PARASITOLOGICAL INVESTIGATIONS IN THE VIEW OF TRICHINELLOSIS ON WILD ANIMALS

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Abstract. The studies have shown that the distribution of this parasite is globally vast, and it is present in both temperate, tropical and arctic areas. The research aimed at isolating and examining the *Trichinella* spp. larvae from samples of muscle tissue collected from wild animals from the forest enviroment in the Dolj County (Radovan forest): fox *Vulpes vulpes* (Linnaeus, 1758), (2 samples), wild boar *Sus scrofa* (Linnaeus, 1758), and common rat *Rattus norvegicus* (Berkenhout, 1769) (a sample), mentioning that the disease was reported in 1999 in other species of wild animals raised in captivity (*Panthera leo* - lion) (Linnaeus, 1758) – The Zoo from the "Nicolae Romanescu" Park in Craiova, and the causes that led to the death of the animal being frequent feeding with infested horse meat. The experiments were performed using the equipments and devices of the parasitology laboratory within the L.S.V.S.A – Dolj, by two methods: the artificial digestion method and the *Trichinella* spp. parasite detection method by trichinelloscopic examination. Trichinelloscopic examination allows the differentiation of *Trichinella* and *Sarcocystis* cysts, the latter being arranged among the muscle fibers not being delimited by fibrillar layers that form a secondary cystic wall. *Trichinella spiralis* is the *Trichinella* species with the highest infectivity of domestic pigs *Sus scrofa* (Linnaeus, 1758), *Rattus norvegicus* rats (Berkenhout, 1769) and *Mus musculus* mice (Berkenhout, 1769). It can be said that that the wild environment can still be considered a reservoir of *Trichinella* spp. for domestic animal species. The results of the performed investigations may provide information for taking action regarding the diffusibility of trichinellosis in the forest environment such as the proper lifting and disposal of the wild animal carcasses from forests, rodent control around farms or households.

Keywords: parasites, *Trichinella* spp., wild animals.

Rezumat. Investigații parazitologice în direcția trichinelozei la animale sălbatice. Studiile au arătat că distribuția acestui parazit la nivel global este vastă, acesta fiind prezent atât în zonele temperate, tropicale cât și în zonele arctice. Cercetarea a avut ca scop izolarea și examinarea larvelor de *Trichinella* spp. pe probe de țesut muscular recoltat de la animale sălbatice provenite din mediul forestier de pe raza județului Dolj (pădurea Radovan): vulpe *Vulpes vulpes* (Linnaeus, 1758), (2 probe), porc mistreț *Sus scrofa* (Linnaeus, 1758) (o probă), șobolan comun *Rattus norvegicus* (Berkenhout, 1769) (o probă), menționând că parazitul a fost semnalat și în anul 1999 și la alte specii de animale crescute în captivitate (*Panthera leo* - leu) (Linnaeus, 1758) - Grădina Zoologică din incinta Parcului ”Nicolae Romanescu” din Craiova, cauzele care au dus la moartea animalului fiind hrănirea frecventă cu carne de cal infestată. Experimentele s-au efectuat utilizând echipamentele și aparatura laboratorului de parazitologie din cadrul L. S. V. S. A – Dolj, prin două metode: metoda digestiei artificiale și metoda detecției parazitului *Trichinella* spp. prin examen trichineloscopic. Examenul trichineloscopic permite diferențierea chiștilor de *Trichinella* și cei de *Sarcocystis*, cei din urmă fiind dispuși printre fibrele musculare, nefiind delimitați de straturi fibrilare care să formeze un perete chistic secundar. *Trichinella spiralis* este parazitul cu cea mai mare infectivitate a porcilor domestici *Sus scrofa* (Linnaeus, 1758), șobolanilor *Rattus norvegicus* (Berkenhout, 1769) și șoarecilor *Mus musculus* (Berkenhout, 1769), aceștia fiind principalii vectori transmițători. S-a constatat că mediul sălbatic, poate fi considerat în continuare rezervor de *Trichinella* spp. pentru speciile de animale domestice. Rezultatele investigațiilor efectuate pot furniza informații pentru luarea de măsuri în privința difuzibilității trichinelozei în mediu silvatic cum ar fi ridicarea și eliminarea corespunzătoare a cadavrelor animalelor sălbatice din păduri, combaterea rozătoarelor din jurul fermelor sau a gospodăriilor populației.

Cuvinte cheie: paraziți, *Trichinella* spp., animale sălbatice.

