Veterinary Parasitology 194 (2013) 175–178

Contents lists available at SciVerse ScienceDirect

Veterinary Parasitology

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

Freeze-tolerance of *Trichinella* muscle larvae in experimentally infected wild boars

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ARTICLE INFO

Keywords:
Trichinella spiralis
Trichinella britovi
Freezing
Wild boars
Sus scrofa

ABSTRACT

Freeze-tolerance of encapsulated *Trichinella* muscle larvae (ML) is mainly determined by *Trichinella* species, but is also influenced by host species, the age of the infection and the storage time and temperature of the infected meat. Moreover, the freeze-tolerance of the encapsulated species appears to be correlated to the development of thick capsule walls which increases with age. An extended infection period and the muscle composition in some hosts (e.g. herbivores) may provide freeze-avoiding matrices due to high carbohydrate contents. The present experiment compares freeze-tolerance of *Trichinella spiralis* and *Trichinella britovi* ML in wild boar meat 24 weeks post inoculation (wpi). Three groups of four wild boars were infected with 200, 2000 or 20,000 ML of *T. britovi* (ISS 1575), respectively. Additionally, three wild boars were inoculated with 20,000 ML of *T. spiralis* (ISS 004) and two animals served as negative controls. All wild boars were sacrificed 24 wpi. Muscle samples of 70 g were stored at −21 °C for 19, 30 and 56 h, and for 1–8 weeks. Larvae were recovered by artificial digestion. Their mobilities were recorded using Saisam® image analysis software and their infectivities were evaluated using mouse bioassays. Samples frozen for 19, 30 and 56 h allowed recovery of mobile ML, but samples frozen for 1 or 2 weeks did not. Correspondingly, only *T. spiralis* and *T. britovi* larvae isolated from wild boar meat frozen for 19, 30 and 56 h established in mice. This study showed that freezing at −21 °C for 1 week inactivated *T. spiralis* and *T. britovi* ML encapsulated in wild boar meat for24 weeks.

1. Introduction

Freeze-resistance of *Trichinella* muscle larvae (ML) is influenced by the *Trichinella* species, the host species, the age of the ML, the temperature, and the duration of freezing (Pozio et al., 1994). The nurse cell which harbours the ML is surrounded by a collagen capsule that continually thickens during infection (Sacchi et al., 2001). Muscle larvae may therefore be better protected from freezing in older infections. The most remarkable example is *Trichinella nativa*, which is able to survive for years at −18 °C in muscles of arctic foxes (Kapel et al., 1999); however, it loses its tolerance to freezing when encysted in rodent muscle tissues (Malakauskas and Kapel, 2003). The factors which favour the development of freeze-tolerance is not fully elucidated, but the development of a thick capsule wall in the nurse cell has been suggested. Indeed, freeze-tolerant *T. nativa* present in raccoon dog muscle for 12 weeks

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http://dx.doi.org/10.1016/j.vetpar.2013.01.049

have a significantly thicker capsule than freeze-sensitive *Trichinella spiralis* encapsulated in the same host (Sukura et al., 2002), although the capsule thickness of *T. nativa* and *T. spiralis* are comparable when either is encapsulated in mice (Evensen et al., 1989). In horse meat stored at −18 °C, 12-month-old *T. spiralis* ML survived 4 weeks (Hill et al., 2009) and 20-week-old *T. spiralis*, *Trichinella britovi* and *Trichinella pseudospiralis* ML survived up to 8 weeks (Kapel et al., 2004). This contrasts with the lack of survival observed for *T. spiralis* ML in 40-week-old pork infections stored at −18 °C (Kapel et al., 2004; Lacour et al., 2009).

In Europe, *T. spiralis* and *T. britovi* are the most widespread etiological agents of *Trichinella* infection in wild and domestic animals (Pozio, 2007). The presence of both species is revealed occasionally in human trichinellosis due to consumption of wild boar meat (Gallardo et al., 2007; Romano et al., 2011). As the lifespan of wild boars is likely longer than production pigs, *Trichinella* infections may also be up to several years old in such game. Consequently, the ML complex may be allowed time to develop maximal freeze-tolerance. This study focuses on the freeze-resistance of *T. spiralis* and *T. britovi* isolated from long-term infections in wild boars.

