years after the bite of infected female sandflies of the *Phlebotomus (P.)* genus. Leishmaniae from inapparently infected to naive dogs might be transmitted with vectors or as a rare exception with bite, blood or semen. The parasite is frequently imported from endemic to sandfly-free countries of Europe (e.g. Germany, Netherlands) with infected dogs, but these cases have no epidemiological importance in the absence of competent vectors. Emerging concerns follow the current northward spread of vector sand fly species in Europe, that already caused former non-endemic territories (e.g. continental Northern Italy) to become endemic. The process is thought to be driven by current complex changes in the climate and the socio-economic as well as ecological environment.

Hungary is traditionally regarded to be free of *L. infantum* and its vectors, despite the fact that specimens of a vector sand fly species were already collected in a southern county in the early 1930s. Reliable data however are missing, because no investigations were either before or since then dedicated to elucidate the occurrence of these insects in the country. Available human (HumL) as well as canine leishmaniosis (CanL) records in Hungary were scarce: a single case of canine leishmaniosis imported from Greece was known, and with the exception of a single uncertain case only reports of a few dozen HumL cases imported from the Middle-East (31), and Croatia (1) were known.

Hungary on the other hand is adjacent to endemic countries of the Balkan-peninsula and is regarded therefore to be threatened by spread of *L. infantum* and its vectors. At least 3 species of phlebotomine sand flies (*P.*

neglectus, *P. perfiliewi* és *P. tobbi*) with vector capacity are known to occur in Croatia and Serbia. Northward spreading sand flies that might invade Hungary could transmit leishmaniae imported with infected traveling dogs or wandering wild canids (foxes, golden jackals) of the Balkan as well as infected travellers to naive hosts endemically during their active period. Updated informations about the occurrence of vector sand fly species and/or *L. infantum* infected dogs in the country are therefore of high importance.

Our study was partly managed in the frame of and funded by the EDEN (GOCE-2003-010284, Emerging Diseases in a changing European eNvironment) FP6 project that explored the changes in the distribution of several pathogens and their vectors in Europe in relation to environmental changes. Our investigations however, were carried out in several counties the main focus of our efforts fell on the southern parts of the country, where the occurrence of leishmaniosis as well as vector sand flies is the most feasible. Our investigations were oriented by the following two objectives:

1. We were searching all over the country for dogs infected within or out of the borders of Hungary with *L. infantum* or other leishmaniae with the serological testing of dogs' blood and with a questionnaire sent to veterinary clinics.
2. We searched for the occurrence of sand fly species of leishmania-vector capacity with different trapping methods in multiple counties of the country

2. Materials and Methods

2.1. Serological survey of dogs

Serum samples of 705 dogs in 21 municipalities and 6 counties were collected with the help of local veterinarians and examined with the immunofluorescent antibody test (IFAT) method. Threshold of positivity was 1:80 according to the recommendations of OIE.

2.2. Veterinary questionnaire

A questionnaire and an informational letter dealing with canine leishmaniosis were sent to 216 veterinary clinics in 19 counties via post in the May of 2008. With the questionnaire we asked the vets whether they have encountered any cases of canine leishmaniosis during their practice.

2.3. Examinations in a kennel in Paks

A dog was taken to the local vet in Paks who recognized symptoms that might have been associated with canine leishmaniosis in October 2007. After euthanasia smears were prepared from the liver, spleen and a lymphnode. Histopathological examination of the smears were carried out by Imre Biksi (SZIU, Veterinary Faculty, Large animal Clinic, Üllő). Blood samples of the dog were tested in a German laboratory for the presence of specific antigens and Leishmania DNA. Both tests proved the infection with *L. infantum*. We got informed about the case and visited the kennel in the May of 2008. At that occasion whole as well as EDTA blood samples were collected from the remaining 19 dogs living in the kennel. One of the dogs showed swellings of the lymph nodes and deformities of the skin. Symptoms of the dog became severe later that year and fine-needle aspiration biopsy samples were collected. Sera extracted from the whole blood samples were tested with IFAT. After DNA extraction (QIAamp Mini Kit, Blood and body fluid protocol, QIAGEN GmbH, Hilden, Germany) all the EDTA blood samples, the biopsy samples as well as the tissue smears of the first diseased dog were tested for the presence of *L. infantum* kinetoplast (kDNA) and ribosomal small subunit

(ssurDNA) DNA with a single step and a nested PCR method, respectively. Positive kDNA PCR products of the dogs showing clinical symptoms were sequenced (Macrogen Inc., Seoul, Korea) and aligned (NCBI BLAST) to GenBank references. Immunohistochemical examination of the spleen of the first infected dog was carried out by Levente Szeredi (NFCSO, VDD). The dogs as well as their parents were born in the same kennel that they haven't ever left, while new dogs haven't been introduced to the flock.