INTRODUCTION

The Radovan Forest is located in the Oltenia Region, 30 km from Craiova, on the border between the Băileşti Plain and the Strehaia Platform (Getic Piedmont). It is located at about 80 m altitude, and it is part of the Protected Natural Area of National Interest Rea Valley - Radovan, a protected natural land area with an area of 20ha (Fig. 1). The forest consists largely of deciduous species of oak, downy oak and ash (*Quercus cerris*, *Q. frainetto*, *Q. pubescens* and *Fraxinus angustifolia),* being the trophic base of many animals. The study was conducted in 2019 on muscle tissue belonging to three species of wild animals: *Vulpes vulpes, Sus scrofa, Rattus norvegicus* (GOGA, 2012), from the Radovan Forest, with the aim of isolating and examining the larvae of *Trichinella* spp. The purpose of these investigations was to demonstrate that the disease exists in the current forest environment (with the spread and maintenance of farm domestic outbreaks, households, population). The causative agent of trichinellosis is a parasitic worm that cannot be see with the naked eye, that is located as an adult in the intestine and as a larva in the striated muscles in both humans and animals. (CIROCEANU & ISPAS, 2002; BORONTEA, 2009). The symptoms of trichinellosis can be confused with the symptoms of several infections and they are difficult to diagnose clinically (CAPO & DESPOMMIER, 1996; WUTIWES et al., 1998), so preventive measures have been proposed to combat it, such as examination in specialized laboratories before the meat is consumed and marketed. At the level of slaughterhouses, depending on the origin of the slaughtered animals and their traceability, it is preferable to perform trichinoscopic examination in the form of artificial digestion. In authorized pig farms, an immunodiagnostic surveillance plan shall be drawn and complied and the result shall condition the trichinoscopic examination. Samples from hunted wild animals are examined by artificial digestion and the presence of various parasites can be confirmed or denied (*Trichinella* spp., *Alaria alata, Toxocara* sp.) (ROTARY & DAN, 2005).

Figure 1. Location of the Radovan forest in the Dolj County. (Source: https://maps.google.com, July 2021).

MATERIAL AND METHODS

The artificial digestion method is accredited according to the international standard ISO 18743 first edition 15-09-2015 - Microbiology of the food chain. Detection of Trichinella larvae in meat by the artificial digestion method. (Fig. 2). The artificial digestion method is accepted as an international reference method (according to the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, OIE, 8th edition, chap. Trichinellosis (infection with *Trichinella* spp. Chap.3.1.20) and is based on the enzymatic degradation of fibers muscle in a fluid composed of pepsin and hydrochloric acid and then by sedimentation and washing processes, where the presence or absence of these larvae is identified. For each individual sample, the artificial gastric juice was prepared and four digestions were performed, calculating separately the required amounts of pepsin and hydrochloric acid depending on the amount of sample according to the following table, taking into account that two liters of artificial gastric juice made of two liters of water, 16 ml of hydrochloric acid and 10 g of pepsin are required for 100 g of striated muscle (SR ISO 18743) (Table 1).

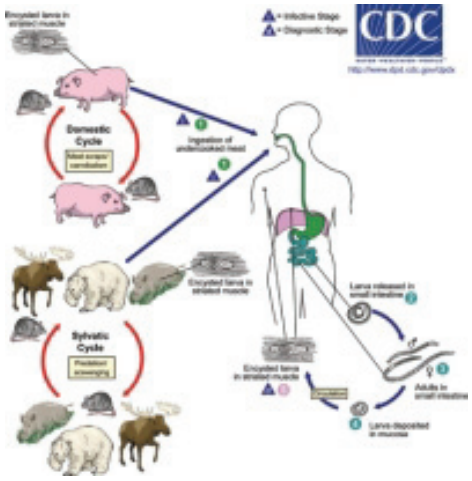

Figure 2. Life cycle of *Trichinella* spp.
(https://www.sciencedirect.com/topics/food-science/trichinella).

Table 1. Necessary reagents for the 4 samples subjected to artificial digestion.

Type of the sample	Number of the sample	Total quantity of the sample	Pepsin	Hydrochloric Acid	Water
Fox (*Vulpes vulpes*)	1	6.1 grame	0.61g	0.976 ml	122 ml
Fox (*Vulpes vulpes*)	2	4g	0.4g	0.64 ml	80ml
Rat *Rattus norvegicus*	3	2g	0.2g	0.32ml	40ml
Wild boar *Sus scrofa*	4	50g	5g	8ml	1 000 ml

The preparation of gastric juice for each sample was performed in a container of ideal capacity in which 122ml, 80ml, 40ml and 1000 ml of preheated tap water at 45 ± 2º C were introduced for each digestion, over which the corresponding amount of 25% hydrochloric acid was added, i.e. 0.976ml, 0.64ml, 0.32ml, and 8ml respectively. After homogenization, the required amount of pepsin was added for each sample, i.e. 0.61g, 0.4g, 0.2g, 5g respectively. A stirring rod was inserted into the beaker and then the digestion vessel was placed on a preheated plate and stirring started until the pepsin was completely dissolved (Figs. 3, 4, 5, 6, 7, 8, 9, 10).

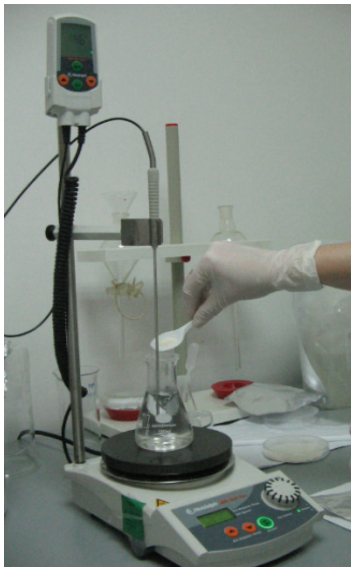

Figure 3. Adding pepsin – fox sample 1 (Photo: Tîmburescu, 2019).

