One Health 11 (2020) 100191

Contents lists available at ScienceDirect

One Health

journal homepage: www.elsevier.com/locate/onehlt

Human toxocariasis, a silent helminthic disease revealed in Savannakhet, Lao PDR

Megumi Sato [a,1,*], Marcello Otake Sato [b,*,1], Jitra Waikagul [c], Tiengkham Pongvongsa [d], Surapol Sanguankiat [c], Tipparayat Yoonuan [c], Sengchanh Kounnavong [e], Satoru Kawai [b], Hiroshi Yamasaki [f], Kazuhiko Moji [g]

[a] Graduate School of Health Sciences, Niigata University, Niigata, Niigata, Japan
[b] Department of Tropical Medicine and Parasitology, Dokkyo Medical University, Mibu, Japan
[c] Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand
[d] Station of Malariology, Parasitology, and Entomology of Savannakhet Province, Savannakhet, Lao PDR
[e] National Institute of Public Health, Ministry of Health, Vientiane, Lao PDR
[f] National Institute of Infectious Diseases, Tokyo, Japan
[g] Graduate School of International Health Development, Nagasaki University, Japan

ARTICLE INFO

Keywords:
Mekong River basin
Neglected tropical diseases
Toxocariasis
Helminth

ABSTRACT

Toxocariasis is a zoonotic helminthiasis caused by the migrating larvae of *Toxocara canis* and *T. cati*, common roundworms of dogs and cats. Our previous study in Savannakhet Province of Lao PDR showed an infection rate of 44.1% of *Toxocara* spp. in dogs. Thus, we investigate if this previous high prevalence in the definitive hosts influenced the occurrence of human toxocariasis. For that we used a 38 kDa recombinant protein derived from *T. canis* larvae excretion secretion products in ELISA. Human serum samples were collected in the Lahanam area of Savannakhet province. The population attending the study in Lahanam village were aged from 7 to 59 years old (y/o) 65.9% (54/82) were male and 34.1% (28/82) were female. The total percentage of seropositivity to *Toxocara* sp. was 30.4% (25/82). Males were more likely to test positive for toxocariasis with a risk ratio of 2.70 (CI95 0.87–4.93). No significant differences between ages were seen. However, it was possible to observe an increase of optical density (OD) values in ELISA according to age. The awareness of the health system on the high prevalence of seropositivity to *Toxocara* sp. in Savannakhet can prevent irreversible consequences as permanent vision loss and seizures caused by this silent chronic disease revealed in the Lahanam area.

1. Introduction

Toxocara canis and *Toxocara cati* are common ascarids of the small intestine of cats and dogs, with worldwide distribution. Toxocariasis has a higher prevalence in areas where these animals are not dewormed, roam free and/or in rural areas [1–3]. Humans are infected when embryonated eggs are accidentally ingested and the infective larvae, which are not able to complete the life cycle, migrate through different organs causing visceral larva migrans or ocular larva migrans when *Toxocara* larvae migrate into the eyes [4,5]. Moreover, it is suggested that toxocariasis frequently presents itself as a syndrome comprising chronic weakness, abdominal pain, different signs of allergy, and hypereosinophilia and has been associated with different neurologic syndromes [4,6].

The direct diagnosis of human toxocariasis is done by detecting infective *Toxocara* larva(e) or DNA material in biopsy samples. These procedures are difficult to implement in the routine diagnostic activities of health centers. The best alternatives for the diagnosis of toxocariasis relies on immunological tests to detect excretory-secretory antigens from *T. canis* larvae (TES) by immunoblotting [7–9] and commercial kits like the ToxocaraCHEK [10]. Nevertheless, TES antigens can present cross-reactivity with helminth infections caused by other species, jeopardizing the test reliability [8,9,11,12]. A recombinant antigen based on a 38 kDa protein of *T. canis* second-stage larvae (RecTcanis) was developed, clearing the cross-reactivity problem and standing as a good alternative for a specific diagnosis [13]. The RecTcanis was successfully

* Corresponding authors.
E-mail addresses: satomeg@clg.niigata-u.ac.jp (M. Sato), marcello@dokkyomed.ac.jp (M.O. Sato).
[1] Contributed equally for this work.

https://doi.org/10.1016/j.onehlt.2020.100191
Received 18 August 2020; Received in revised form 23 October 2020; Accepted 23 October 2020
Available online 27 October 2020

used in different countries for diagnosing toxocariasis in suspected clinical cases and field-surveys [14–16].

