Predilection sites of *Trichinella spiralis* larvae in naturally infected horses

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Abstract

A total of 120 muscle tissues from three horses naturally infected with *Trichinella spiralis* were examined. The head was the most infected site. In particular, the muscles harbouring the highest number of larvae were: *musculus buccinator* (12, 411 and 1183 larvae g^{-1}), the tongue (11, 615 and 1749 larvae g^{-1}), *m. levator labii maxillaris* (17, 582 and 1676 larvae g^{-1}), and the masseter (4.9, 289 and 821 larvae g^{-1}). Compared with the diaphragm, the number of larvae per gram was from 3.5 to 6.8 times higher in the tongue, from 3.5 to 6.5 higher in *m. levator labii maxillaris*, and from 2.5 to 4.6 higher in *m. buccinator*. Of the examined muscles, the diaphragm had from the 6th to the 15th highest level of infection (3.1, 166 and 256 larvae g^{-1}). Published data from experimentally infected horses confirm these results, suggesting that efforts to detect predilection sites should focus on the head muscles.

Introduction

In France and Italy, horse meat continues to represent the main source of *Trichinella* infection in humans and, between 1975 and 1998, about 3300 cases were reported in 13 outbreaks (Ancelle, 1998; Anon., 1998). Furthermore, in the last two years, imported horses naturally infected with *Trichinella* sp. were also detected by routine examination in these countries (Pozio *et al.*, 1998). Three of the outbreaks in France and Italy occurred in 1998 (Anon., 1998; Haeghebaert *et al.*, 1998; Pozio *et al.*, 1998), in spite of the fact that, since 1995, both countries have followed the European Union (EU) regulations, which mandate the examination of at least 5 g of selected muscle tissues from all horses by artificial digestion to detect *Trichinella* sp. larvae. In France, the last four human outbreaks of trichinellosis, which occurred in 1993, 1994 and in February and October of 1998, were caused by the consumption of horse meat imported from Canada, Mexico and, for the last two outbreaks, from the former Yugoslavia; the infected horses were certified to have

been investigated correctly according to the EU regulations (Ancelle, 1998). These four outbreaks strongly suggest that the detection of *Trichinella* larvae in horse carcasses needs to be improved; in particular, predilection sites of *Trichinella* larvae require further investigation.

The aim of the present study was to identify the predilection sites of *T. spiralis* muscle larvae in horses by studying the distribution of infecting L_1 larvae in muscles from naturally infected animals.

Materials and methods

Muscle tissues were collected from three naturally infected horses in Italy. The first infected horse, imported from Romania, was detected at the abattoir in the town of Barletta (southern Italy) during routine examination in November 1996 (Pozio *et al.*, 1997). The second animal, imported from Poland, was detected at the abattoir in the town of Brescia (northern Italy) during routine examination in January 1998 (Pozio *et al.*, 1998). The third horse, imported from the former Yugoslavia, was detected at the abattoir in the town of Poggio Imperiale (southern Italy) during routine examination in April 1998.

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Table 1. Number of muscle larvae collected or estimated (underlined numbers) by the Poisson regression analysis per gram of tissue in three naturally infected horses imported from Poland, Romania and the former Yugoslavia in 1996 and 1998.