Figure 4. Adding the fox sample 1 (Photo: Tîmburescu, 2019).

Figure 5. The artificial digestion of the fox sample 1 (Photo: Tîmburescu, 2019).

Figure 6. Fox sample 2 and the pepsin necessary for the process (Photo: Tîmburescu, 2019).

Figure 7. Fox sample 2 and hydrochloric acid (Photo: Tîmburescu, 2019).

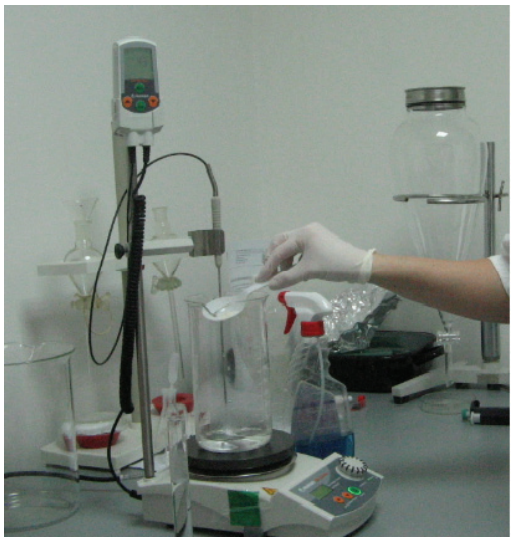

Figure 8. Fox sample 2
adding the pepsin (Photo: Tîmburescu, 2019).

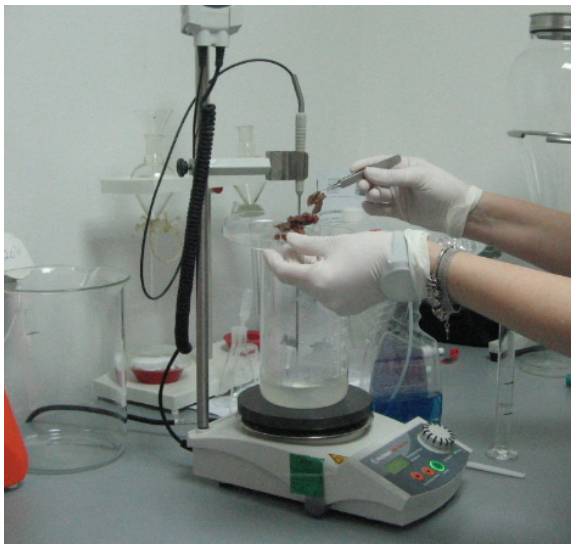

Figure 9. Fox sample 2, adding the chopped meat
(Photo: Tîmburescu, 2019).

Figure.10. Meat sample digestive Fox sample 2 (Photo: Tîmburescu, 2019).

During stirring, the digestion fluid was spun at a speed high enough to form a deep vortex, but without causing splashes, and thus stirred until the pieces of meat were completely gone (about 30 minutes). After the completion of the digestion operation, the filtration step follows so that the digestion liquid obtained from each sample was poured through a special sieve with a porosity of 180µ in the sedimentation funnel. The digestion fluid was left to rest for 30 minutes, long enough to filter the entire amount of fluid. After the expiration of the sedimentation time, the reading of the samples follows, so that 40 ml of the digestion liquid were poured into the graduated test tube and left to rest for 10 minutes. From the 40 ml tube, 30 ml of the supernatant was extracted with a Pasteur pipette and a volume of 10 ml of sediment remained. The remaining 10 ml sediment sample corresponding to each digestion was transferred to a counting basin provided with a trench for examination with a trichinoscope.

The samples with the digestion liquid were clarified as follows: out of the final sample of 40 ml, after its collection in a graduated cylinder and a 10-minute rest, about 30 ml of supernatant were removed, leaving a volume of 10 ml. This volume of 10 ml is increased to 40 ml by adding tap water. After a settling period of 10 minutes, 30 ml of the supernatant is extracted and the remaining 10 ml of sediment is placed in a counting basin with a groove for the examination and counting of *Trichinella* spp. larvae (Fig. 11). The final amount of supernatant, as well as the remaining amount of digested sample was also clarified and examined for all four samples.

The blade compression method and optical trichinoscope examination (TPE) (Figs. 12, 13, 14) - Trichinoscopic examination establishes the detection of *Trichinella* spp. larvae and cysts in muscle tissue samples using the trichino-projector. A working sample of a compressor consisted of 28 sections in which 28 samples with the size of an oat grain were carefully cut and placed. A drop of clarifying solution - 10% potassium hydroxide - was added over each field, and after about 5 minutes - the time allotted for clarifying the sample, the upper plate of the compressor was carefully fixed, and with the palm of the hand was pressed evenly over the entire length of the compressor for performing compression. After that, the nuts were screwed on, and the compressor was ready for examination at the trichino-projector (OIE MANUAL, 2018).