There is a lack of reported human cases of toxocariasis in Lao PDR, despite the high prevalence of *Toxocara* in dogs reaching more than 40% [3]. This is probably because of the difficulty in diagnosing this zoonosis by clinical symptoms as well as in applying diagnostic tests for toxocariasis in suspected cases. In this study, we used a highly specific ELISA toxocariasis diagnostic test to determine the extension of the disease in humans living in Savannakhet Province in Lao PDR.

2. Material and methods

2.1. Study area and human sampling procedures

The study was conducted in Songkhone District, Lao PDR in 2011. The area is located in Savannakhet province and is bordering with Mukdaham (Thailand) (Fig. 1).

All the villagers in Lahanam (Songkhone District) were invited to join this study through a call from the health agents in the community. All the participants granted approval to engage in this study. The participants received a detailed explanation of the study, given in the local language to assure proper understanding, affirmed informed consent, acknowledged the right to opt out the study at any time, joined the study voluntarily, and then received instructions regarding the manner of sample collection. Blood sampling for serological diagnosis was done by venipuncture by local staff nurses at the district health centers from 82 villagers of Lahanam, in Songkhone District. Serum samples were brought to the laboratories in Thailand and Japan for further processing and analysis.

2.2. Serodiagnosis for toxocariasis

The recombinant *T. canis* antigen (RecTcanis) was prepared as previously described [13]. Briefly, the recombinant antigen was expressed in a bacterial system, induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) and purified by using Talon® metal affinity resin (Takara Bio Inc. Japan). The antigen concentration was estimated using Bio-Rad Protein Assay Kit® (Bio-Rad Laboratories, Inc. USA) and stored at −80 °C until use.

RecTcanis-ELISA was performed as previously reported [13] with slight modifications as follows: Microplates (Maxisorp®, Nunc, Copenhagen) were coated with 100 μl of recombinant *T. canis* antigen at a

