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Characterization of host-parasite interactions during the experimental *Trichinella spiralis* infection in pigs

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Summary

The aim of the present experiment was to assess the clinical, haematological, biochemical and immunological responses of pigs experimentally infected with *Trichinella spiralis*. One group of 6 pigs was infected with 1500 larvae/kg body weight and one group of 7, was maintained as control. The highest larval density was in the diaphragm (504.3 ± 8.2). During the experimental *T. spiralis* infection in pigs, increased values of total granulocyte number, eosinophils, blood glucose, K+ concentration, CK, ALT, AST and ALP were registered in different stages of infection. A slight increase of the phagocytic activity was registered 14 days p.i., in the infected group. Significantly increased values in lymphocyte activity, in infected group, was observed 30 and 60 days p.i. comparative with control. The specific IgG antibodies were detectable by ELISA at 14 days p.i. The results obtained in this study provide a better understanding about complete interactions occurring during *Trichinella* infection in pigs.

Keywords: *Trichinella spiralis*; RCI, phagocytosis ; blastic transformation; ELISA

Introduction

Trichinosis is a reportable zoonotic disease, because of the recurring sporadic outbreaks in humans who have consumed undercooked or raw infected meat from swine or wild mammals. Most of the human infections and deaths around the world are caused by *Trichinella spiralis* and its pathogenicity is higher than that of other species due to the higher number of newborn larvae produced by the females (Pozio *et al*., 1992) and a stronger immune reaction induced in humans relative to other genotypes (Pozio *et al*., 1993; Bruschi *et al*., 1999; Gomez Morales *et al*., 2002).
Trichinella spiralis has a worldwide distribution, because it has been passively imported into most continents due to its high infectivity to swine and synanthropic rats (Pozio, 2001a). This species has also been detected in wildlife in temperate regions because of the improper human behavior (Pozio, 2001b). According to a survey of the International Commission on Trichinellosis completed in 2004, Romania has the most cases of human trichinellosis in the world (Blaga *et al*., 2007). Both the domestic and the sylvatic cycles of *Trichinella* are present in Romania (Pozio, 2001a)
The parasitic nematode is unique in that it lives most of its life intracellularly (Despommier, 1983). Molting, growth and development culminating in sexually mature adult worms occur within the cytoplasm of columnar epithelial cells in the small intestine (Wright, 1979). Newborn larvae are shed by the female into this niche, from where they migrate and finally penetrate the striated skeletal muscle cell (Despommier, 1983). Here they induce the formation of a specialized unit known as the nurse cell (Purkerson & Despommier, 1974).
The aim of the present study was to evaluate clinical, biochemical, haematological and immunological changes in swine following *T. spiralis* infection.

Materials and methods

Experimental design
A total of 13 Large White pigs were divided in two groups, as infected (6; 3 males and 3 females) and control (7; 3 males and 4 females). The animals used in this experiment were treated in accordance with the animal ethics laws of the EU. The pigs were between 2 – 3 months and they had a body weight ranging between 6 and 15 kg. During the experiment the pigs were maintained in swine breeding facility belonging to the Faculty of Veterinary Medicine in Cluj-Napoca under normal housing conditions. They were fed with special swine concentrated formula and the water was provided ad libitum.
The parasites were isolated from muscles of a naturally infected pig by artificial digestion (Gamble *et al*., 2000)

Table 1. Versions for assessing the phagocytic activity

	Negative control[1]	Positive control[2]	Sample[3]
Blood	0.5 ml	0.5 ml	0.5 ml
China ink*	1.5 µl	1.5 µl	1.5 µl
PHA**	-	6 µl	-
AgTr***	-	-	9 µl

** PHA – phytohaemagglutinin, was used as a positive control, because of its lymphocyte stimulation capacity
*** AgTr – *Trichinella* antigens, prepared by sonication of freshly digested larvae as showed in a previously published protocol (Perrudet-Badoux *et al*., 1975)
150 µl of the [1,2,3] mixture were passed in 3 ml saline solution, centrifuged for 5 min at 1500 rpm; the results were evaluated by spectrophotometry at 535(540)nm at 0 minutes (T0), 30 minutes (T30), 60 minutes (T60)