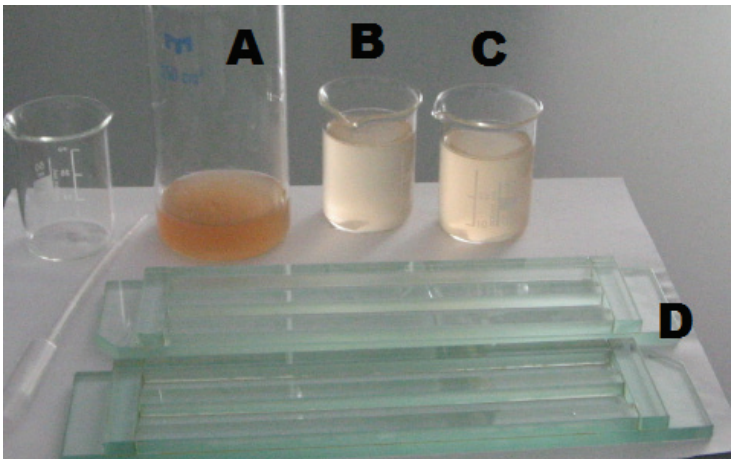

Figure 11. Digestion fluid total amount of the rat sample (A), supernatant - rinse 1 (B), sediment - rinse 1 (C), counting basins (D) (Photo: Tîmburescu, 2019).

Figure 12. Rat display sample (Photo: Tîmburescu, 2019).

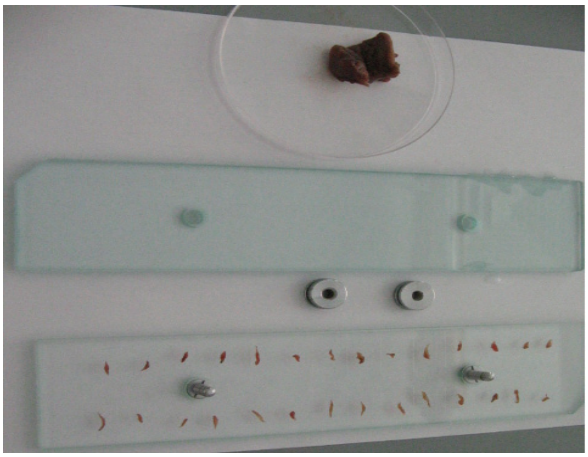

Figure 13. The clarification of the rat display sample (Photo: Tîmburescu, 2019).

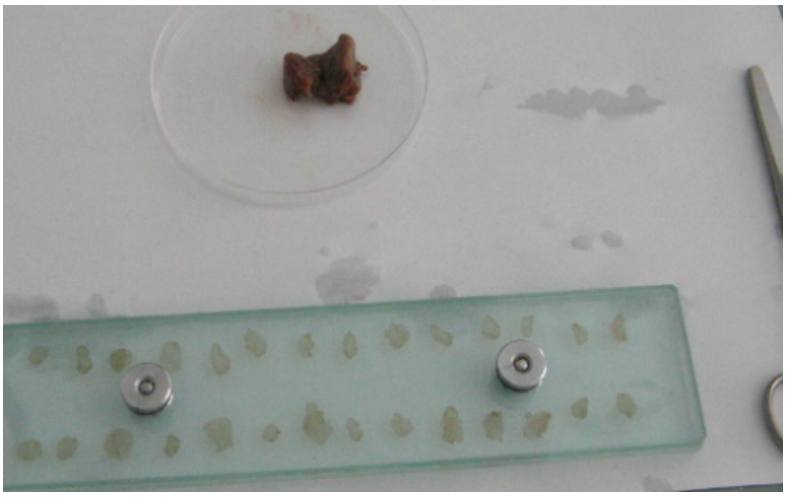

Figure 14. Rat sample compressor ready for examination (Photo: Timburescu, 2019).

RESULTS AND DISCUSSIONS

All the samples examined by the artificial digestion method had a positive result. The total amount of digestion fluid calculated according to the total sample introduced into work was fully examined to create a correlation between the number of larvae counted and the amount of sample and the number of larvae counted per gram. For the samples of fox muscle tissue after a digestion of 30 min, filtration, sedimentation time of 30 min, rinsing and reading, a total number of 118 larvae per the six grams introduced in the work were counted. If from the total of six grams subjected to digestion we found 118 larvae of *Trichinella* spp.., we deduce that in an amount of one gram of sample we find 20 larvae of *Trichinella* spp. Respecting the analogy and the calculation method for the second sample of fox muscle tissue we get a total number of 2318 *Trichinella* spp. larvae per four grams of digested meat and a number of 580 *Trichinella* larvae per gram. It can be deducted that *Trichinella* spp. infection of the second animal is much stronger (Table 2).

In the rat muscle tissue sample, the following values were obtained for the four grams artificially digested, namely 1812 larvae of *Trichinella* spp., and for a quantity of one gram we found 453 larvae of *Trichinella* spp. (Table 2) (MURRELL & BRUSCHI, 1994). For the wild boar sample, the number of *Trichinella* larvae counted per gram of meat introduced into the work was 336 larvae, resulting in a number of 16.800 larvae per a quantity of 50 grams, so we can conclude that the infection of this animal was massive (Table 2).

