Article available at http://www.parasite-journal.org or http://dx.doi.org/10.1051/parasite/200108s2216

INFECTIVITY, PERSISTENCE AND SEROLOGICAL RESPONSE OF NINE *TRICHINELLA* GENOTYPES IN RATS

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Summary :

Domestic and sylvatic *Trichinella* genotypes were evaluated for infectivity, muscle larvae persistence, and host antibody responses in rats. Groups of rats were inoculated with *T. spiralis*, *T. nativa*, *T. britovi*, three genotypes of *T. pseudospiralis* (from USSR, USA, and Australia), *T. murrelli*, *Trichinella* T6, and *T. nelsoni*, respectively. The muscle larvae intensity (larvae per gram), total larval burden (lpg x rat weight), and the antibody levels were determined at necropsy 5, 10, 20, and 40 weeks post inoculation. All *Trichinella* genotypes were established in the rats, but infectivity and persistence differed significantly: *T. spiralis* established and persisted in high numbers, the three *T. pseudospiralis* genotypes were also highly infective but differed significantly in persistence, *T. britovi* and *T. nativa* had limited infectivity and persistence, *Trichinella* T6 had low infectivity and very low persistence, and *T. murrelli* and *T. nelsoni* were almost non-infective. Except for *T. spiralis*, initial total muscle larval burdens declined significantly for other genotypes during the experiment. A high initial serological response was detected for all genotypes, but the antibody levels decreased rapidly in relation to decreasing larval burdens. After 20 w.p.i. the antibody levels remained high only in *T. spiralis* and *T. pseudospiralis* infected rats. The high infectivity and persistence of *T. pseudospiralis* in rats, suggests that in addition to *T. spiralis*, this species might be of significant importance in the domestic cycle of trichinellosis.

KEY WORDS : *Trichinella* genotypes, rats, infectivity, persistence, antibody response, ELISA.

Trichinellosis is characterised by two main life cycles: a domestic cycle involving *T. spiralis* and a sylvatic cycle involving all known *Trichinella* genotypes. Epidemiological studies indicate that rats have an important role in the transmission of *T. spiralis* to pigs, and might be a vector between domestic and sylvatic ecosystems (Murrell *et al.*, 1987; Leiby *et al.*, 1988). Besides *T. spiralis*, *T. britovi* and *T. pseudospiralis* have been detected in naturally infected rats in Europe and Asia (Pozio *et al.*, 1996; Britov, 1997; Pozio, 2000). Prior to the recognition of multiple species in the genus (Pozio *et al.*, 1992a) experimental studies with different geographical *Trichinella* isolates of undefined species demonstrated variation in infectivity and pathogenicity in rats (Nelson.G.S & Mukundi J, 1963; Kozar & Kozar, 1965; Nelson *et al.*, 1966; Siddiqi & Meerovitch, 1976a; Siddiqi & Meerovitch, 1976b; Belosevic & Dick, 1980; Ooi *et al.*, 1986; Leiby & Bacha, 1987). However, only one study compared infectivity of well-defined *Trichinella* genotypes in rats (Pozio *et al.*, 1992b). Recent experimental studies in domestic pigs and wild boars demonstrated significant differences in infectivity and persistence of well characterised domestic and sylvatic *Trichinella* genotypes in these hosts (Kapel & Gamble, 2000; Kapel, 2001). Persistence of muscle larvae is probably the most important factor for *Trichinella* transmission as it increases the opportunity for parasite propagation through predation and cannibalism. Different infectivity among *T. pseudospiralis* genotypes has been observed in mice, guinea pigs, domestic pigs, and wild boars (Pozio *et al.*, 1992c; Webster *et al.*, 1999; Kapel & Gamble, 2000; Kapel, 2001), but studies in rats on this genotype have received little attention.

The aims of the study reported here were to determine infectivity, muscle larvae persistence, and antibody response of well-characterised *Trichinella* genotypes in rats. Such knowledge is important to understand interaction of sylvatic and domestic life cycles, and for risk factor analysis in conventional and outdoor pig production.