| Body region	Muscle	Larvae g^{-1} (95% confidence interval)		
		Former Yugoslavia	Poland	Romania
Head	m. levator labii maxillaris	582 (346–981)	1676 (995–2822)	17 (11–27)
	m. hyoideus transversus	162 (149–200)	497 (418–590)	16 (14–17)
	m. buccinator	411 (325–553)	1183 (646–2166)	12 (7–21)
	linguae	615 (562–658)	1749 (1553–1971)	11 (8–16)
	m. temporalis	169 (146–196)	486 (409–578)	5 (4–6)
	m. masseter	289 (255–320)	821 (710–950)	4.9 (4–7)
	m. pterygoideus	154 (60–398)	444 (172–1145)	4.5 (2–11)
	m. cricothryroideus	153 (131–179)	440 (367–528)	4 (3–6)
	m. larynx	86 (24–301)	246 (70–867)	2.5 (1–9)
	m. orbicularis oculi	86 (24–301)	246 (70–867)	2.5 (1–9)
Neck	m. obliquus capitis	137 (50–374)	394 (145–1075)	4 (2–11)
	m. splenius	109 (94–125)	312 (280–349)	3.9 (2–5)
	m. longus colli	17 (13–23)	49 (38–66)	1.5 (1–2)
	m. sternocephalicus	17 (12–22)	47 (36–63)	1.1 (1–2)
	m. brachiocephalicus	26 (19–33)	127 (106–133)	0.7 (0.5–0.8)
	m. omotransversarius	24 (2–252)	69 (7–725)	0.7 (0.5–1)
	m. cutaneus colli	21 (2–260)	59 (5–749)	0.6 (0.0–8)
Pectoral	m. pectoralis	26 (21–34)	75 (61–95)	1.5 (1–2)
	m. pectoralis profundus	30 (24–37)	85 (69–105)	1.0 (0.5–2)
	m. pectoralis transversus	10 (7–15)	29 (20–42)	0.3 (0.2–0.4)
Lumbar	m. iliocostalis thoracis	110 (36–335)	315 (284–345)	3.2 (1–10)
	m. longissimus thoracis	29 (23–37)	83 (68–104)	1.5 (1–2)
	m. longissimus dorsi	8 (7–25)	67 (48–73)	1.3 (1–2)
Pelvis	m. gemelli	29 (23–37)	84 (68–104)	1.0 (0.5–2)
	m. gluteus profundus	34 (5–246)	99 (14–708)	1.0 (0.5–7)
	m. gluteus accessorius	17 (13–23)	49 (38–65)	1.0 (0.7–2)
	m. gluteus superficialis	10 (6–19)	30 (16–54)	0.6 (0–4–1)
	m. quadratus femoris	31 (4–246)	89 (11–708)	0.9 (0.1–7)
	m. gluteus medius	17 (13–23)	49 (37–65)	0.5 (0.3–0.7)
Posterior leg	m. pectineus	45 (8–252)	128 (23–724)	1.3 (1–7)
	m. sartorius	26 (21–33)	75 (60–94)	1.0 (0.5–2)
	m. semitendinosus	12 (9–21)	110 (86–118)	0.9 (0.7–1.2)
	m. quadriceps femoris	6 (4–12)	57 (47–70)	0.8 (0.6–1.2)
	m. semimembranosus	44 (36–54)	128 (107–152)	0.8 (0.6–1.4)
	m. gracilis	31 (25–39)	90 (73–110)	0.7 (0.4–0.8)
	m. adductores	20 (14–26)	56 (43–73)	0.7 (0.0–7)
	m. biceps femoris	21 (2–260)	59 (5–749)	0.6 (0.0–8)
Distal region of posterior leg	m. flexores digitorum	103 (33–325)	296 (93–935)	3.0 (1–9)
	m. extensores digitorum	41 (34–50)	117 (99–142)	2.5 (1–3)
	m. extensor dorsalis digitorum	51 (42–61)	146 (124–172)	1 (1–2)
	m. gastrocnemius	34 (5–246)	99 (14–708)	1.0 (0.5–7)
Thorax	diaphragma (pillars)	166 (97–183)	256 (236–288)	3.1 (3–4)
	m. intercostales	116 (66–199)	175 (143–198)	3.5 (2–4)
	m. serratus ventralis	103 (33–325)	295 (93–935)	3.0 (1–9)
	m. serratus dorsalis	41 (7–249)	118 (20–717)	1.2 (1–7)
Abdomen	m. trasversus abdominis	59 (50–71)	171 (147–199)	2 (1–2)
	m. obliquus externus	38 (28–52)	109 (79–151)	1.0 (0.5–2)
	m. obliquus internus	34 (5–246)	99 (14–708)	1.0 (0.5–7)
	m. abdominis	20 (15–27)	58 (45–75)	1.0 (0.5–2)
	m. rectus abdominis	35 (25–37)	85 (74–106)	0.8 (0.6–1.2)
Lumbar-iliac	m. psoas minor	44 (37–54)	128 (107–152)	1.0 (0.5–2)
	m. psoas major	13 (11–31)	85 (60–89)	1.0 (1)
	m. rectus femoris	26 (21–33)	75 (60–94)	1.0 (0.5–2)
Tail	m. sacrococcygeus	191 (162–215)	538 (455–636)	1.5 (1–8)
Thoracic girdle and arm	m. trapezius	130 (82–136)	231 (211–268)	3.5 (2–4)
	m. supraspinatus	64 (40–55)	118 (100–142)	0.7 (0.5–0.9)
	m. infraspinatus	24 (2–252)	69 (7–725)	0.7 (0.0–7)
	m. subscapularis	49 (41–60)	143 (121–167)	0.6 (0.5–0.8)
	m. deltoideus	45 (37–54)	129 (108–152)	0.6 (0.5–0.8)
Anterior leg	m. triceps brachii	86 (74–101)	248 (219–280)	2.5 (2–3)
	m. biceps brachii	131 (115–150)	381 (342–418)	0.9 (0.7–1.2)
Distal region of anterior leg	m. extensores digitorum	54 (45–65)	154 (133–182)	3.0 (1–4)
	m. flexores digitorum	62 (52–73)	178 (154–206)	1.8 (1–2)
	m. flexores profundus digitorum	50 (42–61)	145 (123–171)	1 (1–2)
	m. flexores carpi radialis	42 (34–51)	120 (101–144)	2.0 (1–3)
	m. extensores carpi radialis	24 (19–37)	427 (402–456)	0.9 (0.6–1.2)
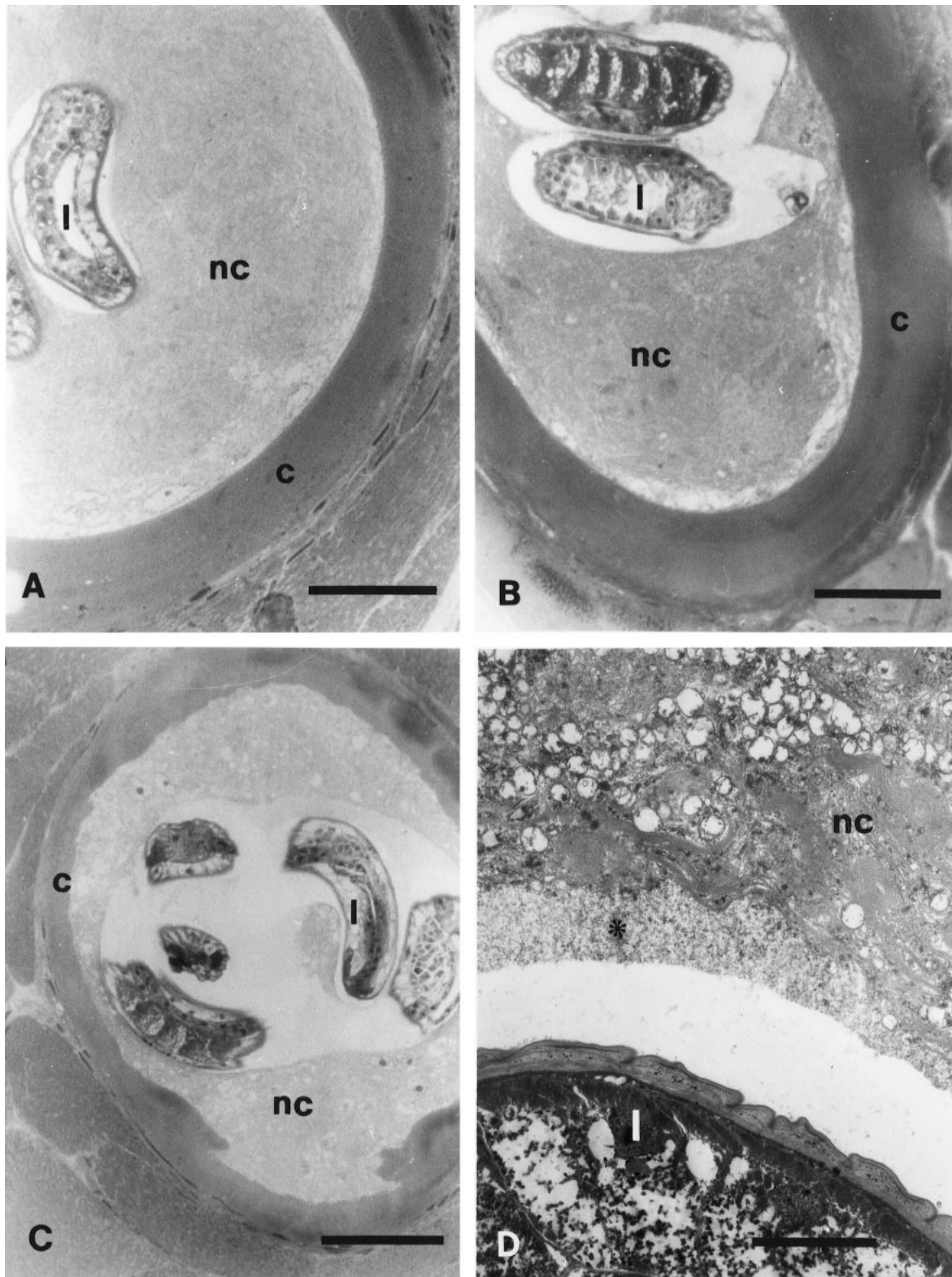
Fig. 1. Semi-thin sections of *Trichinella spiralis* larvae encapsulated within muscles of infected horses imported from Romania (A), the former Yugoslavia (B) and Poland (C). Transmission electron micrograph (D) of an encapsulated L1 larva from a muscle of the Polish horse showing a large amount of endoplasmic reticulum, swollen mitochondria and an amorphous material (asterisk) scattered in the nurse cell–parasite interface. c, Collagen capsule; l, larva; nc, nurse cell. Scale bars: A and B = 50 μm; C = 62.5 μm; D = 6.5 μm.

