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Original Article

Survival of *Trichinella spiralis* and *T. pseudospiralis* in Experimentally Infected Wild Boar Muscle Tissue under Freezing and Environmental Conditions

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Abstract

Background: The aim of this study was to investigate the survival of *Trichinella spiralis* and *T. pseudospiralis* in decaying wild boar tissue and assess their freezing tolerance in experimentally infected animals.

Methods: The present study was conducted in Buenos Aires City, Argentina during the 2018-2019 period. Two wild boars were used, one infected with 20,000 muscle larvae (ML) of *T. spiralis* and the other with *T. pseudospiralis*. Both animals were euthanized 19 weeks post-infection. Limbs from each boar were placed over soil in plastic containers to assess ML survival in decaying tissue, under natural temperature and humidity, shielded from rain. Weekly samples were taken for artificial digestion, and the ML were inoculated into mice to determine their reproductive capacity index (RCI). Additionally, to evaluate the freezing tolerance of the ML, muscle samples were stored at -18°C. Six samples were taken and digested after 2, 4, 7, 9, 11, and 14 days, with subsequent inoculation into mice to assess RCI.

Results: *T. spiralis* remained infective in decaying wild boar tissue for 11 weeks, while *T. pseudospiralis* remained infective for only 4 weeks. The freezing tolerance assay showed that *T. spiralis* ML remain infective for 9 days. However, *T. pseudospiralis* ML remain infective for only 2 days at -18°C.

Conclusion: The findings highlight the survival strategies of *T. spiralis* and *T. pseudospiralis* in different environmental conditions, which may have implications for understanding their transmission dynamics in wild animals.

Introduction

A wide range of animals are involved in the maintenance of *Trichinella* spp. infection in wildlife (1). The probability of muscle larvae (ML) being ingested by carnivores or scavengers increases in direct proportion to the ML ability to remain viable in decaying tissue (2). Environmental conditions, such as humidity and temperature, play a key role in the spread of *Trichinella* spp., as they directly affect the survival of the larvae in host carcasses (3,4).

The ability of ML to survive in tissues exposed to low or high temperatures may be influenced by the presence or absence of a collagen capsule surrounding the ML. The collagen capsule may provide some protection and therefore increase the survival time of these larvae in decaying tissues (5 - 7). It may also confer some freeze tolerance (8). Studies have shown that the tolerance temperature of the ML is influenced by both the host species and the *Trichinella* species, among other factors (9 - 12).

Wild boars (*Sus scrofa*) are known to be a source of *Trichinella* spp. infection for humans (13 - 16). Consumption of infected meat from these animals has been found to be one of the main causes of human trichinellosis (3,14, 17 - 22).

Trichinella spp. have diverse freeze tolerances that vary depending on each species. *T. nativa* has survived in arctic fox tissues (*Alopex lagopus*) at -18 °C for up to 4 years (20), *Trichinella T6* has survived in grizzly bear tissues (*Ursus arctos*) at -6.5 to -20 °C for up to 34 months (9), and *T. britovi* has survived in laboratory rat muscles at -5 °C for up to 1 week (12). This tolerance also varies depending on the host species involved (23). Therefore, as *Trichinella* species have diverse freeze tolerances, relying solely on freezing meat as a preventive method could pose risks to food safety.

The purpose of this study was to determine whether *T. spiralis* and *T. pseudospiralis* can survive in decaying wild boar tissue and to assess the freezing tolerance of these *Trichinella* species in experimentally infected animals.

Materials and Methods

Animals

Two 60-day-old castrated wild boars were use in this study. They underwent a 30-day adaptation period to the new environment where the study was to be conducted. Clinical and hematological parameters were evaluated to assess their health condition (24). The present study was conducted in Buenos Aires City during the 2018-2019 period. All animals were housed in an indoor pigpen with plastic slatted floors at the experimental facilities of the Faculty of Veterinary Sciences, University of Buenos Aires, Argentina.

Ethics approval

The study was approved by the Institutional Committee for Use and Care of Laboratory Animals of the Faculty of Veterinary Sciences, University of Buenos Aires (CICUAL), permit number 2015/16.