2. Materials and methods

2.1. Animal experimentation

Three groups of four 8-week-old wild boars were infected with 200, 2000 or 20,000 ML of *T. britovi* (ISS 1575). An additional group of three wild boars were inoculated with 20,000 ML of *T. spiralis* (ISS 004); two wild boars were included as negative controls. All wild boars were sacrificed 24 weeks post-infection (wpi). All experimental procedures were conducted according to the guidelines of the appropriate Local Animal Experimentation Ethics Commission.

2.2. Parasitological examination

Trichinella muscle larval burden was determined for each wild boar from 50 g of tongue, masseters, diaphragm, triceps brachii or supraspinatus muscle using the artificial digestion method using a magnetic stirrer as described in the European Commission Regulation (EC) No. 2075/2005 (EC, 2005).

2.3. Freezing treatment and assessment

Triceps brachii and supraspinatus muscles were removed from each wild boar infected with *T. spiralis* or *T. britovi*. Samples of 70 g were frozen at −21 °C ± 2 °C for 19, 30 or 56 h and 1 or 2 weeks. The duration of the freezing began when the centre of the sample reached the target temperature. Temperature was monitored using a probe and a controled system (Evisense, AES Chemunex, Bruz, France). After thawing, ML were recovered using artificial digestion. Larval motility was recorded after 2 h at 37 °C using Archimed® and replay® software for image analysis (Microvision, Evry, France). The infectious capacity of frozen ML was assessed by infecting mice with recovered ML as described by Kapel (2001). After 6 weeks of infection, the presence of ML in mouse muscles was analysed using the artificial digestion method. The infectivity index (number of larvae recovered in mice relative to the number of larvae inoculated) was then calculated.

Table 1
Infectivity indexes of *T. spiralis* and *T. britovi* after different times of freezing.

	T. spiralis		*T. britovi*	
	Median	SD	Median	SD
Before freezing	158.2	16.7	32.2	5.2
19 h	15.4[a]	0.1	24.9	2.0
30 h	0.0	5.6	20.3	3.7
56 h	0.04	5.6	6.3	0.8
1 week	0.0	0.0	0.0	0.0
2 weeks	0.0	0.0	0.0	0.0

Infectivity index: larvae recovered/larvae inoculated in mice.
[a] Statistical test could not be applied due to $n = 2$.

2.4. Statistical analysis

The effect of the *Trichinella*-infecting dose on wild boar weight was assessed using the mixed model procedure for analysis of repeated measures data with the statistical software package SAS (SAS version 9.1, SAS Institute Inc., Cary, IN, USA). Differences in the species' infectivity indexes were assessed with a permutation test for independent samples using StatXact software (StatXact version 3.0.2, Cytel Inc., Cambridge, MA, USA). The freezing time required to decrease the infectivity index by one-half was assessed using a linear regression with infectivity index as the dependent variable and freezing time (up to 56 h) as the explanatory variable for each *Trichinella* species using R (R development Core Team, 2009).

3. Results

Two wild boars, belonging to the 200 ML and 2000 ML *T. britovi*-infected groups, died after 2 and 3 weeks of infection. These were the smallest of the lot and had lost weight since their arrival at the animal facilities. Autopsy did not show any lesions that could have been related to the *Trichinella* infection. Each wild boar was weighed before infection, and again at 12 and 24 wpi. The average daily gain was 164.1 ± 10.8 g/day. No significant effect of the infecting *Trichinella* dose was observed on wild boar weight ($p = 0.12$).

Motile *T. britovi* and *T. spiralis* ML were observed after digestion of samples frozen for 19, 30 and 56 h but not for 1 or 2 weeks. Furthermore, mice given *T. spiralis* and *T. britovi* ML recovered from wild boar meat frozen for 19, 30 and 56 h were indeed infected; however, mice given meat frozen for longer time periods tested negative for ML *T. spiralis* showed a higher infectivity index than *T. britovi* (158.2 ± 16.7 *T. spiralis* ML vs. 32.2 ± 5.2 *T. britovi* ML; $p = 0.0001$) (Table 1); however, *T. britovi* was more resistant to freezing than *T. spiralis* where only half of the *T. britovi* ML were inactivated by treatment for 35 h at −21 °C (confidence interval [CI]: 23–58 h) whereas only 25 h of