MATERIALS AND METHODS

PARASITE GENOTYPES AND HOSTS

Nine *Trichinella* genotypes comprising both domestic and sylvatic origin from different geographic regions were studied. The genotypes used have the following *Trichinella* Reference Centre codes (Pozio *et al.*, 1989): *T. spiralis* (T1, ISS004, Mary-

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land, USA, *Sus scrofa*), *T. nativa* (T2, ISS042, Alaska, USA, *Ursus maritimus*), *T. britovi* (T3, ISS100, Italy, *Canis lupus*), three genotypes of *T. pseudospiralis* (T4 USSR, ISS013, Caucasus, USSR, *Procyon lotor*; T4 USA, ISS470, Alabama, USA; *Coragyps atratus*, T4, AUST, ISS141, Australia, *Dasyurops maculatus*), *Trichinella murrelli*. (T5, ISS035, Pennsylvania, USA, *Ursus americanus*), *Trichinella* T6 (T6, ISS034, Montana, USA, *Ursus arctos*), *T. nelsoni* (T7, ISS037, Tanzania, Africa, *Phacochoerus aethiopicus*). The parasite genotypes were propagated through several passages in outbred Ssc:CF1 mice. Two hundred sixteen females inbred Fisher 344 rats from two to three months of age, weighing 150-199 g at the beginning of the experiment were used. The rats were housed in plastic cages and were given water and standard laboratory animal pellets ad libitum. Experimental animals were divided into nine groups of 24 rats in each. For inoculation of rats, muscle larvae were recovered from mice inoculated 35 days before by artificial digestion. Skinned and eviscerated mice were cut into pieces with scissors mixed 1:10 with artificial digestion fluid (1 litre 45°C water, 10 ml conc. 37 % HCl, 10 g pepsin 1:10 000 NF) and digested in an incubator at 45°C temperature for two hours using a magnetic stirrer. Digests were poured through a 500 µm mesh sieve into conical pilsner glasses and left 20 minutes for sedimentation. After settling the supernatant was poured of and the glasses were filled up with tap water again. Washings were repeated several times until the supernatant was clear as water. After the last washing, the sediments were poured into 50 ml tubes for larvae concentration. Larvae were counted in chambers under a stereo microscope and subsequently inoculation doses were adjusted. Each experimental group of rats was inoculated per os with one *Trichinella* genotype, administering 2,000 larvae suspended in 0.5 ml water to each CO_2-anaesthetised rat. Because of lack of larvae only three rats in the *T. pseudospiralis* USA group were inoculated with 2,000 larvae/rat, while other three rats were inoculated with 1,200 larvae/rat .

Six rats (n=6) from each experimental group were necropsied 5, 10, 20, and 40 w.p.i. After each necropsy a number of muscle larvae per gram of muscle tissue (lpg) in individual rats was counted after digestion of 2-5 g of muscle tissue from diaphragm, front legs, and the masseters. Muscle larvae were recovered as described above. To determine the persistence of muscle larvae in the growing rats, the total larval burden (weight of rat x lpg) was estimated at necropsy.

Four rats died from other diseases during the experiment: two *T. pseudospiralis* AUST and USA rats during the first week p.i.; one *T. murrelli* and one *T. pseudospiralis* USA three months after the beginning of the study.

SEROLOGY

Blood samples were collected from the periorbital plexus from anaesthetised rats three days before inoculation. Afterwards blood samples were collected only at necropsy 5, 10, 20, and 40 w.p.i. Sera were isolated by centrifugation and subsequently frozen at -20°C temperature. Individual serum samples were tested by ELISA for the presence of *Trichinella* antibodies using excretory-secretory (ES) antigens of *T. spiralis* prepared as described by Kapel and Gamble (Kapel & Gamble, 2000). The sera collected from rats before inoculation were pooled and used as negative control. The sera collected from nine groups of rats at 20 w.p.i. were pooled by adding equal amounts of each genotype and used as a positive control. ELISA plates (NUNC Maxisorb 96 wells) were coated with 100 µl ES antigen (Excretory/Secretory products, 1 mg protein/ml, from *in vitro* cultured muscle larvae) diluted 1:1000 in 0.1 M Sodiumcarbonate buffer pH 9.6 and left in the fridge at 4°C overnight. Plates were emptied and washed five times in washing buffer (Phosphate buffer with 0.5 M NaCl and 0.1 % Tween 20; 0,5 ml Tween in one liter PBS). One hundred ml serum samples diluted 1:4000 in Phosphatebuffer, 0.1 % Tween were added in duplicate and left for one hour on a rocking table at room temperature. Afterwards plates were washed five times as described above. One hundred microlitres of goat-anti-rat IgG (HRP) diluted in 1:1000 in PBS, 0.1 % Tween with 1 % normal goat serum was added to each well. Plates were incubated for one hour on a rocking table at room temperature and washed five times again. Subsequently 100 µl of the substrate solution (OPD tablets dissolved in citrate buffer in dark on a shaking table for 10-20 minutes and added hydrogenperoxide 30 %) was applied to each well. After 10 min in the dark the colour reaction was stopped by adding 100 µl 0.5 M sulphuric acid to each well. The optical density was read at 490 nm with 650 nm as reference on an automatic microplate reader (Multiscan EX, Labsystem). Control positive and negative rat sera were included in each assay. The cut off value was defined as the mean of the negative control OD values plus 3.5 standard deviations (cut off value = mean OD + 3.5 SD) of 11 plates.