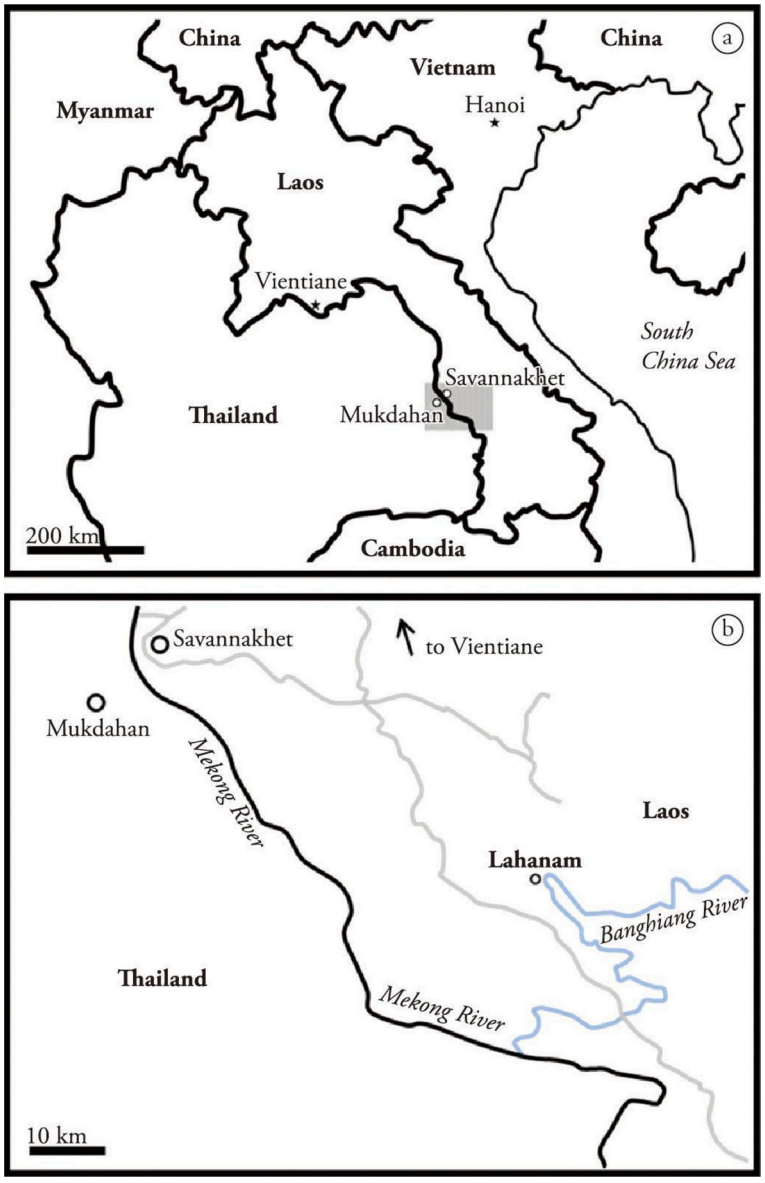

Fig. 1. Maps showing Lao PDR, its neighboring countries, and the study area. a) Map of Lao PDR and neighboring countries. Star "★" corresponds to Vientiane (Capital of Lao PDR) and Hanoi (Capital of Vietnam). Savannakhet City (Lao PDR) and Mukdahan City (Thailand) are indicated by big white circles"○". b) Amplification of the hachured area of the "a" panel, with main routes (grey lines). Mekong River (thick black line) delineates Lao PDR/Thailand international border. Lahanam Village is indicated as a small white circle "○", near Banghiang River (blue line). Adapted from "Nematode infection among ruminants in monsoon climate (Ban-Lahanam, Lao PDR) and its role as food-borne zoonosis". by Sato et al. Rev. Bras Parasitol Vet. 2014;23(1):80–4. (CC BY 4.0). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

concentration of 0.5 μg/ml in carbonate-bicarbonate coating buffer (overnight at 4 °C), blocked with Tris-HCl/Casein buffer (BB) and washed with PBS/Tween 20 washing buffer (WB). Serum samples diluted 1:200 in BB, were applied (100 μl/well) in duplicates and incubated at 37 °C for 1 h. After washing twice in WB, the plates were incubated with 100 μl/well of recombinant protein G conjugated with peroxidase (Invitrogen, USA) diluted 1:1000 in BB at 37 °C for 1 h. For color development, the plates were incubated with 100 μl of peroxidase substrate ABTS™ (Sigma-Aldrich) for 15 min at room temperature. Optical density (OD) was determined at 405/655 nm for each well, in a microplate reader (Immuno Mini NJ-2300, Biotec, Japan). Serum samples giving OD values at 405 nm that were greater than the 4 x OD of a pool of 40 normal human control samples (np40) from Southeast Asian countries, added in each tested plate, were considered to be seropositive. One sample from a confirmed toxocariasis case was added as a positive control for each plate.

2.3. Data analysis

Data collected in paper forms were tabulated and analyzed in Excel 2016 (Microsoft) and EpiInfo version 7.1.5.0 (Centers for Diseases Control and Prevention, Atlanta, GA, USA). Risk predictor variables for *Toxocara* seropositivity were analyzed by the Mid-p exact test at 95% confidence interval with *p*-values of less than 0.05 considered as statistically significant.

2.4. Ethics

This study was approved by the National Ethical Committee for Lao Health Research of the Ministry of Health Lao PDR under the number 172/NECHR.

