

**Guideline 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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1. Introduction

According to the Commission Regulation (EC) No 2075/2005 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 holdings applying for *Trichinella*-free status coexist, or for a region 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 animal and detection technique, by sampling as wide a number of animals and taking as large a meat sample as is feasible; parasites detected in wildlife are identified at species level at the CRLP or NRL; the CRLP can assist by preparing a standardised protocol for a wildlife monitoring programme. Historical data may be used for the fulfilment of the requirements.

Since nematodes of the genus *Trichinella* are primarily parasites of wildlife, the source of all infections in domestic animals can be traced back to wild animals, i.e. to the sylvatic cycle which, however, may also be influenced by human behaviour. *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 (almost exclusively *T. spiralis*) is transmitted by a domestic cycle in pig herds. In addition, the prevalence of infection could be high in regions and countries where *T. spiralis* occurs, whereas it is very low where only *T. britovi* and/or *T. pseudospiralis* circulate in wildlife.

The aim of the wildlife monitoring program is first to determine the presence/absence of these pathogens in susceptible animals of a certain area and if they are detected, to establish the prevalence of the infection load .

2. Target animals for an epidemiological surveillance

The selection of the target animals for an epidemiological surveillance is of great importance, because even if the four *Trichinella* species (*T. spiralis*, *T. pseudospiralis*, *T. nativa* and *T. britovi*) circulating in Europe have a broad host spectrum, not all mammals play the same role as reservoir for the different parasite species. The preferential muscles that should be tested are different as well as their digestibility among swine, carnivores, rodents and other susceptible mammals . In addition, the amount of the muscle tissue which should be tested in wildlife is greater than the amount used to test fattening pigs. 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 (or less) and 1.0 larvae/gr in preferential muscles. It follows that technicians performing the test in the laboratories assigned for the epidemiological surveillance should be able to detect a low number of larvae by one of the approved methods.

- sows and boars – In areas where sows and boars represent the only susceptible species, they can play an important role as indicator animals for *Trichinella* spp., since they have a longer life span than fattening pigs and since they show a more aggressive behaviour and outdoor access for food resources than fattening pigs,. According to the current legislation, all sows and boars should be tested and at least 2g of muscle tissue of the diaphragm (or 4 g in the absence of the diaphragm pillars) should be examined by an approved method; however, since in this case sows and boars are tested for the epidemiological surveillance, the amount of grams of muscles which should be tested is that reported in the ANNEX 3. 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 as extremely rare and only due to a wild animal entered accidentally in the pig herd. *Trichinella nativa* does not develop in swine. The muscles of choice to be digested are the pillars of the diaphragm which show a good balance between the number of larvae per gram and ease of digestibility; however, the muscle of the tongue harbours a higher number of larvae mainly in *T. spiralis* and *T. britovi* infected pigs (see ANNEX 2).
- free-roaming and backyard pigs – These categories of pigs are those at higher risk for *Trichinella* infection in the domestic habitat and consequently they are important indicator animals. Indeed, they are in contact with wildlife and can be more easily tested than wild animals. Today, almost all human infections occurring in the former EU countries are caused by the consumption of these categories of pigs. The three species, *T. spiralis*, *T. britovi* and *T. pseudospiralis*, can be detected in these swine. However, *T. spiralis* is the species more frequently detected, even if there are country differences. The muscles 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 harbours a higher number of larvae mainly in *T. spiralis* and *T. britovi* infected pigs (see ANNEX 2).
- 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 horses has been detected in Europe including horses as a source of infection for humans and horses detected positive at the slaughterhouse since 1975. The origin of *Trichinella*-infected horses was always related to a country with a very high prevalence of this infection in domestic pigs and wildlife. Horses cannot be considered as a suitable target species for epidemiological surveillance in an established area, but positive horses are indicators of highly infected geographical areas.
- synanthropic rats (*Rattus norvegicus*) – The brown rat can act as the link between the domestic and wild habitat, favouring the transmission of these pathogens from wild to domestic animals and vice versa, even if

