Section 3.1

Multiple species

CHAPTER 3.1.23

TRICHINELLOSIS (INFECTION WITH *TRICHINELLA* SPP.)

SUMMARY

Eating raw or undercooked meat of domestic animals or game containing infective Trichinella *spp. larvae can cause trichinellosis in humans. Animals can become infected by feeding on* Trichinella*-infected tissues. Ingested infective larvae mature and reproduce in the small intestine of host species including humans, pigs, rats, bears, walruses, horses and many other not-strictly herbivorous mammals, birds and reptiles. The adult worms survive less than 1 month. The larvae they produce migrate to and persist in the muscles of their hosts, serving as a source of infection for susceptible new hosts.*

Detection and identification of the agent: *Tests for detecting* Trichinella *spp. fall into two categories: 1*) *direct detection of first-stage larvae encysted or free in striated muscle tissue, and 2*) *indirect detection of infection with, or exposure to,* Trichinella *spp. by tests for specific antibodies.*

Tissue digestion and tissue compression methods have been used for the direct detection of Trichinella *larvae in tissues.* Trichinella *larvae usually localise in higher concentrations in preferred muscles, which may vary by host species. It is important that preferred muscles be sampled to maximise test sensitivity. For example, in pigs, the diaphragm* (*crus*), masseter *and tongue muscles are the most preferred sites in order of preference, whereas in horses, the tongue, masseter, and diaphragm typically harbour the most larvae, followed by neck muscles.*

The artificial digestion methods involve enzymatic digestion of individual or pooled muscle tissue samples incorporating mechanical homogenisation or grinding, stirring, and incubation. This is followed by filtration and sedimentation procedures to recover and concentrate any larva that are released from the muscle tissue during digestion. Samples processed by these methods are examined under a stereomicroscope for the presence of larvae. Digestion tests can detect <1 larva per gram (*lpg*) *of tissue, but at these low levels of infection, the amount of digested muscle and uneven distribution of larvae within tissues* (*as well as reduced digestibility of some tissues and frozen or otherwise non-optimal wildlife samples*) *are limiting factors. This can be compensated for by testing larger samples per carcass, such as a minimum of 3–5 g for pigs and 5–10 g for horses, game and indicator wildlife species such as foxes. Digestion methods are recommended for the inspection of individual carcasses of food animals such as pigs, horses and game.*

The compression method (*trichinoscopy*) *is less sensitive than artificial digestion and is not recommended as a reliable test for inspection of carcasses for either food safety or surveillance purposes.*

Serological tests: *Serological assays are used for indirect detection. The sensitivity and specificity of serological methods are mainly dependent upon the type and quality of antigens used. Most serological test performance* (*validation*) *data are from pigs. False-negative serological results may occur 1 week or longer after muscle larvae become infective in pigs with light or*

moderate infections. False-positive results have also been reported for serological tests. For surveillance or verification of Trichinella*-free herds, serological methods are acceptable. For individual carcass inspection, only direct methods can be recommended. Pigs harbouring as few as one larva/100 g of tissue have been detected by enzyme-linked immunosorbent assays (ELISA). Excretory/secretory antigens collected by short-term (18 hours) maintenance of* T. spiralis *muscle larvae* in vitro *currently provide the most specific source. It is critical that appropriate positive and negative control sera be used to ensure that ELISAs being performed have acceptable sensitivity and specificity. Positive results obtained by ELISA should be confirmed by Western blot. The digestion of 100 g or more of tissue is also recommended as a confirmatory test for serologically positive animals.*

Requirements for vaccines: *There are no available vaccines for* Trichinella *infection in food animals.*