STATISTICAL ANALYSIS

Statistical calculations were performed with the software package GraphPad PRISM for Windows®, Version 2.01. Comparisons of lpg, total larval burden, and antibody level among genotypes were conducted using parametric one-way analysis of variance (ANOVA). Data were log-transformed according to log (x+1) before analysis. Pairwise Tukey's Multiple Comparison

Test was used to compare the nine *Trichinella* genotypes at times of necropsy, and Bonferroni's Multiple Comparison Test to evaluate changes over time for each genotype. Differences were considered significant when $P<0.05$.

RESULTS

Both infectivity and persistence of muscle larvae varied significantly among the genotypes (Table I). At 5 w.p.i., *T. spiralis* had established in very high numbers, the three *T. pseudospiralis* (USSR, USA, AUST) genotypes had established in high numbers, *T. britovi* and *T. nativa* established in moderate numbers, *Trichinella* T6 and *T. murrelli* in low numbers, whereas *T. nelsoni* established in very low numbers. Significant differences between lpg means were detected: *T. spiralis* and *T. pseudospiralis* USSR, USA and AUST versus all the other genotypes; *T. nativa* and *T. britovi* versus *T. murrelli*, *Trichinella* T6 and *T. nelsoni*; and *Trichinella* T6 versus *T. nelsoni*. One *T. spiralis* rat was excluded, because of the extremely high larval burden (18,482 lpg), which thought to be a result of immunological disorder. For the other rats of the same group lpg ranged from 2,659 to 3,688. Larvae were detected only in three out of six rats in the *T. murrelli* group. Only a few *T. nelsoni* larvae (lpg = 0.04) were detected in the entire carcasses. Five and 10 w.p.i. *Trichinella* T6 larvae were detected in five out of six rats. At 20 w.p.i., only two *T. nativa* rats harboured larvae (lpg 0.19 and 5.5), while five of six *T. britovi* rats were still infected. At 40 w.p.i., only in one of three rats infected with 1200 larvae/rat of *T. pseudospiralis* USA isolate, muscle larvae were found (0.7 lpg), while in two rats infected with 2,000 larvae/rat, 622 and 3.7 lpg were recovered, respectively.

From week 5 to week 40 p.i., larval intensity in muscle tissue decreased for all genotypes (Figure 1a). From 5 to 40 w.p.i., the mean lpg was reduced with 27 % for *T. spiralis*, 47 % for *T. pseudospiralis* USSR, 62 % for *T. pseudospiralis* AUST, and 96 % for *T. pseudospiralis* USA. For the others genotypes the lpg decreased by approximately 90 % from 5 to 10 w.p.i. and no larvae were recovered from rats muscle tissue after 40 w.p.i.

During experimental period the total larval burden was constant only for *T. spiralis*, while a significant decreased was found for all other genotypes (Fig. 1b). From 5 to 10 w.p.i. the total larval burden decreased significantly for *T. nativa, T. britovi*, and *T. murrelli*, from 10 to 20 w.p.i. for *T. nativa, T. britovi, T. pseudospiralis* AUST and *Trichinella* T6, and from 20 to 40 w.p.i. for *T. britovi* and for *T. pseudospiralis* USSR and USA. Significant differences among *T. pseudospiralis* genotypes were observed at 10 w.p.i. for USSR and AUST versus USA, 20 w.p.i. for USSR versus USA, and 40 w.p.i. USSR and AUST versus USA.

