

Guidelines for the identification and development of sampling methods and design of suitable protocols for monitoring of *Trichinella* infection in indicator species

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Summary. According to the European Community Regulation No. 2075/2005, a risk-based program should be implemented in those areas where wildlife and pig holdings applying for *Trichinella*-free status coexist, or for region where the risk of *Trichinella* in domestic swine is officially recognised as negligible. *Trichinella* nematode species are primarily parasites of wildlife which can transmit the infection to pigs intended for human consumption, acting as reservoir of the parasite. Since not all mammals play the same role for the different *Trichinella* species, the selection of the target animals for epidemiological surveillance is of utmost importance. This document provides indications on the most suitable wild animals to be targeted for epidemiological surveillance according to the four *Trichinella* species circulating in Europe, together with technical specifications on the muscles of choice and their amount to be analysed for each host species considered.

Key words: European Union, *Trichinella*, monitoring programmes, wildlife, muscles of choice.

Riassunto (*Linea guida per l'identificazione e lo sviluppo di metodi di campionamento e per la progettazione di protocolli finalizzati al monitoraggio di specie animali indicatori d'infestazione da Trichinella*). Il Regolamento della Comunità Europea No. 2075/2005 stabilisce che debbano essere attuati programmi di monitoraggio nelle aree nelle quali coesistano animali selvatici ed allevamenti suini ritenuti esenti da *Trichinella*, o nelle regioni in cui il rischio di infezione nei suini domestici sia trascurabile. I nematodi del genere *Trichinella* parassitano essenzialmente gli animali selvatici, che possono trasmettere l'infezione ai suini destinati al consumo umano, fungendo da serbatoio del parassita. Poiché le diverse specie di animali selvatici suscettibili a questi parassiti svolgono ruoli di diversa importanza a seconda della specie di *Trichinella* trasmessa, la scelta della specie animale da monitorare ai fini di una sorveglianza epidemiologica dell'infestazione riveste una notevole importanza. Il presente documento fornisce indicazioni su quali selvatici siano più adatti a monitorare la presenza delle quattro specie di *Trichinella* presenti in Europa, insieme ai dettagli tecnici relativi ai muscoli d'elezione ed alla loro quantità da esaminare a seconda della specie ospite considerata.

Parole chiave: Unione Europea, *Trichinella*, programmi di monitoraggio, animali selvatici, muscoli d'elezione.

INTRODUCTION

According to the European Community Regulation (EC) No. 2075/2005 [1] on *Trichinella* in meat intended for human consumption, a risk-based wildlife monitoring programme should be put in place in those areas where wildlife and pig holdings applying for *Trichinella*-free status coexist, or for regions where the risk of *Trichinella* in domestic swine is officially recognised as negligible. The monitoring programme should optimise parasite detection by applying the most suitable indicator animals and detection techniques, by sampling as wide a number of animals and taking as large a meat sample as is feasible. The Community Reference Laboratory for

parasites (CRLP) can assist by preparing a standardised protocol for a wildlife monitoring programme. Moreover, parasites detected in wildlife can be identified at species level at the CRLP.

Since nematodes of the genus *Trichinella* are primarily parasites of wildlife, the source of all infections in domestic animals should be brought back to wild animals, *i.e.* to the sylvatic cycle which, however, may also be influenced by human behaviour [2, 3]. *Trichinella* infections in domestic pigs are sporadic when there is a direct transmission of these pathogens from wild animals to pigs (*e.g.* free roaming pigs, backyard pigs), whereas the prevalence increases considerably when the parasite (al-

most exclusively *Trichinella spiralis*) is transmitted by a domestic cycle in pig herds [3]. In addition, the prevalence of infection could be high in regions and countries where *T. spiralis* occurs, whereas it is very low where only *Trichinella britovi* and/or *Trichinella pseudospiralis* circulate in nature [4].

TARGET ANIMALS FOR EPIDEMIOLOGICAL SURVEILLANCE

The selection of the target animals for epidemiological surveillance is of great importance, because even if the four *Trichinella* species circulating in Europe have a broad host spectrum, not all mammals play the same role of reservoir for the different parasite species. The preferential muscles that should be tested are different from swine and carnivores, as well as the digestibility of muscle tissues [5]. In addition, the amount of the muscle tissues which should be tested is greater than that used to test fattening pigs [6]. As a general rule, the number of larvae per gram of muscle in naturally infected animals is very low, most of infected animals harbouring between 0.1 and 1.0 larvae/gr in preferential muscles. It follows that the test performed in the laboratories assigned for the epidemiological surveillance should validate the test/s to detect *Trichinella* larvae using meat samples from wildlife, which show greater difficulties to be digested [5, 6].