3. Results and discussion

The population attending the study in Lahanam village were aged from 7 to 59 years old (y/o) (average of 38 y/o) 65.9% (54/82) of them were male and 34.1% (28/82) were female. The total percentage of seropositivity to *Toxocara* sp. was 30.4% (25/82). The potential for cross-reactivity in the used RecTcanis is lower than the TES, though some degrees of cross-reaction could be seen in RecTcanis in schistosomiasis japonica, paragonimiasis, gnathostomiasis, spirometriasis [13]. Such cross-reactivity could be a confounding factor that might inflate the estimated seroprevalence for *Toxocara* sp., despite that the studied area is not endemic for schistosomiasis japonica and there are no reports of human cases of the other cross-reacting infections in this region.

Toxocara seroprevalence in humans has been reported from several countries, and the recent studies are showing varied positivities from 8% to 59.9%. The disease occurs in both developed and underdeveloped countries. In the Netherlands, for instance, an 8.0% *Toxocara* seroprevalence was reported [17], whereas in Poland, seroprevalence in children tested for toxocariasis was 3.0% for preschool children and 7.7% for school children [18]. In the State of São Paulo in Brazil, 6.41% and 2.53% of seroprevalence were reported in individuals less than 15 years old and for older subjects respectively [19]. In Iran, 7.2% of seroprevalence was reported [20]. Our seroprevalence result of 30.4% is more related to other studies done in rural areas of under developed countries, such as China, with 14.4 to 40.6% positivity in children in Jilin and Shandong provinces [21], Another study of ethnic groups in this region showed seropositivity of 14.21 to 20.58% [22]. On the other hand, a study performed in Gabon presented 59.9% of seropositivity [23]. Higher prevalence is related to the presence of risk factors e.g. living in rural areas, close contact with dogs and cats' litter, younger age, and geophagy [20]. The persistence of helminthic NTDs in the environment and animal hosts make eradication a very difficult task [24].

The occurrence of visceral larva migrans by *T. canis* in humans is related to the environmental contamination with eggs spread by its definitive hosts, such as domestic dogs [4,25]. In the studied area, dogs are mainly companion animals, but they breed as free-roaming animals [3]. The animals have free access to kitchens, usually located at the external area of the houses in Lahanam.

In our previous study from Lahanam, *Toxocara* prevalence in dogs was 44% by fecal examination [3]. Although *Toxocara* eggs are easily diagnosed in the feces of definitive hosts and effective anthelmintic drugs are available, infection rates are high in areas where dogs are let free to roam considering diagnosis and treatment of animals are rarely done. The presence of infected dogs roaming free contaminates the environment with *Toxocara* eggs favoring the infection and reinfection of susceptible hosts. Humans are likely to be acquiring this zoonosis without noticing once, up to now, there is no report of clinical toxocariasis in Savannakhet.

3.1. Age and human toxocariasis by serology

No significant difference in seropositivity for gender and age were seen (Figs. 2 and 3). However, higher OD values were observed in the ELISA test as age increased, indicating the possibility of continual exposure to the pathogen (Fig. 2).

Toxocara adult worms have high fecundity, and their eggs are viable for a long time in the environment, even after sludge treatment [25,26]. The resistance of eggs causes cumulative environmental contamination. Therefore, the environmental contamination poses higher infection risk for humans and animals than the one suggested by the infection rate of dogs, as observed in another study in Brazilian municipalities, where the prevalence of *T. canis* was 5.54% in dogs, with 23% rates of environmental contamination with eggs [27].