All rats were sero-converted 5 w.p.i. and specific antibody titres remained above cut-off level for the duration of the study (Fig. 2). The antibody dynamic differed significantly among the genotypes. Five w.p.i. antibody titres in serum samples were significantly higher for: *T. spiralis* versus *T. pseudospiralis, T. murrelli, T. nelsoni* and *Trichinella* T6 genotypes; *T. britovi* and *T. nativa* versus *T. pseudospiralis* AUST. For all genotypes antibody level increased from 5 to 20 w.p.i. and the highest antibody level was detected 20 w.p.i. At this time the only significantly lower antibody titre was detected for *T. nelsoni* versus *T. spiralis* genotype. From week 20 to week 40 the antibody levels

Trichinella genotypes	Mean[a] muscle larvae in rats ± S.D.			
	5 w.p.i.[c] (187 g)[e]	10 w.p.i. (199 g)	20 w.p.i. (215 g)	40 w.p.i. (240 g)
T. spiralis	3,138 ± 375	4,638 ± 2,100	4,199 ± 2,200	2,283 ± 1,373
T. nativa	109 ± 71	12 ± 13.1	0.94 ± 2.2	0
T. britovi	2,08.5 ± 151	16.6 ± 8.8	9.7 ± 21.5	0
T. pseudospiralis USSR	2,287 ± 778	2,046 ± 320	1,495 ± 189	1213 ± 320
T. pseudospiralis USA	1,829 ± 666	650[b] ± 285	454 ± 467	125[b,d] ± 277
T. pseudospiralis AUST	2,060[b] ± 535	1834 ± 598	900 ± 471	773 ± 199
T. murrelli	4.2 ± 4.7	0	0	0[b]
Trichinella T6	23.9 ± 36.8	1.8 ± 2	0	0
T. nelsoni	0.04	0	0	0

[a] number of larvae per gram muscle tissue for 6 rats per group except [b] where 5 rats per group.
[c] weeks post infection
[d] three rats each inoculated with only 1200 larvae
[e] mean weight of rats

Table 1. – Infectivity and persistence of nine *Trichinella* genotypes in rats.

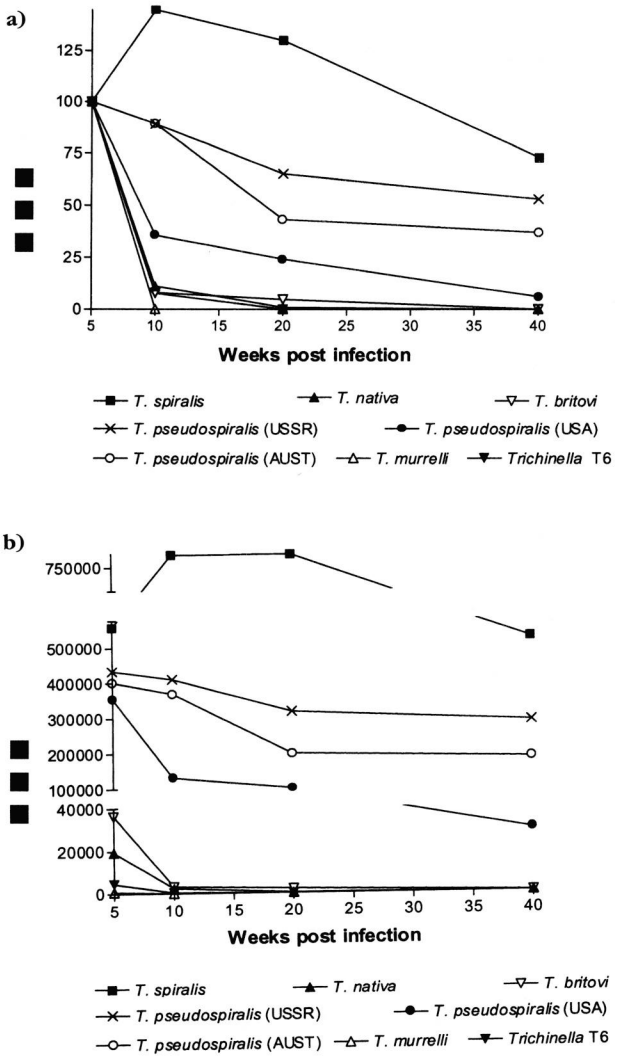

Fig. 1a+b. – Larval intensity (a) and total larval burden (b) of nine *Trichinella* genotypes in rats 5, 10, 20, and 40 weeks post infection.