Sows and boars

Since sows and boars have a life span longer than that of fattening pigs present in the same farm, and a more aggressive behaviour for food resources than that of fattening pigs, they can play an important role as reservoir and indicator animals for *Trichinella*. According to the current legislation [1], all sows and boars should be tested and at least 5g of muscle tissues should be examined by an approved method. Even if *T. spiralis*, *T. britovi* and *T. pseudospiralis* can develop in farmed sows and boars, only *T. spiralis* is well adapted to the farm environment. Thus the occurrence of the other two species should be considered extremely rare and only due to wild animals entered accidentally in the pig herd. *Trichinella nativa* does not develop in swine [3].

The muscle of choice to be digested are the pillars of the diaphragm, showing a good balance between the number of larvae per gram and the digestibility; however, the muscle of the tongue harbours a higher number of larvae mainly in *T. britovi* and *T. pseudospiralis* infected pigs [5, 6].

Free-roaming and backyard pigs

These categories of pigs are those at higher risk for *Trichinella* infection and even if they are frequently out of market, because they are consumed directly by the owner and its relatives, they represent the most important indicator animals. Indeed they are easily in contact with wildlife, they can be more easily tested than wild animals, they are spread almost everywhere in a

country, and they are slaughtered in a well defined period along the year. Today, almost all human infections caused by pork consumption in the EU countries, are caused by the consumption of these pigs.

Horses

Trichinella sp. in horses is a low frequency infection with high human risk; a prevalence of only four infected horses per one-million slaughtered animals has been detected in Europe, including both horses that were the source of infection for humans and positive horses detected at the slaughterhouse since 1975 [2]. The origin of *Trichinella*-infected horses was always related to country with a very high prevalence of this infection in domestic pigs and wildlife. Consequently, horses cannot be considered as a target species for epidemiological surveillance purposes.

Synanthropic rats

The brown rat (*Rattus norvegicus*) does not play any role as reservoir of *T. spiralis* but it is only a victim. This figure means that in farms, garbage dumps and slaughterhouses with low sanitation, rats can acquire this infection and can represent the source of infection for pigs bred nearby or for wild animals [3]. Indeed, there are no reports of *T. spiralis* infection in brown rats in areas where pig populations have been found to be negative. This indicates that brown rats alone cannot maintain the infection, if *T. spiralis* is not conveyed into their populations from other host species. However, the parasite transmission in a pig farm may involve rats as an important source of infection when this synanthropic animal is exposed to pork scraps or cannibalism under unique circumstances such as high population pressure. Infected rats represent an offshoot of the domestic cycle, being recipients of infection [3]. *Trichinella* infection in rats can be considered as a “symptom” of the occurrence of this parasite in swine and the real source of infection for both pigs and rats to be scraped and offal of hog carcasses. *Trichinella britovi* and *T. pseudospiralis* have been also detected in brown rats, suggesting that in particular epidemiological situations not only *T. spiralis* can reach these rodents, but these infections were always detected when rats got in contact with wildlife [3].

Stray dogs and cats

As for the above reported categories, these animals can act as a link between the domestic and wild habitat, favouring the transmission of these pathogens from wild to domestic animals and vice versa. Even if the four *Trichinella* species circulating in Europe can develop in these hosts, dogs and cats are most frequently infected by *T. spiralis* and *T. britovi*. The muscles of choice are the tongue and the masseter and, in dogs, also the anterior tibial.

Wild boars

The wild boar (*Sus scrofa*) is one of the best indicator species of the presence of *T. spiralis* and *T.*

pseudospiralis, whereas its role as host of *T. britovi* is less important than that of wild carnivores. The wild boar does not play any role as reservoir of *T. nativa* [3]. The muscle of choice to be digested are the pillars of the diaphragm, which show a good balance between the number of larvae per gram and the digestibility; however, the muscle of the tongue shows a higher number of larvae mainly in *T. britovi* and *T. pseudospiralis* infected wild boars [6].

Red fox

The red fox (*Vulpes vulpes*) is one of the best indicator of the presence of *T. britovi* and *T. nativa*, whereas its role as host of *T. spiralis* is less important than that of swine. The presence of *T. pseudospiralis* in the red fox is exceptional [7].