3.2. Gender and human toxocariasis by serology

There was a significant difference ($p < 0.05$) between genders on the seropositivity for *Toxocara* specific antibodies with 65.9% (54/82) for males and 34.1% (28/82) for females (Fig. 3). Males are more likely to test positives for toxocariasis with a risk ratio of 2.70 (CI95 0.87–4.93). Although we have not evaluated epidemiological features, the results of this study corroborate with similar studies done in the Netherlands where authors reported that increased anti-*Toxocara* IgGs in male subjects presenting older age contact with soil, ownership of cats, cattle or pigs, hay fever, low education, high income and non-Western ethnic origin [17]. The lifestyle in the studied area is predominantly rural, where the men usually take care of livestock, mainly goats and bovines [28], this condition may expose more men to the contaminated environments [24,29]. Considering the behavioral differences as posed by Brei and Fish (2003) [30], males are more likely to be exposed to the pathogens in the environment than females, so the reinfections could increase the humoral response to *Toxocara* as we observed in the age-increasing OD pattern.

4. Conclusions

This is the first survey on human toxocariasis in Laos and we observed that *Toxocara* sp. is in active transmission in Savannakhet, with high seroprevalence in humans which can cause permanent loss of vision, seizures and cases of chronic weakness, abdominal pain and allergy. The presence of infected definitive hosts and a favorable environmental condition for the infection of animals and humans requires urgent action to control the human infection especially in children. It includes health education in order to inform the risks and the need of collecting dogs feces from soil/public areas, improving the environmental conditions to diminish the risk of contact with *Toxocara* eggs and treating the pregnant bitch, puppies and dogs/cats with anthelmintics to reduce environmental contamination. Nevertheless, awareness of the

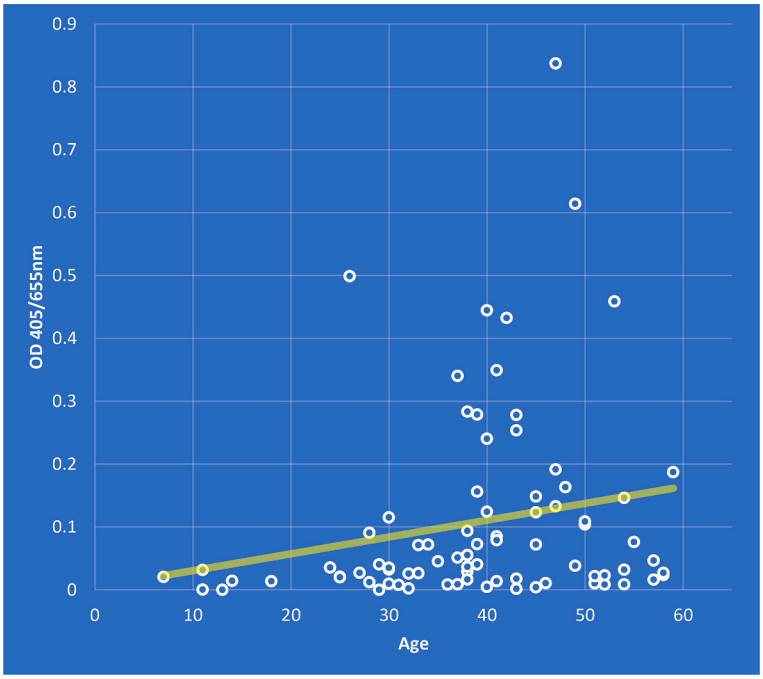

Fig. 2. Results of OD values by age by ELISA using recombinant antigen (RecTcanis-ELISA) for toxocariasis in Lahanam, Savannakhet, Lao-PDR.