A. INTRODUCTION

Clinical signs of *Trichinella* infection are not generally recognised in animals, and its main importance is as a zoonosis. Eating raw or undercooked meat of domestic animals or game containing infective *Trichinella* spp. larvae can cause trichinellosis in humans (Gajadhar *et al*., 2006). The short-lived adult worms reside in the small intestine of host species including humans, pigs, rats, bears, walruses, horses, many other not-strictly herbivorous mammals, and some birds and reptiles. The parasite has a direct life cycle completed within a single host. Within hours following consumption of tissue with infective larvae by a suitable host, first-stage muscle larvae (L1) are released by digestion and burrow into the villi of the small intestine. They develop into adults (males up to 1.8 mm long, females up to 3.7 mm long) and survive for less than 1 month. The ovo-viviparous females release new-born larvae (NBL), which migrate via venules and lymphatics into the general circulation. The NBL are distributed throughout the body where they invade striated muscle cells and develop into infective first-stage larvae, with a predilection for specific muscle groups, which vary by host species.

For example, in pigs, the diaphragm pillar, masseter and tongue usually contain the highest concentrations of larvae and are thus sampled in that order of preference, while in horses the order of preference is tongue, masseter, diaphragm, and neck muscles. Predilection sites vary by host species, but in most species, diaphragm, masseter and tongue are optimal sites for sampling. Current knowledge on predilection sites is available for several host species (Gajadhar *et al*. 2019; ISO, 2015). In cases of severe infection, striated muscles contain high numbers of larvae. The larvae of most *Trichinella* species become encapsulated in collagen in host musculature where they can remain infective for years.

Within the genus *Trichinella*, thirteen taxa have been identified, ten of which have been designated as species (Pozio & Zarlenga, 2021; Zarlenga *et al*., 2020). Taxa in this genus are separated into two groups (clades); one characterised by larvae that encapsulate only in mammalian muscles, and one characterised by larvae that do not encapsulate in the muscles and infect both mammalian and avian hosts or mammalian and reptilian hosts. Encapsulating taxa include the following: *Trichinella spiralis* (T1), which has a widespread distribution and is commonly associated with domestic pigs. It is highly infective for domestic and sylvatic swine, mice and rats, but it has also been detected in mammalian carnivores and horses; *Trichinella nativa* (T2) occurs commonly in mammalian carnivores of arctic and sub-arctic regions of North America, Europe and Asia (Oksanen *et al*., 2022). It is highly resistant to freezing and has been experimentally shown to have poor infectivity for pigs, however it has been found in wild boar; *Trichinella britovi* (T3) is found predominantly in wild mammals and pigs, and occasionally in horses and occurs in temperate regions of Europe, Western Asia, and in Northern and Western Africa. *Trichinella murrelli* (T5) is found in mammalian carnivores of North America. It has low infectivity for domestic pigs, and has been reported in horses (Scandrett *et al*., 2018). *Trichinella* T6 is cold-climate adapted, is closely associated with *T. nativa* in northern North America, and is also highly resistant to freezing (Zarlenga *et al*., 2020). *Trichinella nelsoni* (T7) has been isolated from mammalian carnivores and sporadically from wild pigs in Eastern and South Africa. *Trichinella* T8 has been detected in mammalian carnivores of Namibia and South Africa and *Trichinella* T9 in mammalian carnivores of Japan (Zarlenga *et al*., 2020). T8 and T9 share some intermediate characteristics with *T. britovi* and *T. murrelli*, respectively. *Trichinella patagoniensis* (T12) has been isolated from mountain lions of Argentina, and experimentally shown to have poor infectivity for pigs and rodents (Krivokapich *et al*., 2012). In 2020, a new taxon, named *Trichinella chanchalensis* (T13), was described from wolverines (*Gulo gulo*) of north-western Canada (Sharma *et al*., 2020). Non-encapsulated taxa include the following: *Trichinella pseudospiralis* (T4) is widespread in distribution and has been recovered from raptorial birds, wild carnivores and omnivores, including domestic and wild pigs, and rats and marsupials in Asia, North America, Europe and Australia (Pozio, 2016); *Trichinella papuae* (T10) has been reported from wild and domestic pigs and farmed crocodiles in Papua, New Guinea, Thailand and Australia; *Trichinella zimbabwensis* (T11) has been described in farmed and wild