The larvae of *Trichinella* from wild boar samples were prelevated and send to the Institute of Hygiene and Veterinary Public Health for determination of the *Trichinella* genome. According to the confirmation through the *Trichinella* genome method, and the official analysis papers from the Institute of Hygiene and Veterinary Public Health, 2 species of *Trichinella* spp. were frequently isolated, namely *Trichinella britovi* and *Trichinella spiralis*. *Trichinella spiralis* is the one which is predominating and it is also known that *Trichinella spiralis* is the trichinella species with the highest infectivity of domestic pigs Sus scrofa (Linnaeus, 1758), *Rattus norvegicus* rats (Berkenhout, 1769) and *Mus musculus* mice (Berkenhout, 1769) (MURRELL et al., 2000). Still the most important source of human infection worldwide is the domestic pig *Sus scrofa* (Linnaeus, 1758), but in Europe, horse and wild boar meat has played a significant role during outbreaks in the last 3 decades (KOCIECKA, 2000).

Table 2. The results of four samples subjected to artificial digestion.

Result	**Sample / artificial digestion time**			
	Fox 1/60 min of digestion	**Fox 2 / 60 min of digestion**	**Rat/ 60 min of digestion**	**Wild boar/ 60 min of digestion**
Number of *Trichinella* spp. larvae counted / amount of sample subjected to artificial digestion	135/ 6g	2318/4g	1812/4g	16 800 larve/50g
Number of *Trichinella* spp. larvae / 1g	27	580	453	336

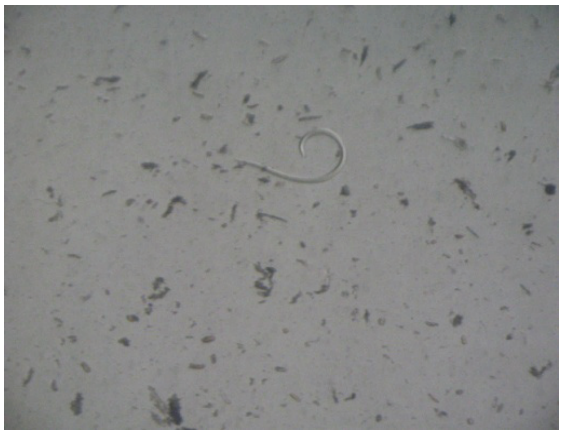

Figure 15. *Trichinella* spp. Larvae - fox sample 1 (Photo: Borontea, 2019).

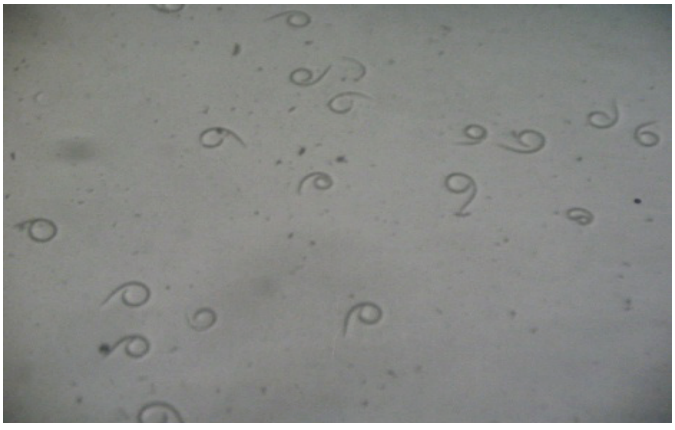

Figure 16. *Trichinella* spp. larvae - fox sample 2 (Photo: Borontea, 2019).

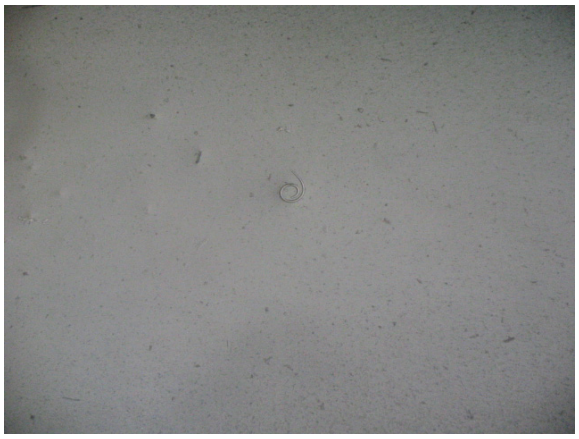

Figure.17. *Trichinella* spp. larvae rat sample
(Photo: Borontea, 2019).

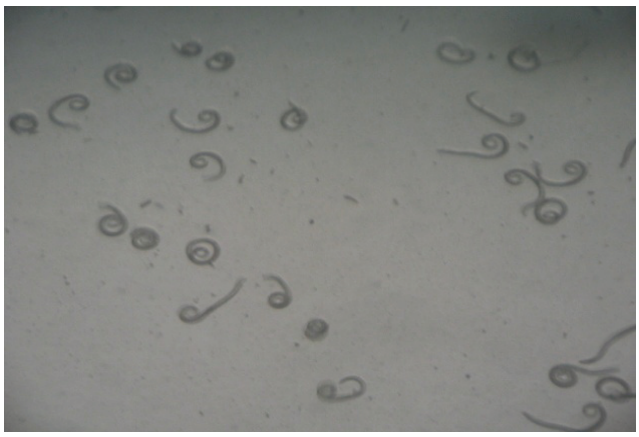

Figure 18. *Trichinella* spp. larvae wild boar sample
(Photo: Borontea, 2019).