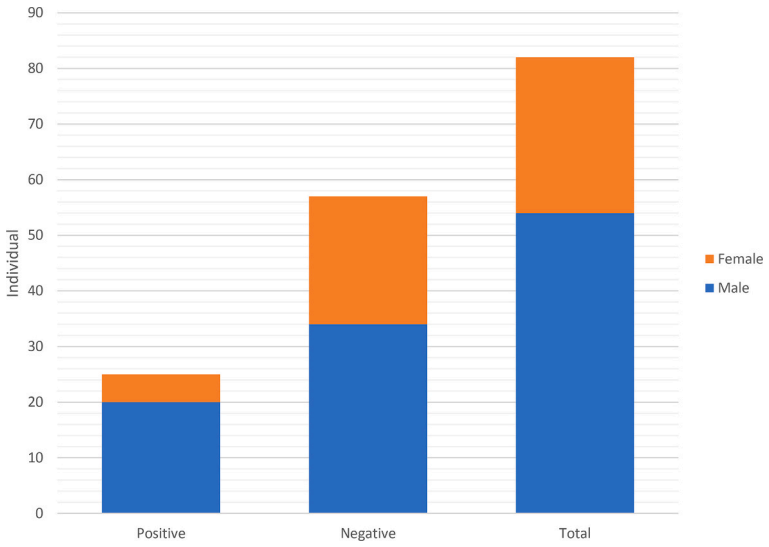

Fig. 3. Seropositivity and gender by ELISA using recombinant antigen (RecTcanis-ELISA) for toxocariasis in Lahanam, Savannakhet, Lao-PDR.

health system about the high prevalence of toxocariasis in Savannakhet can prevent irreversible consequences caused by this silent chronic disease revealed in Lahanam area.

Declaration of Competing Interest

None.

Acknowledgments

We are thankful to Mr. Nirandorn Homsuwan of the Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, Mrs. Vongphaka Boutsyhalath of the Health Centre, Lahanam Village, Sonkon District, Savannakhet Province, Laos, for their generous help in the field. Thanks to Dr. Carol Bain of the Department of Pathology, School of Medicine, Indiana University for kindly reading and improving the manuscript. This research was partially supported by the RIHN research project on "Environmental Changes and Infectious Diseases in Tropical Asia" (R-04) from the Research Institute for Humanity and Nature, Japan (for Kazuhiko Moji), and the Thailand Research Fund (BRG 5180005) from the Faculty of Tropical Medicine, Mahidol University, Thailand (for Jitra Waikagul).

References

[1] G. Acosta-Jamett, S. Cleaveland, A.A. Cunningham, B.M. Bronsvoort, Demography of domestic dogs in rural and urban areas of the Coquimbo region of Chile and implications for disease transmission, Prev Vet Med 94 (2010) 272–281, https://doi.org/10.1016/j.prevetmed.2010.01.002.

[2] R. Ngui, S.C. Lee, N.J. Yap, T.K. Tan, R.M. Aidil, K.H. Chua, et al., Gastrointestinal parasites in rural dogs and cats in Selangor and Pahang states in peninsular Malaysia, Acta Parasitol. 59 (2014) 737–744, https://doi.org/10.2478/s11686-014-0306-3.

[3] M. Otake Sato, M. Sato, T. Yoonuan, T. Pongvongsa, S. Sanguankiat, S. Kounnavong, et al., The role of domestic dogs in the transmission of zoonotic