crocodiles of Zimbabwe, South Africa, Ethiopia and Mozambique, in monitor lizards of Zimbabwe and in mammalian carnivores of South Africa. Experimentally, it has high infectivity for a wide spectrum of mammalian hosts including pigs and rats (Pozio & Zarlenga, 2021). Most species and genotypes of *Trichinella* have been detected in humans, and it is generally accepted that all taxa of *Trichinella* are highly infective for people, representing a significant public health risk. The risk of establishing *Trichinella* infection in pig herds is presented mainly by *T. spiralis,* and to a lesser degree by *T. britovi, T. nelsoni, T. pseudospiralis, T. papuae*, and *T. zimbabwensis,* whereas there is no evidence that other species and genotypes can play such a role.

Human trichinellosis can be a debilitating disease and may result in death. The short-lived adult worms in the intestine can cause transient gastroenteritis, but the most severe signs and symptoms result from the migration and establishment of the larvae in striated muscle. The disease is transmitted primarily by eating meat of infected pigs or game that has not been sufficiently cooked (or otherwise treated to inactivate the parasite). Although the prevalence of *Trichinella* infection in horses is low, consumption of raw or undercooked horsemeat is a well documented source of human trichinellosis (Boireau *et al.*, 2000). Prevention of human infection is accomplished by meat inspection, by adequate processing (thorough cooking is the most reliable means of inactivating *Trichinella* spp.; freezing or curing of meat can also be effective depending on the genotype being targeted and if a validated procedure is used), and by preventing the exposure of food animals to tissues harbouring *Trichinella* larvae, including uncooked food waste, rodents and wildlife (Gajadhar *et al.*, 2006; Noeckler *et al.*, 2019). Meat from non-strictly herbivorous game species should always be considered a potential source of infection, and should be tested or properly cooked. *Trichinella* found in game meats (mainly *T. nativa*, T6, and to a lesser degree *T. britovi*) may be resistant to freezing and therefore untested, frozen game poses a public health risk. *Trichinella* parasites circulate mainly among wild animals; among domestic animals, they usually infect only free-ranging and backyard pigs and rarely horses; *Trichinella*-infected horse and game meat has been implicated in outbreaks linked to international trade. The illegal importation of pig and wild boar meat in personal baggage has been the source of many trichinellosis outbreaks (Pozio, 2021).

Testing methods for the detection of *Trichinella* infection in pigs and other species include either: (a) direct demonstration of the parasite in tissue samples; or (b) indirect demonstration of the presence of, or exposure to, the parasite by detecting specific antibodies to *Trichinella* spp. in blood, serum or tissue fluid samples (Gajadhar *et al.*, 2009).

Laboratory manipulations should be performed with appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). The risk of laboratory-acquired infection for analysts is minimal if good laboratory practices are followed. Transmission occurs by the ingestion of muscle larvae in tissues or freed by artificial digestion. Naked larvae die quickly when exposed to the environment or commonly used disinfectants. Contaminated glassware and other surfaces should be cleaned with water at ≥85°C or other suitable processes to lyse and remove all larvae. Laboratory waste, including sample remnants, should be treated by boiling, autoclaving, incineration or other suitable processes to kill larvae and prevent their re-introduction into the environment. This is particularly critical when testing proficiency samples containing live larvae in a non-endemic region.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for detecting Trichinella *infections in pigs and their purpose*

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of positive cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent[a]						
Artificial digestion	+	–	–	+++	+++	–
PCR[b]	–	–	–	++	++	–
Detection of immune response						
ELISA	+++	+	+	–	++	–
Western blot[c]	++	+	+	–	++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;

+ = suitable in very limited circumstances; – = not appropriate for this purpose.

PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.

[a]A combination of agent identification methods applied on the same clinical sample is recommended.

[b]PCR is used as a confirmatory test and for species-determination.

[c]Western blot is used as a confirmatory test for ELISA positives.