One animal was orally infected with 20,000 *T. spiralis* L1 larvae (ISS1097), while the other was infected with 20,000 *T. pseudospiralis* L1 (21). These *Trichinella* species were maintained by serial passages using CF1 mice, and ML were recovered by artificial digestion (24). After 19 weeks post-infection (p.i.), the wild boars were euthanized (26). To determine the muscle larval burden in each animal, 100 g of upper forelimb and 100 g of upper hindlimb were artificially digested (26). These tissue amounts were taken from different parts of both. The recovered larvae were then inoculated into three mice to assess their infective capacity (day 0).

Persistence

One forelimb and one hindlimb were severed from each wild boar and placed over a

layer of soil in plastic containers. All containers were covered with metallic mosquito net screens, protected them from rain, exposed to natural environmental temperature and humidity conditions. The study was carried out during the Argentine autumn and winter seasons for 16 weeks. The environmental temperatures and relative humidity ranged from 9.0 to 16.6 °C and 33.75 to 99.25%, respectively. This data was obtained from the National Weather Service in Argentina (27).

Decaying muscle from limbs infected with *T. spiralis* and *T. pseudospiralis* in wild boars was analyzed weekly for 16 weeks. A 50-gram sample from each limb was taken weekly and artificially digested (26). These samples were taken from different points of both limbs due to uneven ML´s distribution. Then, three BALB/c mice were orally inoculated with 300 larvae in 15 µl. After 6 weeks p.i., the mice were sacrificed, and the reproductive capacity index (RCI) was assessed to determine the infectivity of the recovered larvae (28). The RCI was calculated as the number of larvae recovered after digestion divided by the number of larvae orally inoculated.

The data about temperature and humidity were obtained from the National Weather Service in Argentina (27).

Freezing tolerance

To study the freezing tolerance of the ML of *T. spiralis* and *T. pseudospiralis*, one hindlimb and one forelimb from each wild boar were used. From these muscle groups, six 2 cm thick, 200 g samples were taken and placed in individual plastic bags. All samples were stored at -18 °C for 14 days. At each time point (2, 4, 7, 9, 11, and 14 days), a sample of each muscle group was thawed at 4 °C in the refrigerator and then artificially digested. The recovered larvae were counted, and four BALB/C mice were orally inoculated with 300 larvae. After 6 weeks pi., the mice were sacrificed, and the RCI was assessed. Both meat samples at each point of analysis were digested in combination.

Statistical analysis

Mean RCI values for *T. spiralis* and *T. pseudospiralis* were assessed in two experiments: persistence and freezing, at different time points. Prior to ANOVA, normality of the data was confirmed via Shapiro-Wilk test. ANOVA was then employed to compare mean RCI values between species. The Tukey test compared means at significant time points ($\alpha = 0.05$). All statistical analyses were performed using InfoStat™ software.

Results

Larvae were recovered up to week 16 and week 10 from *T. spiralis* and *T. pseudospiralis*, respectively, from decaying tissues. In the freezing tolerance experiment, larvae from both *Trichinella* species were recovered after 14 days at -18 °C.

The highest mean RCI value found in the persistence study was 89.50 for *T. spiralis* in tissues decaying for 0 weeks, while for *T. pseudospiralis*, it was 75.14 in tissues decaying for 0 weeks.

The infective larvae recovered from the decaying tissues remained viable until week 11 for *T. spiralis* and week 4 for *T. pseudospiralis*, as shown in Table 1. After these periods, the RCI tested negative for both *Trichinella* species. The last positive RCI mean value obtained for decaying tissues infected with *T. spiralis* was 0.0056, and for decaying tissues infected with *T. pseudospiralis*, it was 15.48.

Although *T. spiralis* larvae were recovered from decaying wild boar tissues for 16 weeks, from the 11th week on, all larvae showed significant cuticle damage, absence of internal content, and no motility after being maintained for 1 hour at 37 °C. The RCI values from that point on were consistently negative.