and maintained in mice. *T. spiralis* was confirmed by PCR according to a previously described protocol (Zarlenga *et al*., 1999).The experimental infection was done orally with 1500 larvae of *T. spiralis*/kg. All the infected pigs were individually housed in cages for avoiding parasite passage. During the experiment, animals were closely monitored, observing any changes in clinical status and weight gain. In the first 30 days post inoculation, the body temperature, cardiac and respiration rates were measured every 48 h.
Blood samples were collected by jugular venipuncture at 0, 4, 14, 30, 60 days after experimental infection for assessing the biochemical and haematological changes.
On day 60 p.i. the pigs were slaughtered and immediately dissected. Muscle tissue from diaphragm, tongue, intercostals, gastrocnemius muscle, front leg (biceps) and neck (splenius) were collected for the assessment of larval burden. The larvae were recovered using the artificial digestion (Gamble *et al*, 2000) from 20 g of each muscle sample. Reproduction capacity index (RCI) was calculate by multiplying the LPG (larvae per gram of muscle) with the animal total weight and divided by infection dose (RCI = no. of muscle larvae recovered / no. of infective larvae given) (Bolas-Fernandez & Wakelin, 1989).

Laboratory procedures

Haematological analysis

The haematological analyses were performed with 25 µl of whole blood for each sample using the Auto Veterinary Haematology Analyzer Abacus Junior Vet. The following haematological parameters were determined : white blood cell (WBC), lymphocyte (LYM), granulocyte (GRA), red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), platelet (PLT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDWc), platelet percentage (PCT), mean platelet volume (MPV), platelet distribution width (PDWc). The white blood cell differential count was performed using a simple glass slide with a drop of blood stained with May-Grümwald Giemsa technique.

Biochemical analysis

The sera obtained by centrifugation (2000 x g) were stored at -20ºC until analysis was made. All the biochemical analyses were performed using the spectrophotometer Screen Master Plus.
The blood glucose and cholesterol were determined in visible spectrum at 576 and 505 nm wavelength. The potassium ions were determined by turbidimetric method in UV spectrum at 405 nm.
The enzymatic analysis: aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), creatine kinase (CK), alkaline phosphatase (ALP), were made using the kinetic method in UV spectrum at 340 nm wavelength. The serum creatinine was determined by Jaffe method, in visible spectrum at 505 nm wavelength.

Immunological analysis

Cellular and humoral immune responses were evaluated by *in vitro* phagocytosis assay, phytohemaglutinin (PHA) – stimulated blastic transformation test and enzyme-linked immunosorbent assay (ELISA).

In vitro phagocytosis activity was evaluated using the carbon particle inclusion test. The phagocytic activity was measured with Chinese ink phagocytosis as showed in Table 1.

Table 2. Versions for assessing the blastic transformation

	Control		PHA		AgTr	
SM	200 µl	200 µl	200 µl	200 µl	200 µl	200 µl
PHA	-	-	1 µl	1 µl	-	-
AgTr	-	-	-	-	1.5 µl	1.5 µl
Incubation 48 hours						
CM	12.5 µl		12.5 µl		12.5 µl	
OT	0.5 ml		0.5 ml		0.5 ml	
100 ºC – 8 min						
Reading 610 nm						

PHA stimulated blastic transformation test

Mononuclear cells, sensitized *in vivo* by various antigens, possess the capacity to respond vigorously to the same antigen when contacted *in vitro* (Khokhlova *et al*., 2004).
The blastic transformation test with PHA was performed in 3 double versions: 2 controls, 2 PHA and 2 AgTr, as showed in Table 2. The RPMI 1640 medium was supplemented with fetal calf serum 5 %, penicillin (1000UI/ml) and streptomycin 1000 µg/ml and the mixture was adjusted at pH 7.2 – 7.4 with sodium carbonite 5 %. Every sample (SM) was prepared 1 part blood/3.2 parts medium mixture. The microplate was incubated for 48 hours at 37 ºC in 5 % CO_2 atmosphere. From each double version a common (CM) of 12.5 µl version was made, and finally combined with 0.5 ml ortho-toluidine (OT). The microplate was warmed in shaking water bath at 100 ºC for 8 minutes and

Table 3. Mean values of haematological and biochemical constituents (average ± SD)