1. Detection and identification of the agent

The only recommended procedures for the detection of *Trichinella* larvae in muscle tissues are enzymatic digestion assays. A number of digestion assays are officially recognised in various countries for trade purposes. The International Commission on Trichinellosis (ICT, http://www.trichinellosis.org; Gajadhar *et al.*, 2019; Noeckler *et al.*, 2019) recommends several of these assays, which are documented standards in the European Union (EU), Canada and elsewhere (European Commission, 2015; Forbes & Gajadhar, 1999). Other methods are not recommended because of their lack of efficiency or reliability. Modern diagnostic assays should meet internationally accepted standards of quality assurance, which include scientifically derived validation data and a design that allows routine monitoring and documentation of critical control points. An International Organization for Standardization (ISO) standard (18743:2015) for the detection of *Trichinella* larvae in animals has also been published (ISO, 2015). The digestion assay recommended here is based on desirable innovations inherent in some digestion assays that are accepted for international trade purposes.

1.1. Recommended direct procedure for testing muscle tissue

1.1.1. Sensitivity

The sensitivity of direct testing methods depends on the amount of tissue examined and the site from which the sample was obtained. Direct methods can identify infected pigs, horses or other animals infected with *Trichinella* sp. as early as 17 days after exposure, coincident with the time that muscle larvae become infective for a new host. Direct methods are most sensitive on fresh samples. The number of larvae that can be recovered from samples declines

unpredictably after prolonged storage, putrefaction and freezing (particularly for freeze-susceptible taxa). Samples tested for food safety-related purposes should be stored at 4°C and tested as soon as possible. For wildlife, larger samples (≥10 g) should be tested to compensate for a possible decrease in sensitivity due to unknown variation of predilection sites in these host species, as well as more variable storage conditions. Current methods for testing fresh samples for food safety or individual animal inspection by artificial digestion and employing a 1 g sample have a sensitivity of approximately three larvae per gram (lpg) of tissue, and testing of a 5 g sample increases sensitivity to 1 lpg of tissue (Gajadhar *et al.*, 2019; Noeckler *et al.*, 2019). Where large amounts of tissue (up to 100 g) are available for digestion, the sensitivity is further increased.

1.1.2. Sampling

Tests are conducted on carcass samples collected post-mortem. Muscle samples are taken from predilection sites, usually the diaphragm pillars, masseter, or tongue of pigs, and tongue, masseter, or diaphragm muscles of horses. For wildlife species in which predilection sites are unknown, tongue (preferred), masseter or diaphragm should be taken. The anterior tibial muscle is a predilection site for foxes. The sample sizes for food safety testing are based on the reliable detection of animals harbouring ≥1 lpg in tissue, but for surveillance purposes, a higher sensitivity is required to provide more accurate infection prevalence data and to overcome sampling limitations such as those encountered with wildlife. surveillance samples taken from predilection sites (if known) should be ≥10 g. Samples of 100 g would enable the detection of as low as 0.01 lpg in the source tissue and if the sample was obtained from a predilection site, a low larval burden or negative result would indicate a negligible load in the rest of the carcass, with an associated low risk for transmission. For food safety testing, each digestion assay can accommodate up to 100 g of muscle tissue. Individual samples of 100 g may be taken from a single animal, or multiple samples of lesser amounts may be collected from a number of animals to make a 100 g pool. The size of the samples from each carcass that contribute to the pool will determine the sensitivity of the method per sample. The ICT recommends 5 g samples per pig for testing in endemic areas (Gajadhar *et al.*, 2019). For testing horsemeat, a minimum of 5 g per carcass is required. For horses originating from endemic areas, or if horsemeat is consumed raw, a 10 g sample is recommended (Gajadhar *et al.*, 2019). Testing these amounts of muscle should prevent trichinellosis in humans but will not prevent asymptomatic infections from the consumption of meat infected with very low numbers of larvae.