their role as a true reservoir of *Trichinella* species is still under debate. This indicates that brown rats alone, without external introduction of *T. spiralis* into their population, cannot maintain the infection. However, transmission of the parasite on 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 pig population pressure. Infected rats represent an offshoot of the domestic cycle, being recipients of infection. *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 is usually scrap and offal of hog carcasses. *Trichinella britovi* and *T. pseudospiralis* have been detected also in brown rats, suggesting that in particular epidemiological situations not only *T. spiralis* can infect these rodents. However, these infections were always detected when rats were in touch with wildlife. The detection of *Trichinella* sp. infections in brown rats trapped into a farm or in garbage dumps, can give an information on the circulation of *Trichinella* spp. (mainly of *T. spiralis*) in domestic pigs and/or wildlife, but the lack of infected rats cannot exclude the circulation of these pathogens in the investigated area.

- stray dogs and cats – As for the above reported categories, these animals can act as the 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, they are most frequently infected by *T. spiralis* and *T. britovi*. The muscles of choice are the tongue and the masseter, in dogs also the anterior tibial. According to the current legislation in many EU countries, these animals cannot be killed and consequently, only animals found dead (e.g. killed by cars, poisoned) can be tested.
- wild boars (*Sus scrofa*) – This is one of the best indicator species for the presence of *T. spiralis* and *T. pseudospiralis*, whereas its role as host of *T. britovi* is less important than that of wild carnivores, even if geographical differences exist. The prevalence of infection is generally very low (0.01-0.0001%), but the high number of hunted and tested animals represent a good choice to collect information on the circulation of these pathogens in one area. Wild boars do not play any role as reservoir of *T. nativa*, even if this parasite has been detected in this host species. The muscles 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 harbours a higher number of larvae mainly in *T. spiralis* and *T. britovi* infected pigs (see ANNEX 2).
- red fox (*Vulpes vulpes*) - This is one of the best indicator species for the presence of *T. britovi* and *T. nativa*, whereas its role as host of *T. spiralis* is less important than that of swine, however there are differences

between country. The presence of *T. pseudospiralis* in the red fox is exceptional.

- raccoon dog (*Nyctereutes procyonoides*) – The distribution area of this carnivore is spreading from the far east to the west of Europe, and today this animal is present in 16 EU countries. It is an excellent host for all the four species of *Trichinella* circulating in Europe.
- other sylvatic carnivores - [e.g. 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*)]. These animals 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 a high domestic pig population, 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. In these countries, only animals found dead (e.g. killed by cars, poisoned) can be tested.
- omnivore and carnivore birds – while these animals can be infected with *T. pseudospiralis*, the available information is not (yet) enough to evaluate the role played by birds in the epidemiology of this nematode species. Consequently, the cost/benefit of the examination of these birds is still unknown. It does not mean that screening of a large number of birds cannot add useful information to elucidate the epidemiology of this parasite.
- reptiles – there is no reptile species living in Europe which is susceptible to *Trichinella papuae* or *Trichinella zimbabwensis* infections. Consequently, European reptiles cannot be considered target species for an epidemiological surveillance.

3. Criteria to be followed for animal testing

To estimate 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:

- a. 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; without this information, the surveillance data are not statistically valid;
- b. the *Trichinella* species circulating in the area under study since there are differences in the epidemiology among species;

- c. the sample size of muscles which should be tested;
- d. the costs for the muscle sample collection from target species, the forwarding of muscle samples to the laboratory and the analysis of samples. The investigation should be based on the cost/benefit relationship; the choice of target species can be changed according to the costs.

4. Collection of muscle samples: see ANNEX 1

5. Sample size: see ANNEX 2

6. Ranking of muscle predilection sites: see Annex 2

7. Amount of muscle samples to be collected and tested: see Annex 3

8. Establishment of the number of animals which should be tested: see Annex 4

ANNEX 1

Procedures for the collection, preservation and forwarding of samples

1. Collection of samples

Muscle can be collected from wild animals by active surveillance or passive surveillance, e.g. killed by hunters, by cars, or poisoned, or from carcasses detected on the field and from domestic animals. In many countries, the authorities do not give any permission to kill some wildlife species only for surveillance purpose, even if the EU legislation require this control. It is frequently a political problem.