Raccoon dog

The distribution area of the raccoon dog (*Nyctereutes procyonoides*) is spreading from the far east to the west of Europe, and today this animal is present in 16 EU countries [8]. It is an excellent host for all the four species of *Trichinella* present in Europe [9].

Other sylvatic carnivores

The marten (*Martes martes*), beech-marten (*Martes foina*), badger (*Meles meles*), weasel (*Mustela nivalis*), polecat (*Mustela putorius*), brown bear (*Ursus arctos*), wild cat (*Felis silvestris*), lynx (*Lynx lynx*), wolf (*Canis lupus*), jackal (*Canis aureus*) can play important roles as reservoir of *Trichinella* parasites in some circumscribed areas, but the low consistency of their populations, at least in the areas with high pig farming, reduces their importance as target animals for *Trichinella*. In addition, most of them are highly protected species which cannot be hunted or can be shot only with special permission for a very limited number of specimens.

Omnivore and carnivore birds

Even if these animals can be infected with *T. pseudospiralis*, available information is not enough to evaluate the role played by birds in the epidemiology of this nematode species [10], thus preventing to estimate the cost/benefit of the examination of these animals. Even so, the screening of a large number of birds can add useful information on the epidemiology of this parasite.

Table 1 | Epidemiology of *Trichinella* spp. in the EU countries

Country	<i>Trichinella</i> infection in wildlife	<i>Trichinella</i> infection in domestic pigs ^a	<i>Trichinella</i> risk negligible
Austria	yes	no	no
Belgium	yes	no	no
Bulgaria	yes	yes	no
Czech Republic	yes	no	no
Cyprus	no	no	yes
Denmark	yes ^b	no	yes
Estonia	yes	no	no
France	yes	no	no
Finland	yes	yes	no
Germany	yes	no	no
Greece	yes	no data	no
Hungary	yes	no	no
Ireland	yes	no	no
Italy	yes	no	no
Latvia	yes	yes	no
Lithuania	yes	yes	no
Luxembourg	no data	no data	no
Malta	no	no	yes
Netherlands	yes	no	no
Poland	yes	yes	no
Portugal	yes	no	no
Romania	yes	yes	no
Slovak Republic	yes	no	no
Slovenia	yes	no	no
Spain	yes	yes	no
Sweden	yes	no	no
United Kingdom	Yes ^c	no	no

^aOnly those intended for the market.

^bDetected only in one Baltic island.

^cOnly in Northern Ireland.

Table 2 | Rank of muscle predilection sites of *Trichinella* spp. circulating in Europe according to host species

Host	Swine ^b		Horse		Red fox ^c	
	Ts, Tb	Tp	Ts, Tb	Tp	Ts, Tb, Tn	Tp
<i>Trichinella</i> species ^a	Ts, Tb	Tp	Ts, Tb	Tp	Ts, Tb, Tn	Tp
Tongue base	1	2	1	3	1	9
Diaphragm	2	1	3	1	3	1
Masseter	6	2	2	2	6	7
Tongue tip	3	5	4	4	-	-
Neck	4	4	5	5	7	5
Abdomen	7	8	6	8	-	-
Tenderloin	10	7	7	6	-	-
Intercostals	9	19	10	11	-	-
Upper forelimb	13	9	11	9	4	2
Lower forelimb	14	13	8	14	2	8
Lower hindlimb	12	11	12	15	8	6
Filet	15	15	15	12	9	3

^aTs = *T. spiralis*; Tb = *T. britovi*; Tp = *T. pseudospiralis*; Tn = *T. nativa*.

^bBoth domestic pigs and wild boars.

^cEven if there is no data on the preferential muscles for other carnivores (e.g. wolves, bears, raccoon dogs, badgers), the figure of the fox can be used.

Reptiles

There are no reptile species living in Europe which can be susceptible to *Trichinella papuae* or *Trichinella zimbabwensis* infections; consequently, European reptiles cannot be considered target species for epidemiological surveillance.