1.1.3. Confirmatory testing of pooled digestion samples and serologically positive animals

When a pool of samples from different animals is digested and yields a positive result, additional digestion tests should be used to retest pools of samples from fewer animals and eventually individual animals to determine the identity of the infected animal(s). Animals that are positive on serological testing should have tissues tested by digestion to confirm infection status and to facilitate recovery of larvae and species identification.

1.1.4. Digestion and detection

i) Determine the volume of digestion solution required for the digestion (2000 ml for 100 g of meat, and 1000 ml for 50 g or less).

ii) Digestion solution for 100 g of meat: Prepare the solution, in a 3 litre glass beaker, by adding 16 ml of 25% hydrochloric acid to 2 litres of tap water preheated to 45°C ± 2°C. Stock solutions of hydrochloric acid are available in formulations other than 25% and are to be adjusted accordingly (e.g. use 11 ml of a 37% stock solution). Place a stirring rod in the beaker, place the beaker on a magnetic stirrer, and commence the stirring. Add 10 g of powdered or granular pepsin (1: 10.000 NF) or 30 ml of liquid pepsin (660 U/ml). Once the solution is prepared, retain a sufficient amount in a separate vessel to be used for rinsing as described in step vii below. A serine protease-based *Trichinella* digestion assay kit, that does not use hazardous reagents, is commercially available as an alternative to the pepsin/HCl assay and has been approved by the EU for the testing of pigs only (further information is available from the WOAH Reference Laboratory in Italy[1]).

iii) Remove as much fat, fascia and non-muscle tissue as possible from each sample of meat, including the tongue epithelium when that sample is used.

iv) Weigh the appropriate amount of trimmed meat from each sample. Cut each sample into 1–2 g pieces and pool with other samples into a 100 g amount.

v) Place the pooled meat sample into a blender and add a small amount (50 to 100 ml per 100 g meat) of digest fluid to facilitate homogenisation.

vi) Cover and blend the meat only until it is homogeneous (no chunks of meat should be present and the sample should be the consistency of pureed baby food or coarse paté), do not over-blend as that risks damaging any larvae.

vii) Transfer the homogenised sample into the glass beaker containing the stir bar and digestion solution. To avoid loss of larvae due to adhering muscle tissue, the blending equipment should be thoroughly rinsed with the digest fluid that was set aside in step ii) above and which is then poured back into the beaker.

viii) Place the beaker on a preheated magnetic stirrer hotplate or in an incubation chamber set at 45 ± 2 °C. Cover the beaker with aluminium foil. Activate the stirrer at a sufficiently high speed to create a deep vortex without splashing. Note: If the digest temperature at the beginning of digestion is below 45 ± 2°C, the sample should be allowed to warm to this temperature before the timing of the digestion is started, if feasible. Regularly monitor the temperature of the digestion fluid using a thermometer, particularly when using a hotplate.

ix) Allow the digestion to proceed for 30 minutes. For samples from host species other than domestic pigs, or that are less digestible than diaphragm, the minimum period that enables complete digestion may be longer. As well, if the temperature of the digest has fallen below 45 ± 2°C, additional digestion time may be required to complete the digestion. This can be determined by observing the digestion mixture. If pieces of undigested muscle tissue are present, the digestion time can be increased but should not exceed 60 minutes in total. Care should be taken to ensure that the digestion temperature range is not exceeded (45 ± 2°C).

x) Within 5 minutes of removal from the magnetic stirrer on hotplate or from incubation chamber, pour the digestion fluid through a 180–200 µm sieve into a 2.5 litre or larger separatory funnel with a height to width ratio of about 2:1 and preferably with polytetrafluoroethylene (PTFE) safety plugs (stopcocks). Rinse the beaker with sufficient room temperature tap water from a squirt bottle and pour this through the sieve into the separatory funnel.

xi) Rinse the sieve into the separatory funnel by squirting a small volume of room temperature tap water through the top of the sieve. The digestion process is considered satisfactory if residual debris remaining on the sieve consists primarily of indigestible non-muscle tissue (typically consisting of fascia and connective tissue) of no greater than 5 % of the original sample mass. Allow the fluid in the separatory funnel to settle undisturbed for 30 minutes. (Although not necessary, gentle tapping of the funnel wall [e.g. every 10 minutes] can facilitate the larvae settling to the bottom of the funnel.)