2.4. Trapping of sand flies

We carried out sand fly collecting trials at 46 sites, in 8 counties of Hungary between 2006 and 2010. We applied sticky paper traps at 40, CDC Miniature Light traps and MMX CO₂ baited traps at 44 sites. In the kennel in Paks and its surrounding and sites with warm climate near to animal shelters were chosen for trappings during the night.

2.5. Species identification of trapped sand flies

The species of the collected sand fly specimens were identified after treatment with lacto-phenol and mounting in phenol-balm based on the morphology of the females' cibarium and spermatheca as well as the males' hypopygium and aedeagus.

2.6. Phylogenetic analysis of the collected sand flies

Nucleotide sequences of the Internal Transcribed Spacer (ITS2) locus of *P. neglectus* specimens collected in Hungary, Macedonia, Turkey and Crete, and *P. perfiliewi* specimens collected in Hungary, Macedonia, Italy and Turkey were compared. DNA was extracted from the thorax of 3 specimens from each country with the QIAamp DNA Mini kit ("Tissue" protocol) and amplified with ITS2 specific PCR reaction. Sequences determined by Macrogen Inc. (Seoul, Korea) were compared with the use softwares incorporated into the PHYLIP package, maximum likelihood algorithm. Confidence of genetree was checked with 100 bootstraps. Relevant sequence of *Sergentomyia minuta* was used as an outgroup.

3. Results

3.1. Serological survey of dogs

In two dogs from Baranya county weak positivity was seen with IFAT. Both of these dogs proved to be negative later in the upcoming counter-test.

3.2. Veterinary questionnaire

Only 67 (31%) out of the 216 posted questionnaires had been returned to our Department. Processing of the answers revealed 8 cases of CanL in Hungary. All infected dogs lived before or at the time of the diagnosis in an endemic country. A single case was diagnosed in Hungary.

3.3. Examinations in a kennel in Paks

The kDNA and the ssu rDNA of *L. infantum* were found with PCR in the liver, spleen and lymphnode smears of the first diseased dog. The presence of the parasite was confirmed in the spleen and liver by histological and in the spleen with the immunohistochemical examinations. Both PCR methods proved the infection with the pathogen in the blood sample and in the lymphnode aspirate of the second infected dog. High levels (1:5200) of *Leishmania*-specific antibodies were found in the serum of the same dog. Weak positive (1:80) results were shown in the sera of two additional dogs lacking any clinical signs. In one of these dogs and in another one that was seronegative ssu rDNA-specific PCR proved the presence of the parasite. IFAT gave positive result (1:160) for one of the symptomless dogs. Nucleotide sequences of the first and the second diseased dogs showed the highest similarity (96 and 95 % respectively) to *L. infantum* reference sequences found in the GenBank database.

3.4. Trapping of sand flies

With sticky traps we collected 4 males and 3 females of *P. neglectus* within and around Nagyharsány, Baranya county and 4 males and 3 females of *P. mascitti* at Kapolcs, Veszprém county from 2006 to 2010.

With CDC and MMX traps 124 males and 96 females of *P. neglectus* were collected in the Statue Park near Nagyharsány and in 3 chickencourts in Villánykövesd, Baranya county between 2006 and 2010 and at Törökbálint, Pest county in 2007. One female of *P. mascitti* was collected at each of the later two sites in 2007 and 2008. A sum of 132 males and 235 females of *P. perfiliewi* were collected with CDC traps in a pheasant breeding facility near Földeák and in two chickencourts in Maroslele, Csongrád county in 2008 and 2009. No sandflies were collected in the kennel and the nearby chickencourts in Paks in the summer of 2008 and 2009.

3.5. Phylogenetic analysis of the collected sand flies

Based on our analysis the sand flies examined could be classified in similarity groups referring to geographical classes. Hungarian and Macedonian *P. neglectus* specimens both are related to sand flies in Turkey and Crete but form separated subgroups in themselves. Hungarian *P. perfiliewi* specimens form a subgroup with an Italian specimen within the larger group of Turkish and Italian sand flies.

4. Discussion

No dogs were found to be seropositive for *L. infantum* specific antigens in our 6 county wide survey that implies that infected animals might be very rare or absent in Hungary.