Days p.i.	total granulocyte number (10^9/l)		eosinophils %		blood glucose (mg/dl)		K+ concentration (mEq/l)		CK (U/l)		ALT (U/l)		AST (U/l)		ALP (U/l)	
	Infected	Control	Infected	Control	Infected	Control	Infected	Control	Infected	Control	Infected	Control	Infected	Control	Infected	Control
0	7.9 ± 2.1	10.7 ± 2.7	1	1	114.2 ± 20.5	127.6 ± 35.7	2.7 ± 0.5	3.3 ± 0.5	245.8 ± 50.8	281.8 ± 82.2	76.1 ± 13.3	72.2 ± 13.4	45.1 ± 9.0	27.9 ± 12.9	370.8 ± 145.7	369.4 ± 66.2
4	16.5 ± 4.2	11.4 ± 3.6	1	2	126.9 ± 23.4	149.4 ± 23.5	3.6 ± 0.4	3.1 ± 0.4	373.2 ± 59.0	272.2 ± 45.0	61.6 ± 5.7	69.1 ± 10.9	43.7 ± 17.9	60.7 ± 25.9	424.8 ± 106.6	392.3 ± 87.2
14	13.7 ± 3.9	12.3 ± 3.7	2	1	129.8 ± 19.5	126.6 ± 23.1	4.3 ± 0.6	4.4 ± 1.0	353.8 ± 50.6	263.4 ± 53.8	77.5 ± 14.7	69.1 ± 19.9	41.4 ± 8.4	39.2 ± 13.7	469.8 ± 172.7	467.5 ± 100.9
30	10.2 ± 3.8	11.7 ± 2.3	4.5	1	169.5 ± 53.0	109.3 ± 34.4	3.6 ± 0.1	3.9 ± 0.4	348.3 ± 64.2	260.8 ± 36.5	79.2 ± 8.9	83.1 ± 15.1	43.1 ± 10.3	38.9 ± 3.7	549.2 ± 291.9	435.0 ± 73.4
60	14.4 ± 1.6	16.4 ± 3.3	3	1	108.8 ± 10.1	100.0 ± 15.0	4.6 ± 0.6	4.2 ± 0.2	124.9 ± 70.0	265.6 ± 35.4	92.1 ± 28.2	60.3 ± 6.4	29.8 ± 8.4	30.2 ± 4.0	330.3 ± 73.7	402.0 ± 82.1

than was suddenly chilled. The amount of residual glucose in the medium mixture and in the standard glucose was spectrophotometric evaluated at wavelength of 610 nm. Finally, the transformation index (TI) was calculated after the following formula:
TI= [(MG)SG)/MG] x 100, where MG is the glucose concentration in the initial culture medium, and SG the glucose concentration in the sample after incubation.

ELISA
The specific *Trichinella* IgG antibodies were detected by indirect ELISA (plates coated with excretory-secretory antigens) using two commercial kits: SafePath Trichinae Immunoassay Kit (SafePath Laboratories, LLC, USA) and Elitrich ELISA kit (Pasteur Institute, Bucharest). The procedures followed the specific steps mentioned in manufacturer's instructions.

Statistical analysis
Student's t-test was applied to evaluate significant differences in values obtained in infected animals compared to negative controls animals. A p value < 0.05 was considered significant.

were registered in different stages of infection (Table 3).
The granulocyte number was significantly higher (p = 0.03) in infected group (16.54 ± 4.2) at 4 days after infection than in control group (11.43 ± 3.6). The blood cell differential count revealed an increase of eosinophils percentage from 1 % in day 0 to 4.5 % at 30 days p.i., in the infected group, but the values returned normal at 60 days p.i. In the control group, the eosinophils percentage (1 %) remained constant during the experiment. The percentage of eosinophils was not correlated with the total leucocytes count, which remained in the normal range during the experiment. The other haematological parameters studied did not present any significant differences between the two groups.
Blood glucose increased significantly at 30 days p.i. in infected pigs comparative with uninfected ones (p < 0.05).
K+ concentration increased from day 0 (2.7 mEq/l) to day 14 (4.3 mEq/l), than decrease 30 days p.i. (3.6 mEq/l) and again increase 60 days p.i. (4.6 mEq/l) in infected pigs. Significant differences (p < 0.05) were observed between the 4 collecting days in *Trichinella* inoculated group. Differences in K+ concentration in control group and infected group, were significant (p < 0.05) 4 days p.i. With all these