Muscle tissues were collected from the left, central and right sides of each of the muscles listed in table 1; tissues were sealed in plastic bags and stored at 4°C prior to artificial digestion. Six samples of 10 g each were collected from the distal and medial portion of selected muscles to determine the number of *Trichinella* larvae by artificial digestion using Tricomatic 35® in duplicate. Muscle larvae, recovered after artificial digestion of horse muscles, were identified at species level by the polymerase chain reaction (PCR) analysis using a specific primer set for *T. spiralis* following the protocol of Wu *et al.* (1997), with minor modifications (Pozio *et al.*, 1997). Small pieces of muscle collected from the three infected horses were fixed in 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 4 h at 4°C, rinsed in the same buffer, postfixed with 1% OsO_4 in 0.1 M cacodylate buffer for 1.5 h at 4°C, dehydrated in ethanol, and embedded in Epon 812. For examination with the light microscope, semi-thin sections (0.5 µm) were stained with 0.5% toluidine blue. Thin sections, stained with uranyl acetate and lead citrate, were examined with a Zeiss EM 900 transmission electron microscope. The Poisson regression analysis (Selvin, 1996) was used to estimate the following: (i) the number of larvae present in muscle tissues not examined by artificial digestion; and (ii) the 95% confidence intervals for both the observed and estimated number of larvae for each muscle tissue.