Table 1: Mean RCI of the mice infected with *T. spiralis* and *T. pseudospiralis* ML recovered from decaying muscle tissue

	RCI			
	T. spiralis	*T. pseudospiralis*		
Weeks	Mean +/- SE[a]	Mean +/- SE	F[b]	*P* value
0	89.51 +/- 5.73 A[*]	75.14 +/- 5.73 A	3.14	0.151
1	64.93 +/- 5.98 A	69.47 +/- 5.98 A	0.29	0.6205
2	50.46 +/- 5.17 A	57.07 +/- 5.17 A	0.82	0.4176
3	48.34 +/- 3.52 A	50.96 +/- 3.52 A	0.28	0.6262
4	47.98 +/- 3.35 A	15.49 +/- 3.35 B	46.99	0.0024
5	36.98 +/- 2.02 A	0 +/- 2.02 B	167.64	0.0002
6	34.70 +/- 3.69 A	0 +/- 3.69 B	44.25	0.0027
7	23.01 +/- 4.44 A	0 +/- 4.44 B	13.42	0.0215
8	5.46 +/- 1.9 A	0 +/- 1.9 A	4.12	0.1123
9	1.58 +/- 0.39 A	0 +/- 0.39 B	8.22	0.0456
10	0.0036 +/- 0.0026 A	0 +/- 0.0026 A	1	0.3739
11	0.0056 +/- 0.0026 A	0 +/- 0.0026 A	1	0.3739

[a]Standard Error
[b]F critical values
[*]Means with a common letter are not significantly different (*P*-value > 0.05).

Regarding the freezing tolerance study, the mice infected with *T. spiralis* ML obtained from frozen muscles (-18°C) remained infectious for 9 days (Table 2), while the mice infected with frozen *T. pseudospiralis* ML remained infectious for up to 2 days at -18°C (Table 2). *T. pseudospiralis* larvae remained motile after digestion in samples frozen for up to 2 days, but became immotile thereafter. Both *Trichinella* species recovered from samples frozen for 11 and 14 days showed severe cuticle damage.

The statistical analysis showed significant differences between the RCI mean values for *T. spiralis* and *T. pseudospiralis* for the persistence experiment in weeks 4, 5, 6, 7, and 9 (Table 1). For the freezing experiment, the analysis showed significant differences between the RCI mean values of *T. spiralis* and *T. pseudospiralis* for days 2, 4, and 7 (Table 2).

Table 2: Mean RCI obtained from the mice infected with *T. spiralis* and *T. pseudospiralis* ML recovered from frozen muscle tissue, after different times under freezing conditions

	RCI			
	T. spiralis	*T. pseudospiralis*		
Days	Mean +/- SE[a]	Mean +/- SE	F[b]	*P*-value
0	89.51 +/- 5.73 A[*]	75.14 +/- 5.73 A	3.14	0.151
2	52.84 +/- 6.53 A	17.45 +/- 6.53 B	14.68	0.0186
4	30.42 +/- 3.92 A	0 +/- 3.92 B	30.13	0.0054
7	12.65 +/- 2.82 A	0 +/- 2.82 B	10.1	0.0336
9	1.93 +/- 0.97 A	0 +/- 0.97 A	1.96	0.2341

[a]Standard Error
[b]F critical values
[*]Means with a common letter are not significantly different (*P*-value > 0.05)

Discussion

In the present study, we observed that the ability of ML to survive in decaying and frozen muscle tissue varied according to the *Trichinella* species. Specifically, *T. spiralis* exhibited a longer survival time in both decaying and frozen meat than *T. pseudospiralis*.

The age of the ML has been suggested as a factor that can affect the ML ability to survive in decaying tissue, as previously reported by several authors (5-7). In a study (7), mice infected with *T. spiralis* for 5 weeks had ML surviving in decaying tissue for 1 week, while those infected for 37 weeks had ML surviving for up to 3 weeks under the same conditions. Prolonged infection time may result in the collagen capsule becoming thicker and more fully developed, which could favor encapsulated species like *T. spiralis*. In this study, wild boars were infected for over 19 weeks, and the *T. spiralis* ML remained infective for almost 11 weeks in decaying wild boar tissues, while *T. pseudospiralis* ML only remained infective for 4 weeks in decaying tissues. This finding supports the idea that the age of the ML capsule may be beneficial for its survivability in decaying tissues. In a study conducted in guinea pigs (*Cavia porcellus*) decomposing tissues infected with *T. patagoniensis* (33), the ML could survive for several weeks. In addition, several authors suggest that high temperatures and droughts reduce the survival capacity of these encysted larvae (34, 35).