Table 4. LPG values in 6 muscles groups of the 6 infected pigs and the reproductive capacity index (RCI)

Pig (body weight)	**1 (11 kg)**	**2 (10 kg)**	**3 (6 kg)**	**4 (15 kg)**	**5 (18 kg)**	**6 (8 kg)**	
NAL*	16500	15000	9000	22500	27000	12000	
**** MG**							**Mean LPG[1]**
Diaphragm	498	505.5	510	500.2	495	517	504.2
Tongue tip	472	495	500.5	480	475	502	487.4
Biceps	105.3	117	145	115	107.4	112	116.9
Gastrocnemius	87	97	120.2	102	73	92	95.2
Splenius	110	100.2	107.2	113	107.2	121	109.7
Intercostals	77	87	100.5	92	87	97	90
Mean LPG[2]	224.8	233.6	247.2	233.7	224.1	240.1	
± SD***	202.0	206.8	200.5	198.9	202.6	208.9	
RCI	177	202	274	155	141	240	

* NAL- number of administrated larvae; ** MG- muscular groups; ***± SD- standard deviation
[1] average LPG in a certain muscle group;[2] average LPG per animal

Results

Clinical signs
None of the 6 infected pigs presented any clinical sign during the monitored 60 days. The body temperature, cardiac and respiration rates during the intestinal, systemic and muscular stage of infection were in normal range. Beginning with the third week after infection, the weight gain in infected group (6.6 ± 0.8 kg) was significantly (p = 0.005) lower than in control group (8.8 ± 1.3 kg).

Haematology and biochemistry
During the experimental *T. spiralis* infection in pigs, increased values of total granulocyte number, eosinophils, blood glucose, K+ concentration, CK, ALT, AST and ALP fluctuations, the values of blood glucose and K+ were still in the normal range during the experiment.
The level of blood cholesterol was normal during the 60 days and no differences were noted between the groups and between the collection days. The level of CK, in *T. spiralis* infected group, increased 14 days p.i. (373.2 U/l), remained at this level for a month and decreased 60 days p.i. (124.9 U/l). In the inoculated group, LDH had an emphasized activity 4 and 30 days p.i., but the values were not statistically relevant comparative with control. The activity of ALT increased 60 days p.i. (p < 0.05) comparative with control, and AST decrease in the same interval. The ALP values were increased 30 days p.i (p < 0.05), and values returned normal 60 days p.i.

Immunological analysis

In Figure 1 is presented the correlation between the immunological results obtained. A slight increase of the phagocytic activity was registered 14 days p.i. in the infected group, but the differences between the infected and control groups were not statistically significant ($p > 0.05$).

Significantly increased values in lymphocyte activity in infected group was observed 30 ($p = 0.003$) and 60 ($p = 0.05$) days p.i. comparative with control. The specific IgG antibodies were detectable by Elitrich ELISA (Pasteur) kit at 14 days p.i. in infected group (5 out of 6 serum samples from infected pigs presented a OD greater than 0.3 UDO) and at 30 days p.i. by SafePath Trichinae Immunoassay Kit. The high antibody level persisted throughout the experimental period.