Results

No clinical or pathological disorders were observed in the three infected horses before slaughtering. Muscle larvae collected from the three horses were identified as *T. spiralis*. A total of 120 muscle tissues were examined: 21, 38 and 61 tissues from the horses imported from the former Yugoslavia, Poland, and Romania, respectively. The number of larvae detected after artificial digestion, the number of larvae estimated, and the 95% confidence intervals are reported in table 1. The horses from Poland and the former Yugoslavia showed a high level of infection (256 and 166 larvae g^{-1} in the diaphragm, respectively), whereas the horse imported from Romania showed a lower level of infection (3.1 larvae g^{-1} in the diaphragm).

The examination of muscle sections showed that nurse cells present in the muscle of horses imported from Romania and the former Yugoslavia possessed very thick capsules (average 22.7 µm and 33.5 µm, respectively), suggesting that in both hosts the infection was old (fig. 1A,B). Furthermore, 22% of nurse cell–larva complexes were partly or completely calcified in the muscle tissues of the Romanian horse, indicating that the infection had been acquired a long time before investigation. In contrast, nurse cells of the horse muscle from Poland had thin capsules (average 18.7 µm), suggesting that this animal had acquired the infection recently (<60 days post infection; i.e. late November or early December) (fig. 1C). Ultrastructural observations of this sample showed the presence of amorphous electron-dense material in the nurse cell–parasite interface (fig. 1D), probably generated by immunological defence mechanisms. All samples from the head of the horse imported from Poland tested negative, whereas muscles from the body showed a high level of infection. This was due to the

fact that, at the abattoir, the head of an uninfected horse had been mistakenly identified as that of the infected horse. The infected head of the horse was placed on the market and induced a human outbreak of trichinellosis in Piacenza (northern Italy) in February–March 1998 (data not shown).

The most infected muscles examined or estimated by the Poisson regression analysis in the three horses were *musculus levator labii maxillaris*, *m. buccinator* and *linguae* (table 1). Compared to the diaphragm, the number of larvae per gram was from 3.5 to 6.8 times higher in the tongue, from 3.5 to 6.5 higher in *m. levator labii maxillaris*, and from 2.5 to 4.6 higher in *m. buccinator*. The number of larvae per g in *m. masseter* was from 2.1 to 2.2 times lower than that detected in *linguae*. Of the examined muscles, the diaphragm had from the 6th to the 15th highest level of infection (table 1).