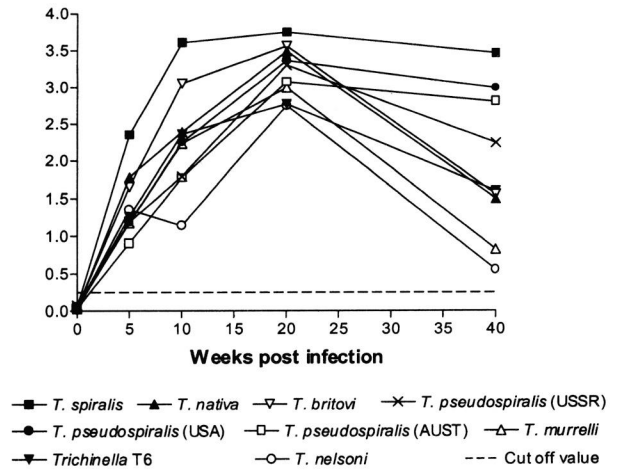

Fig. 2. – Antibody levels in rats after infection with nine *Trichinella* genotypes

decreased significantly for all genotypes but *T. spiralis* and the three *T. pseudospiralis* genotypes induced highest antibody titers.

DISCUSSION

The present study demonstrated profound differences in infectivity and persistence of nine *Trichinella* genotypes in rats. According to infectivity and larval persistence the genotypes may be described as following: *T. spiralis,* highly infective and persistent; *T. pseudospiralis* genotypes, highly infective but with variable persistence (*T. pseudospiralis* USSR and AUST with good persistence, and less so for the USA genotype); *T. britovi* and *T. nativa,* moderately infective and persistent; *Trichinella* T6, low infective and non-persistent; and *T. murrelli* and *T. nelsoni* almost non-infective and non-persistent. The high infectivity found for *T. spiralis*, intermediate infectivity for *T. pseudospiralis*, and low infectivity for other sylvatic genotypes in rats are in agreement with other reports on the various genotypes (Ooi *et al.,* 1986; Pozio *et al.,* 1992a; Pozio *et al.,* 1992b; Yao *et al.,* 1997). The high reproductive capacity of *T. spiralis* in rats, as found here, support the general perception that pigs and rats are the most important hosts in the domestic cycle of infection (Schad *et al.,* 1987; Leiby *et al.,* 1988; Pozio *et al.,* 1996). The increasing larval burdens for *T. spiralis* at 10 and 20 w.p.i. might be explained by the fact that inbread F344 rats strain is weak responder of primary worm expulsion (Bell, 1992). Bell (1992) demonstrated that constant adult worm burden in intestines of this rat strain persisted at least until 10 days p.i., while it was reduced for app. 40 % for other strains. Most likely, larvae produced by female worms in the end of intestinal phase did not reached complete development in the muscles and were digested at 5 w.p.i. (35 days), as they were not resistant to digestion at that time, while in rats necropsied at 10 w.p.i. muscle larvae were resistant to digestion.

The long persistence of *T. spiralis* muscle larvae supports the proposition that this species could be maintained in rat population through cannibalism, even in the absence of other source of infection (Leiby *et al.,* 1990). Similarly, survival of infective *T. spiralis* larvae in the muscle of dead rats for at least 49 days at farm conditions (Barrientos & Torres, 1982) suggests favourable conditions for the parasite maintenance through cannibalism and pose risk for pigs, especially when the rat population is large.

The *T. pseudospiralis* genotype variability in host infectivity has previously been described by Pozio *et al.* (Pozio *et al.,* 1992c) and Webster et al. (Webster