xii) Drain 40 ml of digestion fluid from the separatory funnel into a 50 ml conical tube or measuring cylinder (Pilsner flask) and allow to stand for 10 minutes.

xiii) At the end of 10 minutes use a pipette to remove 30 ml of the upper part of the fluid (supernatant), leaving the bottom 10 ml in the tube undisturbed (do not pour off the upper 30 ml, as this will disturb the sediment). If the analyst considers the remaining digest fluid not clear enough to be examined, a washing step can be performed as follows: add an additional 30 ml of tap water to the 10 ml of digest fluid, allow to stand for another 10 minutes, followed by removal of 30 ml of supernatant.

xiv) Gently swirl the remaining 10 ml of digest fluid and quickly transfer it into a gridded Petri dish or larval-counting basin. Rinse the tube or cylinder into the Petri dish using an additional 10 ml of tap water. The layer of fluid in the Petri dish should not be more than a few millimetres deep.

xv) Wait a minimum of 1 minute to allow larvae to settle to the bottom of the Petri dish or counting basin, then use a stereomicroscope at 10× to 20× magnification to systematically examine each grid for the presence of *Trichinella* larvae. The detection of any suspect larvae must be confirmed by the identification of morphological details at a higher magnification such as 40×. If the sediment is still too cloudy or otherwise difficult to examine at this stage, it will require further clarification as described in step xviii).

xvi) Examination should be performed immediately after digestion; if not possible, the Petri dish containing the digest fluid can be stored refrigerated for examination later that same day.

xvii) If the digest fluid is not examined within 30 minutes of preparation, and is allowed to cool down (or has been refrigerated) it may become too cloudy to examine accurately, and may require clarification as described below.

xviii) Sample clarification: transfer the contents of the Petri dish into a 50 ml conical tube using a pipette. Rinse the Petri dish thoroughly with tap water, adding the rinse water to the conical tube, then cover the Petri dish and set aside. Add additional tap water to the conical tube to bring the volume to 45 ml. Let the contents in the tube settle undisturbed for 10 minutes.

At the end of 10 minutes use a pipette to withdraw the supernatant, leaving the bottom 10 ml undisturbed (do not pour off the supernatant, as this will disturb the sediment). Retain the supernatant for disposal or decontamination after the sample has been examined.

Repeat steps xiv and xv using the same Petri dish that contained the original sample and was set aside in step xviii)

xix) In the event of a positive or suspicious result, an additional sample should be tested from each carcass contributing to the original pooled digest. These should be digested individually or as successively smaller pools until the individual infected animals are identified.

1.1.5. Identification of the larvae

First-stage larvae, digested free from muscle cells, are approximately 1 mm in length and 0.03 mm in width. The most distinguishing feature of *Trichinella* larvae is the stichosome, which consists of a series of discoid cells lining the oesophagus and occupying the anterior half of the worm's body. *Trichinella* larvae may appear coiled (when cold), motile (when warm) or C-shaped (when dead). In case of doubt, larvae should be viewed at higher magnification and further samples should be digested. If the numbers of larvae are high, appropriate dilutions must first be made to obtain accurate counts.

Larvae recovered from muscle digestion may be stored in a small vial (1–2 ml) filled with 70–90% ethyl alcohol (final concentration) for subsequent genotyping by polymerase chain reaction (PCR) (see Section B.1.3) by a qualified laboratory.

1.1.6. Quality assurance

Laboratories using artificial digestion methods should maintain a suitable quality assurance system to ensure test sensitivity. Components of a quality assurance system for digestion testing are described elsewhere and should include regular use of proficiency testing (Forbes *et al.*, 2005; Gajadhar *et al.*, 2009; 2019).