Discussion

In the last two years, seven horses have been found to be positive for *Trichinella* infection in France and Italy: five of them upon routine examination at the abattoir and two after human outbreaks of trichinellosis due to the consumption of horse meat in France (Haeghebaert *et al.*, 1998; Anon., 1998). Muscle larvae from five of these horses were identified at the species level, and all five were found to be infected with *T. spiralis*. This sudden increase of infected horses cannot be completely explained by improvements in diagnostic techniques (i.e. the examination of at least 5 g of selected muscles), and epidemiological factors are assumed to have caused this phenomenon. According to official documents of the infected horses, three of them originated from Poland, three from the former Yugoslavia and one from Romania. In Italy, immediately after the detection of the infection at the abattoir, the veterinary services of the horse's country of origin were alerted and advised to investigate how, where and when the horses had acquired trichinellosis. None of these investigations succeeded in obtaining tangible results. It is beyond doubt that domestic trichinellosis has recently dramatically increased in Eastern Europe (Romania, the former Soviet Union, the former Yugoslavia, etc.). Consequently, it cannot be excluded that in commercial exchanges involving more than two parties ('triangular trades'), all infected horses originated from the same area, even if they arrived in the European Union from different countries. The infected horses which were the source of infection for the human outbreaks that occurred in France in 1998 originated from an area located about 30 km from Vukovar (the former Yugoslavia) where more than 50% of the domestic pig population is infected (A. Marinculic, personal communication). In the area of origin, improper management of horses during the fattening period could result in *Trichinella* infections.

The presence of a thin capsule around larvae in muscle tissues of the infected horse slaughtered in January and the presence of a thick capsule in horses slaughtered in April and October could support the hypothesis that horses acquire this infection in late autumn or winter; they could acquire infection either passively (i.e. by grazing in pastures contaminated by rodent carcasses or pork scraps infected with *Trichinella*), or actively, when the horse farmer feeds horses with infected flesh from infected pigs during the fattening period.

Refining techniques for the detection of this infection in horses is therefore imperative. Since serological diagnosis is not acceptable as an inspection tool in horses (Soulé *et al.*, 1989; Pozio *et al.*, 1997), only parasitological methods can be used. Consequently, if the predilection site is well established, artificial digestion of these muscles is the diagnostic method of choice.

The present results on predilection sites in naturally infected horses are comparable to those of other studies of experimentally infected animals. Gamble *et al.* (1996) examined 15 different muscles (only two from the head) from 12 experimentally infected horses and observed a higher number of larvae in the masseter, compared with the tongue, in animals which received high infecting doses (10,000–40,000 larvae), whereas in animals infected with only 1000 or 4000 larvae, the tongue harboured a higher number of larvae. Soulè *et al.* (1989) detected a higher number of larvae in the tongue, with respect to the masseter, in five of the eight animals examined; in one of the three remaining horses, the masseter had a higher number of larvae per gram than the tongue, and in the other two animals, the diaphragm harboured more larvae per gram than the tongue and masseter. A higher number of larvae per gram in the tongue, compared with the masseter, was also observed by Voigt *et al.* (1997) in seven horses receiving an infecting dose of 5000 larvae, by Smith & Snowdon (1987) in three animals infected with 1000–25,000 larvae, and by Polidori *et al.* (1990) in one animal infected with 10,000 larvae.

The present results and data from the literature concur to show that the predilection sites for larvae of *T. spiralis* in horses are muscles of the head. In particular, *m. buccinator* and *linguae* appear to be the most promising muscles for improving the sensitivity of artificial digestion and protecting consumers from this infection. *Musculus levator labii maxillaris* was observed to be the most infected muscle; however, it was examined in only one horse, and a larger number of infected horses would need to be examined to confirm this muscle as the predilection site for *T. spiralis* larvae. The pillars of the diaphragm, which are the predilection sites for the diagnosis of this infection in pigs, must no longer be considered for trichinellosis in horses, and all efforts must focus on the head muscles. In fact, the choice of predilection sites must be based on the following parameters: (i) the number of larvae per gram; (ii) the ease with which abattoir technicians can single out the muscle; and (iii) the size of this muscle, which must be large enough to allow for collection of several samples of 10–20 g each. Finally, once the muscle of choice has been established, the portion of the selected muscle harbouring the highest number of larvae per gram will need to be determined. Furthermore, horse meat intended to be eaten uncooked should be inspected using the most critical methods possible, which would include testing larger amounts of tissue and applying a high level of quality control in the testing process.

Acknowledgements

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