CRITERIA TO BE FOLLOWED FOR ANIMAL TESTING

To proceed to the evaluation of the number of animals of different species that should be tested for an epidemiological surveillance to apply for *Trichinella*-free holdings, the following information should be available:

- the estimated number of animals of the target species in the area under study (e.g. province, county, region, country); this is an important parameter, because the sampling size is strongly related to it;
- the risk present in the area under study (Table 1):
 - areas with a high prevalence of *Trichinella* infection in wildlife and in pigs intended for the market (Bulgaria, Finland, Latvia, Lithuania, Poland, and Romania);
 - areas with a high prevalence of *Trichinella* infection in wildlife, but no infection in pigs intended for the market (Estonia, Slovak Republic, and Spain);
 - areas with a low prevalence of *Trichinella* infection in wildlife and no infection in pigs intended for the market (Austria, France, Germany, Hungary, Italy, Portugal, Slovenia, and Sweden)
 - areas with a very low prevalence of *Trichinella* infection in wildlife and no infection in pigs intended for the market (Belgium, Czech Republic, Denmark, Ireland, The Netherlands, and United Kingdom);

- areas with no *Trichinella* infection in wild and domestic animals (Cyprus and Malta);
 - areas with no or very few epidemiological information (Greece and Luxembourg);
- the *Trichinella* species circulating in the area under study;
 - the cost of the muscle sample collection from the target species, the forwarding of muscle samples to the laboratory and the cost of tests.

COLLECTION OF MUSCLE SAMPLES

Muscle can be collected from wild animals killed by hunters, by cars, poisoned or from carcasses detected on the field and from domestic animals. Since *Trichinella* larvae survive in the muscle tissues for a long period of time after the death of the host, also very rotten muscles can be collected, with the only limit related to the health security of the workers. Persons who are collecting muscle samples should wear robust gloves and glasses to prevent the risk of transmission of viral or bacterial zoonotic infections. Meat samples should be closed in plastic bags or vials with a code.

Muscle samples should be enclosed with the following information (those with an asterisk are indispensable):

- host name* (common and/or scientific);
- host age and sex;
- place of origin* (name of the locality, longitude and latitude or GIS coordinates);
- date of sample collection*;
- muscle/s collected*;

STORAGE OF MUSCLE SAMPLES

- muscle samples can be stored at room temperature if they are delivered to the laboratory and processed in a short period of time (within one week);

Table 3 | Amount of muscle (in grams) that should be tested according to host species [1]

Host	Minimum amount of preferential muscle	Minimum amount of other muscles
Fattening pig	1	2
Sow and boar	2	4
Frozen pork sample	5	10
Horse	10	20
Wild boar	10	20
Other wild animals	10	20

These amounts in grams are intended to be only of muscle tissues free of all fat and fascia.

- b) for period of time between 1 and 3 weeks, muscle samples can be refrigerated at +4°C;
- c) for longer periods of time, muscle samples can be:
- i. frozen at -20°C. Frozen samples should reach the laboratory still frozen, because freezing and thawing destroy the DNA of *Trichinella* larvae, preventing their identification at the species level by molecular analysis;
 - ii. alternatively, muscle samples can be preserved in a 0.5% merthiolate (thimerosal) solution in plastic vials at room temperature for several months. By this preservative, muscle tissue can be digested and the DNA of larvae is preserved. The disadvantage of merthiolate is its cost and high toxicity.

Muscle tissues fixed by formalin cannot be digested. In addition, formalin destroys the DNA preventing the identification of *Trichinella* larvae at species and genotype level. Samples fixed by formalin can be tested only by histology, but the sensitivity of this method is lower than that of HCl-pepsin digestion.

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PREFERENTIAL MUSCLES

In Table 2, the rank of muscle predilection sites of *Trichinella* species circulating in Europe according to host species are reported. Values from rank 1 to rank 15 are intended as from the muscle with the highest to that with the lowest number of larvae per gram [6].

AMOUNT OF MUSCLE SAMPLES TO BE COLLECTED AND TESTED

As a general rule, higher the amount of muscle grams analysed, higher the chance to detect *Trichinella* sp. larvae in the tested animal. In preferential muscles, about 15-20% of *Trichinella*-positive animals harbour between 0.1 and 1.0 larvae/g, 50% between 1.0 and 10 larvae/g and less than 10% harbour between 10 and 20 larvae/g.

In Table 3, the amount of muscle that should be tested according to the EU Regulation [1], is reported. However, this is a minimalist approach and it is strongly recommended to test a larger amount of muscle to increase the chance of detecting positive animals. In addition, since the collection of samples on the field and their forwarding to laboratories is one of the major costs in surveillance projects, the collection of a larger amount of muscle is strongly encouraged.

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