helminthes in a rural area of Mekong river basin, Acta Parasitol. 62 (2017) 393–400, https://doi.org/10.1515/ap-2017-0047.
[4] D. Despommier, Toxocariasis: clinical aspects, epidemiology, medical ecology, and molecular aspects, Clin. Microbiol. Rev. 16 (2003) 265–272, https://doi.org/10.1128/cmr.16.2.265-272.2003.
[5] S.J. Ahn, N.K. Ryoo, S.J. Woo, Ocular toxocariasis: clinical features, diagnosis, treatment, and prevention, Asia Pac Allergy 4 (2014) 134–141, https://doi.org/10.5415/apallergy.2014.4.3.134.
[6] J.F. Magnaval, V. Galindo, L.T. Glickman, M. Clanet, Human *Toxocara* infection of the central nervous system and neurological disorders: a case-control study, Parasitology 115 (1997) 537–543, https://doi.org/10.1017/S0031182097001558.
[7] D.H. de Savigny, A. Voller, A.W. Woodruff, Toxocariasis: serodiagnosis by enzyme immunoassay, J. Clin. Pathol. 32 (1979) 284–288, https://doi.org/10.1136/jcp.32.3.284.
[8] P. Jacquier, B. Gottstein, Y. Stingelin, J. Eckert, Immunodiagnosis of toxocarosis in humans: evaluation of a new enzyme-linked immunosorbent assay kit, J. Clin. Microbiol. 29 (1991) 1831–1835, https://doi.org/10.1128/JCM.29.9.1831-1835.1991.
[9] J.F. Magnaval, R. Fabre, P. Maurieres, J.P. Charlet, B. de Larrard, Application of the Western blotting procedure for the immunodiagnosis of human toxocariasis, Parasitol. Res. 77 (1991) 697–702, https://doi.org/10.1007/BF00928685.
[10] N. Akao, A.E. Chu, S. Tsukidate, K. Fujita, A rapid and sensitive screening kit for the detection of anti-*Toxocara* larval ES antibodies, Parasitol. Int. 46 (1997) 189–195, https://doi.org/10.1016/S1383-5769(97)00027-5.
[11] S.H. Gillespie, D. Bidwell, A. Voller, B.D. Robertson, R.M. Maizels, Diagnosis of human toxocariasis by antigen capture enzyme linked immunosorbent assay, J. Clin. Pathol. 46 (1993) 551–554, https://doi.org/10.1136/jcp.46.6.551.
[12] N.R. Lynch, L.K. Wilkes, A.N. Hodgen, K.J. Turner, Specificity of *Toxocara* ELISA in tropical populations, Parasite Immunol. 10 (1988) 323–337, https://doi.org/10.1111/j.1365-3024.1988.tb00224.x.
[13] H. Yamasaki, K. Araki, P.K. Lim, N. Zasmy, J.W. Mak, R. Taib, et al., Development of a highly specific recombinant *Toxocara canis* second-stage larva excretory-secretory antigen for immunodiagnosis of human toxocariasis, J. Clin. Microbiol. 38 (2000) 1409–1413, https://doi.org/10.1128/JCM.38.4.1409-1413.2000.
[14] R.A.L. Coêlho, L.B. Carvalho, E.P. Perez Jr., K. Araki, T. Takeuchi, A. Ito, et al., Prevalence of toxocariasis in northeastern Brazil based on serology using recombinant *Toxocara canis* antigen, Am J Trop Med Hyg 72 (2005) 103–107, https://doi.org/10.4269/ajtmh.2005.72.103.
[15] T. Ahn, N. Houki, Y. Ohkura, K. Masui, H. Fukui, K. Shimada, et al., Serologically diagnosed toxocariasis with hemophagocytic syndrome in a patient with primary biliary cirrhosis, Intern. Med. 45 (2006) 31–32, https://doi.org/10.2169/internalmedicine.45.1485.
[16] A. Nkouawa, Y. Sako, S. Itoh, A. Kouojip-Mabou, C. Nadège Nganou, Y. Saijo, et al., Serological studies of neurologic helminthic infections in rural areas of Southwest Cameroon: toxocariasis, cysticercosis and paragonimiasis, PLoS Negl. Trop. Dis. 4 (2010) e732, https://doi.org/10.1371/journal.pntd.0000732.
[17] L. Mughini-Gras, M. Harms, W. van Pelt, E. Pinelli, T. Kortbeek, Seroepidemiology of human *Toxocara* and *Ascaris* infections in the Netherlands, Parasitol. Res. 115 (2016) 3779–3794, https://doi.org/10.1007/s00436-016-5139-6.