The trichineloscopic examination was performed using a screened trichinoscope using the 40X objective. All four samples were tested positive for *Trichinella* spp. At the foxes samples, and wild boar the *Trichinella* spp. larvae were surrounded by a cyst while on the rat sample the *Trichinella* spp. larvae were free. This could mean that the infection with *Trichinella* spp. was recent. (Figs. 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32).

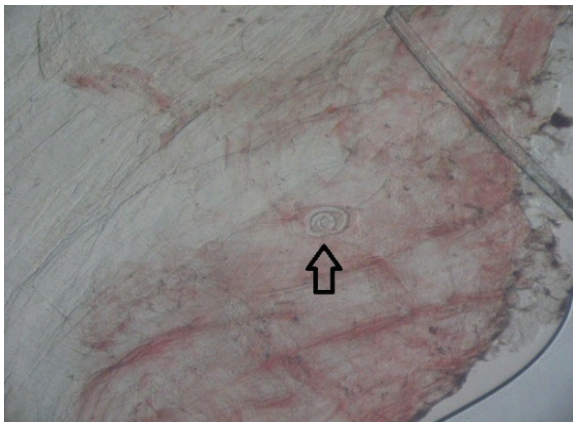

Figure 19. Non cysted *Trichinella* spp. larvae rat sample
(photo: Borontea, 2019).

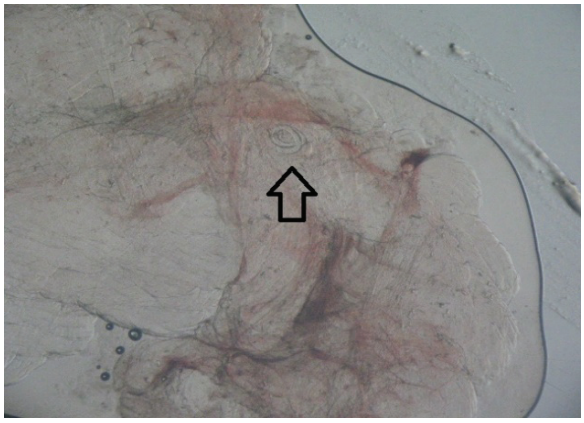

Figure 20. Non cysted *Trichinella* spp. larvae rat sample
(photo: Borontea, 2019).

Figure 21. Non cysted *Trichinella* spp. larvae rat sample (photo: Borontea, 2019).

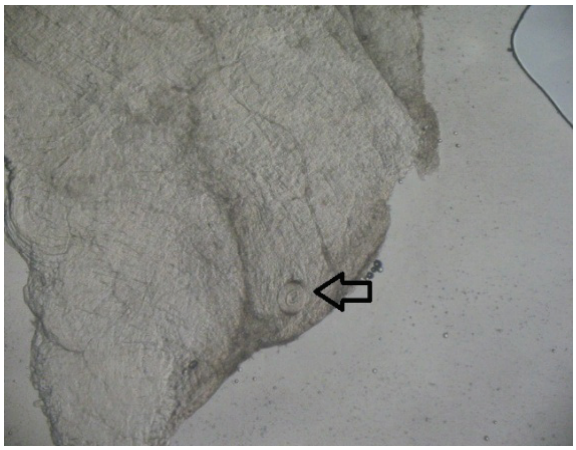

Figure 22. Cyst of *Trichinella* spp. - fox sample 1 (photo: Borontea, 2019).

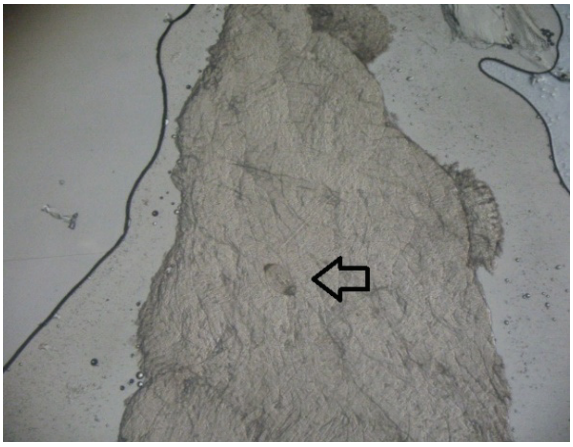

Figure 23. Cyst of *Trichinella* spp. - fox sample 1 (photo: Borontea, 2019).