In our experiment, the ML of *T. pseudospiralis* in decaying tissue were infective for 4 weeks. The shorter survival time observed in *T. pseudospiralis* compared to *T. spiralis* may be attributed to the fact that *T. pseudospiralis* is a non-encapsulated species. Several authors have suggested that the presence of a collagen capsule surrounding the ML benefits its survival in carcasses (5 - 6, 29). Therefore, the increased susceptibility of *T. pseudospiralis* to putrefaction byproducts could be a consequence of the absence of the collagen capsule, which provides greater protection (30 - 31).

Studies conducted with *T. papuae*, a non-encapsulated species, have shown varying results in terms of its survival in carcasses. For instance, its ML could survive for up to 9 days in domestic pig carcasses under the environmental conditions of Papua New Guinea (2). Similarly, it was demonstrated that *T. papuae* remained infective for more than 9 days at a temperature above 20 °C in red fox (*Vulpes vulpes*) carcasses (32).

According to Pozio (36), various factors affect the ability of *Trichinella* ML to survive freezing temperatures, including the host in which they are encysted, the ML age (12, 37), the temperature and time exposed (37, 38). Some *Trichinella* species have been found to survive longer in carnivorous muscles than in pigs or laboratory rodent muscles (37, 39 - 41). Many authors suggest that differential muscle composition, such as the type and amount of lipids present in the tissue, may provide some protection against freezing temperatures in wild animals since they face different environmental conditions than domestic animals (42). Additionally, the ML ability to tolerate freezing temperatures may also be related to the levels of endogenous carbohydrates present in muscle tissue and on larvae glycogen concentrations (43, 44). The temperature range is also a key factor in the ML`s ability to survive in frozen tissues. The optimal temperature range of survival is 0 to -20 ºC, specifically the temperature "below the snow" (9, 34, 35, 45). Finally, the ML survival capacity in freezing environments may be directly linked to the genetic differences between *Trichinella* species (36).

In our experiment, we observed that *T. pseudospiralis* larvae had a low tolerance for freezing, as they only survived for 2 days at -18 °C. (31). Similarly, in a previous study on shredded rat tissues it was reported that *T. pseudospi-*

ralis could survive for less than one week at a freezing temperature of -18°C (12). In contrast, it was demonstrated (46) that *T. pseudospiralis* ML in sheep muscle tissue remained infective for up to 4 weeks at -18 °C.

Differences in the persistence and infectivity of *T. pseudospiralis* populations have been observed in various animal hosts (10,40,41). *T. pseudospiralis* has been found in 18 different mammal species and 12 avian species, as reported by Pozio (36, 48). This wide host range could explain how the species has been able to expand and spread worldwide despite its low tolerance to different environmental conditions (36, 50).

T. spiralis is the *Trichinella* species that is best adapted to domestic and wild swine hosts (49, 51, 52). Kapel et al. (44) studied nine *Trichinella* species and found that none survived over one week at -18°C in pig or wild boar meat. Another study in wild boars showed a survival rate for 56 hours at -21 °C (8). However, our study found that *T. spiralis* could survive for 9 days at -18 °C.

In the present study, decaying limbs were exposed to autumn and winter environmental conditions, which allowed the larvae of *T. spiralis* to survive for 11 weeks and *T. pseudospiralis* for 4 weeks. However, in frozen tissue, *T. spiralis* only survived for 9 days and *T. pseudospiralis* for 2 days. Therefore, the ability to survive in decaying and frozen tissue indicates that these two *Trichinella* species can survive in carcasses and be spread by scavenger animals. These findings are crucial for understanding the epidemiology of *Trichinella* spp., especially given the importance of wild boars as an exotic species widely distributed in Argentina and America.

Conclusion

The findings highlight the survival strategies of *T. spiralis* and *T. pseudospiralis* in different environmental conditions, which may have implications for understanding their transmission dynamics in wild animals.

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Conflict of Interest

The authors declare that there is no conflict of interests.

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