[18] A. Kroten, K. Toczylowski, B. Kiziewicz, E. Oldak, A. Sulik, Environmental contamination with *Toxocara* eggs and seroprevalence of toxocariasis in children of northeastern Poland, Parasitol. Res. 115 (2016) 205–209, https://doi.org/10.1007/s00436-015-4736-0.
[19] P.P. Chieffi, M. Ueda, E.D. Camargo, A.M.C. de Souza, M.L. da Silva Guedes, L. J. Gerbi, et al., Visceral larva migrans: a seroepidemiological survey in five municipalities of São Paulo state, Brazil, Rev Inst Med Trop S Paulo 32 (1990) 204–210, https://doi.org/10.1590/S0036-46651990000300010.
[20] M.R. Rezaiemanesh, M. Afzalaghaee, S. Hamidi, A. Eshaghzadeh, M. Paydar, S. H. Hejazi, Prevalence of toxocariasis and its related risk factors in humans, dogs and cats in northeastern Iran: a population-based study, Trans. R. Soc. Trop. Med. Hyg. 113 (2019) 399–409, https://doi.org/10.1093/trstmh/trz011.
[21] W. Cong, Q.F. Meng, H.L. You, N. Zhou, X.Y. Dong, W. Dong, et al., Seroprevalence and risk factors of *Toxocara* infection among children in Shandong and Jilin provinces, China, Acta Trop. 152 (2015) 215–219, https://doi.org/10.1016/j.actatropica.2015.09.008.
[22] G.L. Yang, X.X. Zhang, C.W. Shi, W.T. Yang, Y.L. Jiang, Z.T. Wei, et al., Seroprevalence and associated risk factors of *Toxocara* infection in Korean, Manchu, Mongol, and Han ethnic groups in northern China, Epidemiol. Infect. 144 (2016) 3101–3107, https://doi.org/10.1017/S0950268816001631.
[23] F. Lötsch, M. Obermüller, J. Mischlinger, G. Mombo-Ngoma, M. Groger, A. A. Adegnika, et al., Seroprevalence of *Toxocara* spp. in a rural population in central African Gabon, Parasitol. Int. 65 (2016) 632–634, https://doi.org/10.1016/j.parint.2016.09.001.
[24] M.O. Sato, P. Adsakwattana, I.K.C. Fontanilla, J. Kobayashi, M. Sato, T. Pongvongsa, et al., Odds, challenges and new approaches in the control of helminthiasis, an Asian study, Par Epidemiol and Control e00083 (2019) 4, https://doi.org/10.1016/j.parepi.2018.e00083.
[25] H. Mizgajska-Wiktor, W. Jarosz, R. Fogt-Wyrwas, A. Drzewiecka, Distribution and dynamics of soil contamination with *Toxocara canis* and *Toxocara cati* eggs in Poland and prevention measures proposed after 20 years of study, Vet. Parasitol. 234 (2017) 1–9, https://doi.org/10.1016/j.vetpar.2016.12.011.
[26] M.I. Black, P.V. Scarpino, C.J. O'Donnell, K.B. Meyer, J.V. Jones, E.S. Kaneshiro, Survival rates of parasite eggs in sludge during aerobic and anaerobic digestion, Appl. Environ. Microbiol. 44 (1982) 1138–1143, https://doi.org/10.1128/AEM.44.5.1138-1143.1982.
[27] J.M. Costa-Cruz, R.S. Nunes, A.G. Buso, Presence of *Toxocara* spp eggs in public squares of Uberlandia city, Minas Gerais, Brazil, Rev Inst Med Trop S Paulo 36 (1994) 39–42, https://doi.org/10.1590/S0036-46651994000100007.
[28] M.O. Sato, M. Sato, K. Chaisiri, W. Maipanich, T. Yoonuan, S. Sanguankiat, et al., Nematode infection among ruminants in monsoon climate (ban-Lahanam, Lao PDR) and its role as food-borne zoonosis, Rev. Bras. Parasitol. Vet. 23 (2014) 80–84, https://doi.org/10.1590/s1984-29612014011.
[29] X. Gao, H. Wang, J. Li, H. Qin, J. Xiao, Influence of land use and meteorological factors on the spatial distribution of *Toxocara canis* and *Toxocara cati* eggs in soil in urban areas, Vet. Parasitol. 233 (2017) 80–85, https://doi.org/10.1016/j.vetpar.2016.12.004.
[30] B. Brei, D. Fish, Comment on "parasites as a viability cost of sexual selection in natural populations of mammals", Science 55a (2003) 300, https://doi.org/10.1126/science.1081430.