et al., 1999) in mice, variability in infectivity and persistence of muscle larvae by Kapel & Gamble (Kapel & Gamble, 2000) in domestic pigs, and by Kapel (2001) in wild boars. The results of the present study also indicate that variation is significant in populations of *T. pseudospiralis* found in Paleoarctic, Nearctic and Australian regions. Although higher infectivity of *T. pseudospiralis* among sylvatic genotypes in rats has been demonstrated previously (Ooi *et al.,* 1986; Pozio *et al.,* 1992b) but persistence of muscle larvae has never previously been studied. The relative high infectivity and persistence of *T. pseudospiralis* suggests that this sylvatic species could also be transmitted by rats in the domestic cycle. We can speculate that *T. pseudospiralis* may be maintained in a rat population through cannibalism and scavenging as *T. spiralis* can and thus could be transmitted to domestic pigs, especially under poor management conditions. This is substantiated by findings of natural *T. pseudospiralis* infections in rats and pigs in the same farm for three consequent years in Kamchatka (Britov, 1997), in rats in Finland (Kapel *et al.,* 2001) and by relatively high establishment in experimentally infected domestic pigs (Kapel & Gamble, 2000) and wild boars (Kapel, 2001). However, because knowledge is lacking on the biological diversity and geographical distribution of the different genotypes of *T. pseudospiralis*, further studies are needed to clarify their role in both the sylvatic and the domestic life cycle.

Even though it is generally found that sylvatic *Trichinella* have low infectivity to rodents, some strain dependent variation have been found both for mice (Wassom *et al.,* 1979; Wakelin, 1980) and rats (Nelson *et al.,* 1966; Siddiqi & Meerovitch, 1976b). Due to such variation it is very difficult to determine which rat strain would best mimic natural host-parasite relationships best. In general, the low infectivity of sylvatic genotypes determined by Pozio et al (Pozio *et al.,* 1992b) is comparable to our study, however there are some disparities. In both experiments, infectivity of *T. britovi* is about twofold compared to *T. nativa*, but in our study these two genotypes have significantly higher infectivity and persistence than the remaining other sylvatic genotypes, while Pozio et al. (Pozio *et al.,* 1992b) demonstrated decreasing infectivity in rats as following: *T. murrelli, T. nelsoni, T. britovi, Trichinella* T6, and *T. nativa.* However, findings of natural infections in rats, suggest that moderate infectivity and persistence of *T. britovi* makes this genotype quite successful in infecting rats, though it cannot be maintained in the domestic cycle (Rosa *et al.,* 1991). A different situation is observed in Africa where *T. nelsoni* is the etiological agent of sylvatic trichinellosis (Pozio *et al.,* 1991; Pozio *et al.,* 1992a; Pozio *et al.,* 1997). Nevertheless, from endemic areas in Kenya none of 1500 rodents, including 428 rats (*Rattus rattus),* were infected, and experimental infections in wild rodents proved very low infectivity of *T. nelsoni* (Nelson.G.S & Mukundi J, 1963).

The peak (around 20 w.p.i.) and the long persistence of IgG antibodies detected in our study are in agreement with Mizuno (Mizuno, 1990), who found similar results with crude muscle larvae and ES antigens in serum of rats infected with *T. spiralis.* Other studies have reported persistence of *T. spiralis* antibodies up to six months p.i. in rat serum using larval metabolic and somatic antigens (Dziemian & Machnicka, 2000). Long lasting antibody response against *T. spiralis* has been demonstrated in pigs and foxes up to 73 w.p.i. (Noeckler, 1995; Noeckler & Voigt, 1998). Our results show that all *Trichinella* genotypes, in spite of the different infectivity, induce various but persistent and detectable antibody response in rats, as it was also observed in other studies in domestic pigs and wild boars (Kapel & Gamble, 2000; Kapel, 2001). However, a high persistent antibody level is maintained only in the presence of antigens released from muscle larvae, as seen in foxes, pigs and wild boars (Noeckler & Voigt, 1998; Kapel & Gamble, 2000; Kapel, 2001) as well as in rats infected with *T. spiralis* and *T. pseudospiralis* in our study. Long lasting antibody response to *Trichinella* in rats could be used for field serological surveys of rats population if needed, and especially in the light of the potential use of antibodies from meat juice.

The obtained data on the infectivity, persistence, and antibody response in rats support the taxonomy of the genus *Trichinella.* Our study suggests that *T. spiralis* and *T. pseudospiralis* are the only genotypes with high infectivity and persistence in rats. Thus, in addition to *T. spiralis,* the non-encapsulated *T. pseudospiralis* has the potential to be a significant risk in the trichinellosis domestic cycle.

ACKNOWLEDGEMENTS

The Danish National Research Foundation is acknowledged for the financial support of the study and the Department of Research and Education under the Ministry of Education and Research of Lithuania for PhD scholarship for A. Malakauskas. Mr. J. van Koler and Mr. K. Taira are thanked for technical assistance.

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