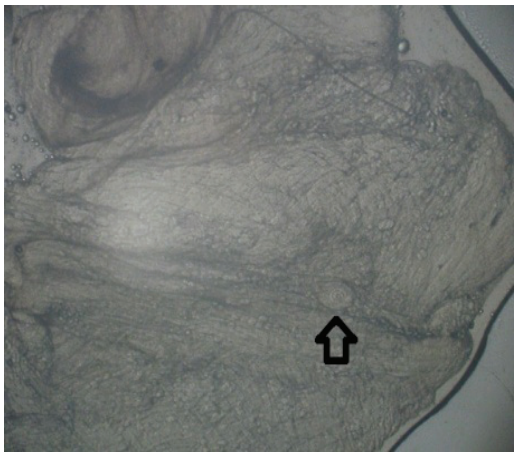

Figure 24. Cyst of *Trichinella* spp. - fox sample 1 (photo: Borontea, 2019).

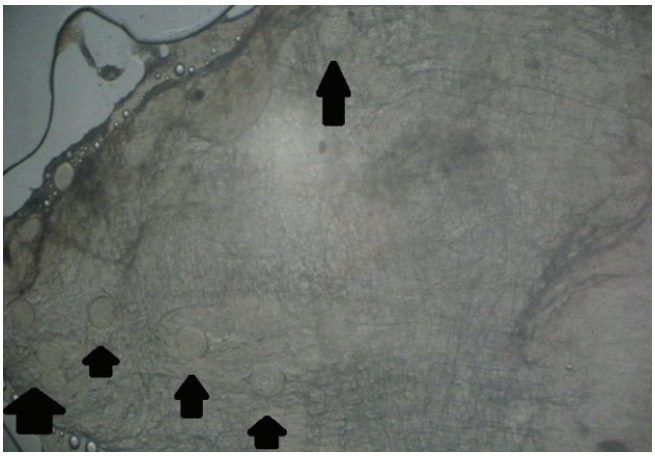

Figure 25. Cysts of *Trichinella* spp. - fox sample 2 (photo: Borontea, 2019).

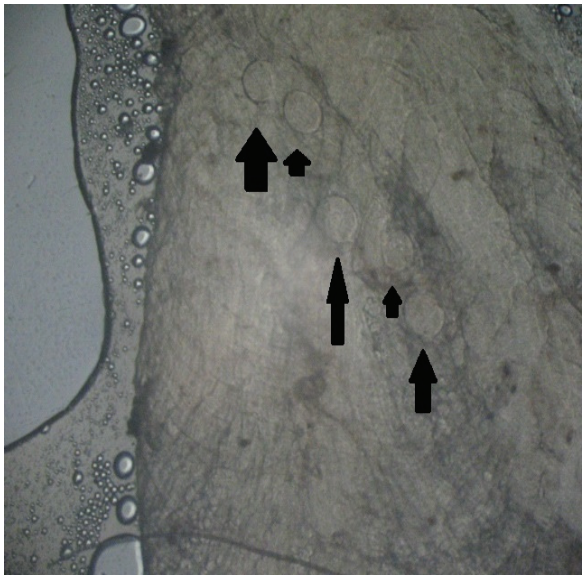

Figure 26. Cysts of *Trichinella* spp. - fox sample 2 (photo: Borontea, 2019).

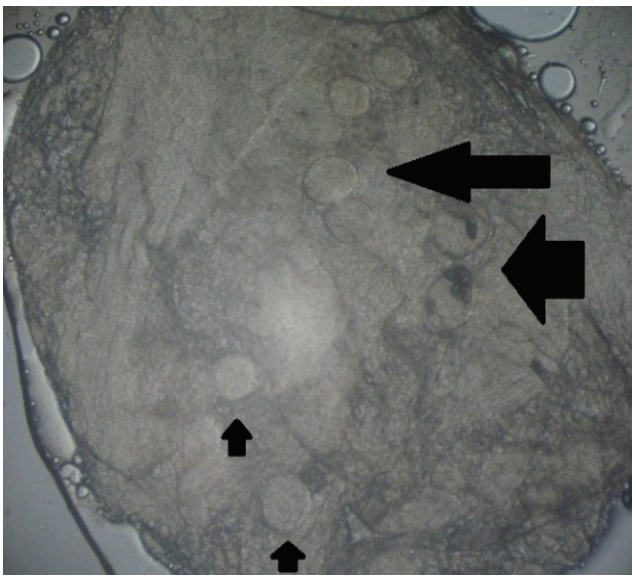

Figure 27. Cysts of *Trichinella* spp. - fox sample 2 (photo: Borontea, 2019).

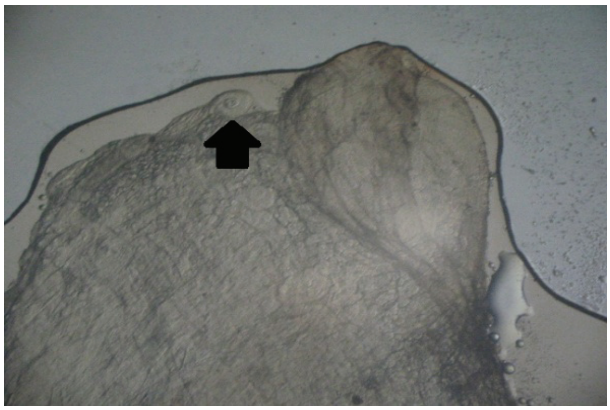

Figure 28. Cyst of *Trichinella* spp. - fox sample 2 (photo: Borontea, 2019).