Beside the 8 imported cases of CanL back up to 1999 that were revealed in the country through returned questionnaires 2 additional cases were reported through informal channels. These cases indicate that *L. infantum* might be imported into the country by foreign tourists travelling with their dogs to Hungary as well as by dogs returning with their owners from holidays or dog exhibitions from any endemic area. Infected wild canids during their migrations (e.g. foxes, golden jackals) and parasite carrier human travelers might also introduce leishmaniae in the country. In the course of our study we contributed with molecular methods to the diagnosis of a HumL case in a Hungarian citizen who acquired the infection during his holidays in Croatia.

We discovered stable populations of vector sand fly species during the years of our trapping surveys in Hungary. *Phlebotomus perfiliewi* might be present in Csongrád county since at least the early 1930s, while *P. neglectus* was collected for the first time in Hungary. The worldwide northernmost occurrence of the latter species was recorded in Pest county. During their period of activity, from early summer till the early autumn, the adults of these species represent an epidemiological risk at the southern parts of Hungary, because they are able to transmit imported *L. infantum* to naive dogs and humans endemically. The role of *P. mascitti* and *P. papatasi* in the transmission of *L. infantum* wasn't proved yet.

Our phylogenetic analysis implies that a population of *P. neglectus* exists in isolation in Hungary for a longer period. More moderate isolation of the Hungarian *P. perfiliewi* population was revealed through our analysis

suggesting involuntary human contribution in the transfer of specimens from great distance (e.g. larvae transported with soil).

Based on the results of our examinations in Paks these CanL cases can be regarded as the first autochthonous cases in Hungary. Because no sand flies were captured in the kennel or its vicinity the roots of infections remained unclear. As a possible way of parasite transmission we can count with the presence of a recent sand fly population in the area that was eradicated before our survey by the regular mosquito control, or the inapparent vertical and horizontal transmission of leishmaniae through several generations of the dogs. Based on our current knowledge we cannot exclude drift of infected sand flies with the prevailing winds or involuntary transport with a vehicle from endemic territories to the area of Paks.

In the light of current studies the picture of the northward spread of *L. infantum* and vector sand fly species as a response to continent wide climatic, socio-economical and ecological changes is drawn in Europe. These changes in the near future can cause the infection of dogs and humans with *L. infantum* to become endemic in countries like Hungary, where they were non-endemic just a few years before.

Our studies provided basic information about the occurrence of CanL and the parasites vectors' in Hungary. Our serological survey encompassed only 6 counties so far, but in the future we plan to extend the serological survey to more dogs in more counties of the country providing more reliable data about possible inapparent CanL cases. To improve the estimations regarding the epidemiological importance of vector sand fly species in the country we have to continue our trapping surveys to elucidate the barriers of their distribution and their period of activity throughout the year.

5. New scientific results

1. None of the 705 randomly selected dogs tested in our survey was found to be infected with *L. infantum* in 6 counties of Hungary
2. Based on returned questionnaires and personnel communications the occurrence of imported CanL cases was revealed in Hungary. These infected dogs might pose epidemiological risk to naïve dogs and humans in the presence of vector sand fly species.
3. Autochthonous transmission of *L. infantum* to dogs was detected for the first time in Hungary. The roots of transmission however remained unclear, because no sand flies could be detected in the kennel and its vicinity in Paks.
4. During our 4 year trapping survey carried out with different types of traps we collected 4 species of phlebotomine sand flies in several parts of Hungary. Two of the collected species *P. perfiliewi* and *P. neglectus* are known vectors of *L. infantum*. The later species along with *P. mascitti* an unproven vector of *L. infantum* and *P. papatasi* known vector of *L. major* were caught for the first time in the country. Our preliminary investigations indicate that these species are mainly present in low densities in the southern counties of the country.
5. Phylogenetic analysis of sand flies collected in our survey and by our partners abroad indicates that the population of these insects lives in moderate isolation in Hungary. Our results however do not exclude the possibility of sand fly migration from nearby leishmania endemic regions into the country that poses epidemiological risk directly to naïve dogs and humans in Hungary.

6. Publications

6.1 In peer reviewed journals, in the topic of the doctoral thesis

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6.2. In peer reviewed journals, in other topics

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Szénási Z, Marton S, Kucsera I, **Tánczos B**, Horváth K, Orosz E, Lukács Z, Szeidemann Zs. **Preliminary Investigation of the Prevalence and Genotype Distribution of *Giardia intestinalis* in Dogs in Hungary** . Parasitology Research Volume 101, Supplement 1. 2007.

Szénási Z., Kovács A.H., Pampiglione, S., Fioravanti, M.L., Kucsera I., **Tánczos B.**, Tiszlavicz L.: **Human dirofilariosis in Hungary: an emerging zoonosis in central Europe**. Wien Klin Wochenschr. 120(3-4):96-102, 2008.