1.2. Other direct detection methods

1.2.1. The double separatory funnel method

This assay is an alternative to the commonly used digestion procedure described above. The method was designed to operate under strict conditions of quality control, minimise technical error, and has been extensively validated for use on pork and horsemeat (Forbes & Gajadhar, 1999; Forbes *et al.*, 2008). It includes a spin-bar digestion technique and sequential separatory funnels for sedimentation of the larvae. The procedure has fewer steps, requires less time and seldom needs further clarification steps. An incubation chamber equipped with transparent glass doors and set at 45°C is used to perform the digestion. The digestion is conducted in 3 litres of digest fluid on a magnetic stirrer. Following digestion, the suspension is poured into a 4 litre separatory funnel through a 177–180 µm sieve, which is rinsed thoroughly into the separatory funnel with tap water. The suspension is allowed to settle for 30 minutes, and the bottom 125 ml then dispensed directly into a 500 ml separatory funnel. The volume in this smaller separatory funnel is increased to 500 ml by adding 375 ml of tap water, and the resultant suspension allowed to settle for 10 minutes. Finally, 22–27 ml of sediment is dispensed into a Petri dish and observed for larvae as previously described.

1.2.2. Mechanically assisted pooled sample digestion method/sedimentation technique

This method uses a heated Stomacher blender for the digestion phase, and a separatory funnel for sedimentation of the larvae (*Equivalent method A, Regulation* [*EC*] *No. 2015/1375*) (European Commission, 2015).

1.2.3. Mechanically assisted pooled sample digestion method/'on filter isolation' technique

This method uses a heated Stomacher blender for the digestion phase, and a Gelman funnel mounted on an Erlenmeyer flask connected to a filter pump for the recovery of the larvae. (*Equivalent method B, Regulation* [*EC*] *No. 2015/1375;* European Commission, 2015).

1.2.4. Automatic digestion method for pooled samples of up to 35 g

This method involves an automated digestion chamber and a membrane filter for the recovery and examination of larvae (*Equivalent method C, Regulation* [*EC*] *No. 2015/1375;* European Commission, 2015). Critical steps in digestion and larval recovery are difficult to control in the automatic method and it is not recommended by the ICT or WOAH.

1.2.5. Magnetic stirrer method for pooled sample digestion/'on filter isolation' and larva detection by a latex agglutination test

This method is only considered equivalent for the testing of meat of domestic swine. The method combines the typical digestion procedure with detection of larvae by latex agglutination (*Equivalent method D, Regulation* [*EC*] *No. 2015/1375*, European Commission, 2015).

1.2.6. Artificial digestion commercial test kit for *in-vitro* detection of *Trichinella* spp. larvae in meat samples

This serine protease-based method is considered equivalent for testing meat of domestic swine only (*Equivalent method E, Regulation* [*EC*] *No. 2015/1375*, European Commission, 2015). The kit shall be used according to the manufacturer's instructions .

1.3. Other tests

1.3.1. Polymerase chain reaction

Limited studies have shown that PCR can be used to detect the nucleic acid of larvae in the musculature of infected animals. However, this method lacks sensitivity and is not practical for routine testing of food animals. Identification of the species or genotype of *Trichinella* recovered from muscle tissue is useful in understanding the epidemiology of the parasite in animals, in assessing the relative risk of human exposure and to trace back infection to the farm of origin. Specific primers have been developed that enable the identification of single larva collected from muscle tissues at the species or genotype level by PCR (Pozio & La Rosa, 2010; Pozio & Zarlenga, 2021). Detailed guidelines for this identification of *Trichinella* muscle stage larvae have been developed by the ICT (http://trichinellosis.org/; Pozio & Zarlenga, 2019). Requests for speciation or genotyping of *Trichinella* larvae can be made to the WOAH Reference Laboratories[2]).