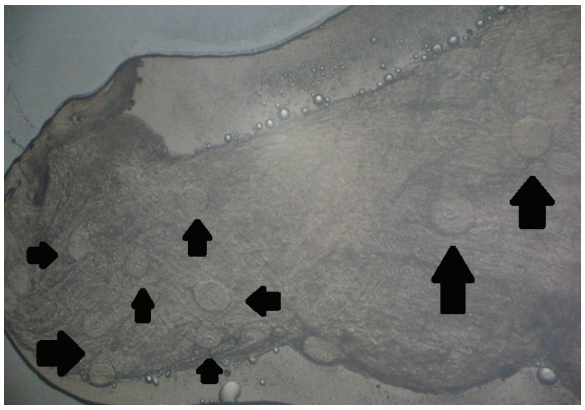

Figure 29. Cysts of *Trichinella* spp. - fox sample 2 (photo: Borontea, 2019).

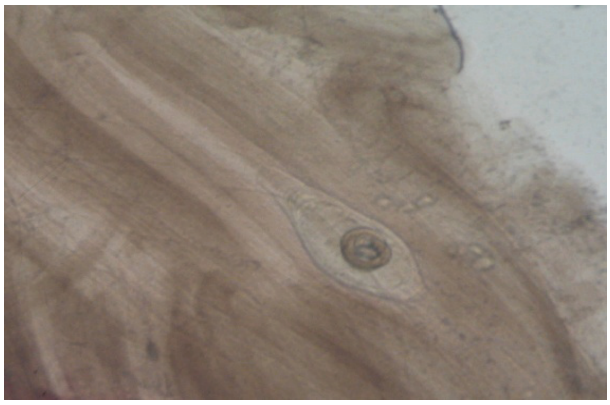

Figure 30. Cyst of *Trichinella* spp. - wild boar sample (photo: Borontea, 2019).

Figure 31. Cyst of *Trichinella* spp. - wild boar sample (photo: Borontea, 2019).

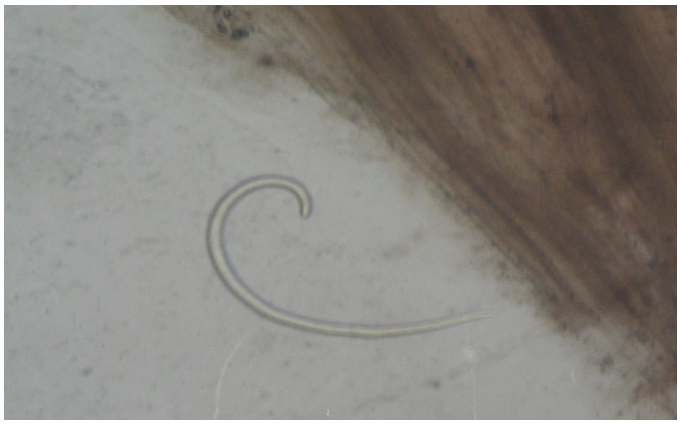

Figure 32. *Trichinella* spp. larvae - wild boar sample - the trichinoscopic examination (photo: Borontea, 2019).

CONCLUSIONS

The used procedures – the detection of Trichinella spp. through the method of artificial digestion and detection of *Trichinella* spp. by the trichinoscopic examination – are effective methods and comply with national and international standards used in the veterinary field.

The results of the performed investigations may provide information for taking action regarding the diffusibility of trichinellosis in the forest environment such as the proper lifting and disposal of the wild animal carcasses from forests, the control of rodents around farms or households.

The isolation of larvae by the digestion method led to the identification of *Trichinella* spp. existing in the Dolj county, results that were confirmed by official test papers of the Institute of Veterinary Hygiene and Public Health.

In the Dolj county, according to the confirmation of this official paper from the Institute of Hygiene and Veterinary Public Health, 2 species of *Trichinella* spp. were frequently isolated, namely *Trichinella britovi* and *Trichinella spiralis.*

Trichinella spiralis is the one which is predominating. *Trichinella spiralis* is the trichinella species with the highest infectivity of domestic pigs *Sus scrofa* (Linnaeus, 1758), *Rattus norvegicus* rats (Berkenhout, 1769) and *Mus musculus* mice (Berkenhout, 1769) .

Trichinella britovi is relatively common in wild carnivores (canids, cats), bears, wild boars, horses and it is very similar to *Trichinella spiralis*, which is why it has recently been recognized as a separate species. It can be said that the wild environment can still be considered a reservoir of *Trichinella* spp. for domestic animal species. Due to the fact that it is a zoonosis that is also transmitted to humans, many countries have focused on controlling and eliminating *Trichinella* from the food chain.

The most important source of human infection worldwide still is the domestic pig *Sus scrofa* (Linnaeus, 1758), but in Europe, horse and wild boar meat has played a significant role during outbreaks in the last 3 decades.

Infection in humans occurs through the ingestion of animal meat containing *Trichinella* cysts or larvae. Early clinical diagnosis of trichinosis is quite difficult because there are no pathognomonic signs or symptoms.

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