Since *Trichinella* larvae survive in muscle tissues for a long time after the death of the host, also very rotten muscles can be collected, with the only limitation related to the health 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 placed in closed plastic bags or vials accompanied by proper documentation. Otherwise, muscles can be also collected from frozen carcasses.

Muscle samples should be accompanied by 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*

2. Storage of muscle samples

- a. muscle samples can be stored at room temperature if they are delivered to the laboratory and processed in a short period of time (within two days);
- b. for a period of time between 1 and 3 weeks, muscle samples can be refrigerated at +4°C;

for longer periods of time, muscle samples can be:

- c. 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;

- d. for carnivore animals which can be the final host of *Echinococcus multilocularis*, it is suggested for safety reasons, to frozen the carcasses at -80°C for at least one week to kill the embryo in the eggs;
- e. alternatively, muscle samples can be preserved in a 0.1% merthiolate (thimerosal) solution in plastic vials at room temperature for several months. This preservative preserves the DNA of larvae. The disadvantage of merthiolate is its cost and high toxicity.

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

Trichinella larvae collected after the HCl-pepsin digestion should be preserved in absolute ethyl alcohol for their identification at the species level. These larvae can be identified by a PCR-derived method at the NRL or CRL for parasites.

ANNEX 2

Ranking of muscle predilection sites of *Trichinella* species circulating in Europe

Ranking of muscle predilection sites of *Trichinella* species circulating in Europe. A ranking as number 1 means that the muscle contains the highest number of larvae per gram. As the ranking number increases (to a maximum of 15), it will be more difficult to detect *Trichinella* larvae.

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

- a) Ts = *T. spiralis*; Tb = *T. britovi*; Tp = *T. pseudospiralis*; Tn = *T. nativa*;
- b) Both domestic pigs and wild boars;
- c) Since there is no data on the preferential muscles for other carnivores (e.g. wolves, bears, raccoon dogs, badgers), the information available for the fox can be used.

Reference

Kapel CMO, Webster P, Gamble HR (2005). Muscle distribution of sylvatic and domestic *Trichinella* larvae in production animals and wildlife. *Vet Parasitol* 132, 101-105.

ANNEX 3**Amount of muscle samples to be collected and tested**

As a general rule, the higher amount of muscle (in grams) tested, the higher the chance to detect larvae of *Trichinella* sp. 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.

The minimum amount of muscle samples (free of fat and fascia) which should be tested according to the Commission Regulation (EC) No 2075/2005, are reported below. However, this is a minimalist approach and a larger amount of muscle is strongly recommended to be tested to increase the chance to detect positive animals. In addition, since a large part of the cost of surveillance projects consists of the collection of samples in the field and the forwarding to the laboratories, the collection of a larger amount of muscle (in grams) is strongly recommended.

For the monitoring of *Trichinella* infection in indicator species, the minimum amount of muscle sample (free of fat and fascia) which should be tested from each tested animal irrespective of the species is:

- **from preferential muscles \geq 10 grams;**
- **from other muscles \geq 20 grams;**

N.B. All the other amount of muscles reported in the Commission Regulation (EC) No 2075/2005 refer to animals tested at the slaughterhouse to prevent the infection in humans, not for epidemiological surveillance.

ANNEX 4**Establishment of the number of animals which should be tested**

The CRLP will develop an electronic form sheet (Excel) that will allow to calculate the number of animals that should be tested according to the animal species, by introducing in the sheet data related to population size, *Trichinella* species circulating in the study area and the expected prevalence of infection (i.e. on the basis of data originating from previous surveys), an algorithm will calculate the statistically significant number for a given epidemiological situation. It will be possible to download the sheet from the CRLP web site, when it becomes available.

Free of charge programs can be download from the web, e.g.

Win episcopo (both English and Spanish versions)

www.clive.ed.ac.uk/cliveCatalogueItem.asp?id=B6BC9009-C10F-4393-A22D-48F436516AC4

Epiinfo

www.cdc.gov/search.do?action=search&queryText=epiinfo&x=16&y=11

ANNEX 5**References**

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Pozio, E., Murrell, K.D. (2006). Systematics and epidemiology of *Trichinella*. *Advances in Parasitology* 63, 367-439.