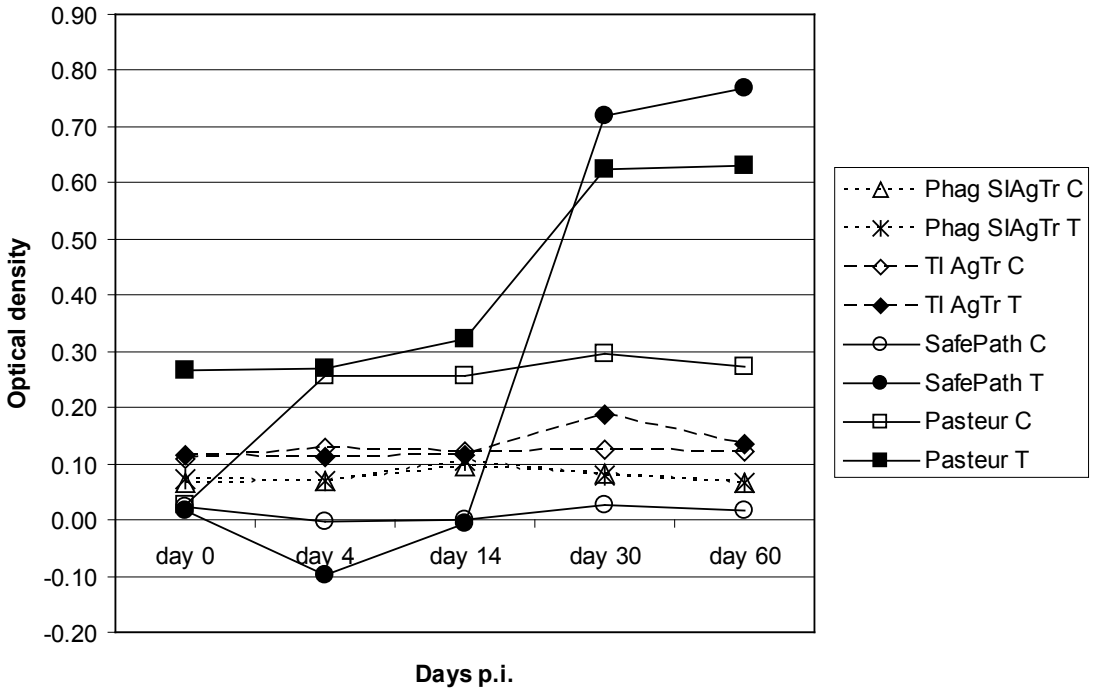

Fig. 1. Correlation between immunological tests in infected (T) and control (C) group
(Phag SIAgTr C – phagocitosis stimulation index with *Trichinella* antigen in control pigs; Phag SIAgTr T – phagocitosis stimulation index with *Trichinella* antigen in *Trichinella* infected pigs; TI AgTr C – transformation index with *Trichinella* antigen in control pigs; TI AgTr T – transformation index with *Trichinella* antigen in *Trichinella* infected pigs; SafePath C – antibody response in control pigs with SafePath kit; SafePath T – antibody response in *Trichinella* infected pigs with SafePath kit; Pasteur C – antibody response in control pigs with Pasteur kit; Pasteur T – antibody response in *Trichinella* infected pigs with Pasteur kit

Larval burden

The LPG values for each of the 6 muscle groups collected are represented in the Table 4. The muscle with the significantly ($p < 0.01$) highest larval density was the diaphragm (504.3 ± 8.2), followed closely by the tongue muscle (487.4 ± 13.3), in all the six infected pigs. The reproductive capacity index was between 141 and 274.

Discussion

In humans, the severity of trichinellosis is usually proportional to the number of larvae ingested, and the disease is characterized by fever, gastrointestinal tract symptoms, myositis, swollen eyelids, and eosinophilia (Murrell & Bruschi, 1994). The clinical signs of *Trichinella* infection in humans (fever, fatigue and myalgia) are not characteristic and the symptoms mimic those of many other diseases (Renier *et al*, 1990; Compton *et al*., 1993; Murrell & Bruschi, 1994; Gottstein *et al*, 2009). In animals, *Trichinella* infection rarely causes clinical signs in the parasite's natural hosts, unless they are infected with a very large number of larvae (Bruschi & Murrel, 2002). Even if an infection dose is quite high, clinical signs do not necessarily appear. Although this parasite can develop in many mammalian species, the main reservoir is represented by domestic and sylvatic swine within which the parasite can attain very high worm burdens (more than 3000 larvae/g) without any adverse physiological effects, suggesting a very good host-parasite relationship (Kapel & Gamble, 2000;). *T. spiralis* infects pigs very efficiently, *T. britovi, T. nelsoni* and *T. pseudospiralis* moderately, and *T. nativa, T. murreli* and *T6* only barely (Kapel & Gamble, 2000; Gottstein *et al*., 2009). In this experiment, beside a delay in the weight gain, no other clinical signs were observed. In other experimental infections in pigs, with 5000 *T. spiralis*/pig, clinical signs such as dyspnea, peri-orbital edema, respiratory problems and reduced weight gain appeared (Ribicich *et al*, 2007).

Regarding the LPG values, the obtained data are similar

with those observed by other researchers (Zimmermann, 1970; Kotula *et al*., 1984; Kapel *et al*, 1998; Nöckler *et al*, 2005).

The evaluation of the RCI in large animals is difficult because digestion of all muscle tissue is not feasible. An approximate total parasite burden was calculated using larval density from selected muscles and the estimated total muscle mass of the infected animal. The RCI values derived from these calculations are estimative but are useful in showing gross differences among different mammalian host species (Kapel *et al*., 2003). Because of this approximate estimation, differences in RCI values can occur even when scientists use the same *Trichinella* species and the same host. According to Theodoropoulos and Petrakos (2010), differences between host bile actions may account for differences in host susceptibility to *T. spiralis*. In other similar studies the *T. spiralis* RCI values in experimentally infected pigs varied from 80 (Medina-Lerena *et al*., 2009) to about 300 (Kapel & Gamble, 2000). In our study the RCI highest value (RCI = 274) was found in the smallest pig (6 kg at the beginning of the experiment) and the lowest value (RCI = 141) in the largest pig (18 kg).

The invasion of the muscles by the migrating larvae can damage the muscle cells, directly or indirectly, with stimulating the infiltration of the inflammatory cells (Bruschi & Murrell, 2002). Eosinophilia and increased levels of muscular enzymes are pathognomonic for this parasitic disease in humans and animals (Bowman *et al*, 1991; Capo & Despommier, 1996; Dupouy-Camet *et al*., 1998; Oksanen *et al*, 2000; Ribicich *et al*, 2007; Gottstein *et al*., 2009; Ashour & Elbakary, in press; Königová *et al*., 2010).

In human infections, eosinophilia is the earliest and most characteristic laboratory finding of trichinellosis (Pawlowski, 1983) and is correlated with the intensity of infection. In the present study, a mild eosinophilia (4.5 %) was observed 30 days p.i.

According to Stewart *et al*. (1978) and Wu *et al*. (2009) *Trichinella* infection induces hypoglycemia in mice. In this case, blood glucose concentration increased 30 days p.i. in infected pigs.

Increased levels of muscle enzymes like CPK, LDH and occasionally AST are characteristic for *Trichinella* infection (Capo & Despommier, 1996; Jonwutiwes *et al.,* 1998; SCVPH, 2001; Ribicich *et al*, 2007). Beside CPK and LDH, the infected pigs, in the present study, presented increased values of ALT and ALP, and the activity of AST was decreased. In another experiment performed with *T. britovi* (15000 larvae) on pigs, CPK, AST and ALT presented increased activity during the infection (Oltean *et al*., 2009).

The irrelevant results obtained in the phagocytic assay could be explained through the results obtained by Pennock *et al*. (1998), Tan *et al*. (2001) and Freitas and Pearce (2010), who demonstrated the presence of a macrophage migration-inhibitory factor (MIF) in *T. spiralis* larvae, very similar to a mammalian cytokine, which might contribute to subversion of host defences. The mammalian MIF is inhibitory for monocyte-macrophage migration, towards glucocorticoid activity (Calandra *et al*., 1995), immunoglobulin synthesis (Tomura *et al*., 1999) and natural killer-cell activity (Apte *et al*., 1998), but not all this properties have been demonstrated yet in MIF produced by *T. spiralis*. Beside this, according to Shupe and Stewart (1991), *T. spiralis* exhibits a decreased neutrophil chemotactic potential, compared with *T. pseudospiralis*.

Regarding the humoral immune response, other experimental studies in pigs demonstrated that between the infecting dose and the onset of seroconversion exists a direct correlation (Kapel *et al*., 1998; Kapel & Gamble, 2000; Kapel, 2001; Oltean *et al*, 2009). At the infective dose used in this experiment, the seroconversion within 30 days p.i., was confirmed by other similar studies (Møller *et al*., 2005; Nöckler *et al*., 2005). Many authors had reported a successful use of *Tricchinella* excretory-secretory antigens in the indirect ELISA for the detection of specific antibodies in various animal species (Smith, 1987; Smith & Snowdon, 1989; Nöckler *et al.,* 1995; Gamble*.,* 1996; Dvorožňáková *et al*., 2010). The test antigen was considered to be an important factor for the identification of specific antibodies which is essential for the specificity of the ELISA result (Nöckler *et al.,* 2000).

Summarizing the results obtained during the *T. spiralis* infection in pigs, with high infective dose, we can conclude that the reaction of the organism is moderate, suggesting the fact that swine has a certain tolerance for this parasite species.

Conflict of interest statement

No financial or personal relationships are maintained with other people or organizations that could inappropriately influence or bias this paper.

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