2. Serological tests

A variety of immunological assays have been described for the diagnosis of *Trichinella* infections in domestic and wild animals (Gamble *et al.,* 2004). An immunochromatographic strip (ICS) assay using excretory/secretory (ES) antigens derived from larval and pre-adult *T. spiralis* to detect infection in pigs has also been recently described (Wang *et al.,* 2021). Only ELISA and Western blot have been validated in accordance with WOAH standards (Bruschi *et al.*, 2019). Requests for reference pig sera can be made to the WOAH Reference Laboratory in Italy (Gomez-Morales *et al.,* 2015). The ICT has provided a uniform set of recommendations for the development and use of serological tests for the detection of circulating antibodies (Bruschi *et al.*, 2019). The ELISA (with confirmatory testing by Western blot as feasible) is the only immunological assay recommended by the ICT. It is only approved as an epidemiological

surveillance tool to detect anti-*Trichinella* antibodies in pigs; it is not reliable for the detection of *Trichinella* infection in individual animals for food safety or other purposes.

Although other serological tests may have some practical applications, the ELISA is generally acknowledged as the test of choice based on economy, reliability, adaptability to good quality assurance practices, increasing body of validation data and good sensitivity and specificity when conducted under appropriate conditions. It is a useful tool for testing populations and is routinely used for surveillance programmes and disease outbreak investigations. Testing by a validated Western blot is recommended to confirm any ELISA-positive results (Bruschi *et al.*, 2019). The digestion of 100 g or more of tissue is recommended as a confirmatory test for serologically positive animals.

2.1. Enzyme-linked immunosorbent assay (ELISA)

2.1.1. Sensitivity and specificity

Infection levels as low as one larva/100 g of tissue can be detected by ELISA in pigs (Gamble *et al.*, 2004). Thus, serological testing by ELISA is useful for detecting anti-*Trichinella* antibodies at the farm level or for more broadly based surveillance programmes. Disadvantages of serology include the low rate of false-negative results observed in infected animals as well as false-positive results, which can be quite high among backyard and free-ranging pigs and wild boar. The false negatives can be attributed to the lag time of the immune response following the ingestion of infective larvae, and false-positive results to cross-reactions with other parasites and microorganisms. Detectable antibody levels are not usually present in pigs until 3–5 weeks or longer following exposure (Gamble, 1996; Gamble *&* Patrascu, 1996; Pozio *et al.*, 2020). For this reason, serological methods are not recommended for individual carcass testing, particularly for food safety purposes. Serological responses in pigs persist for a long time after infection with no decline in titre (Pozio *et al.*, 2020); however, antibodies have been reported to decline in horses within a few months following infection. Serological tests may therefore be of little practical use in horses as antibody titres eventually drop below detectable levels despite the presence of infective larvae in muscle (Hill *et al.*, 2007; Pozio *et al.*, 2002). Little is known of antibody responses to *Trichinella* infection in game animals and other wildlife, but high quality serum samples should be obtained to reduce the likelihood of false positive reactions. Serology validation data are available for domestic pigs, but only limited data have been reported for other animals, including studies on ELISA and Western blot for wild boar and dogs (Bruschi *et al.*, 2019).

2.1.2. Samples

The use of ELISA to detect the presence of parasite-specific antibodies provides a rapid method that can be performed on serum, whole blood, plasma or tissue fluid collected before or after slaughter (Gamble & Patrascu, 1996). The dilutions used are different for serum than for tissue fluid as the antibody concentrations are typically higher in serum than in tissue fluid (Nockler *et al.*, 2005).

2.1.3. Antigens

The sensitivity and specificity of ELISA are largely dependent on the antigens used in the test. TSL-1 (*T. spiralis* L1) antigens are the main components of ES antigens and are specifically secreted from the stichocyte cells of live larvae. TSL-1 bear a common immunodominant carbohydrate epitope, which is recognised by all *Trichinella*-infected animals. The *T. spiralis* ES antigens used in the ELISA appear to be conserved in all species and genotypes of *Trichinella*, even though some differences have been detected, and should therefore enable detection of specific antibodies in pigs or other animals harbouring any of the known taxa. ES antigen preparations have been developed that provide a high degree of specificity for *Trichinella* infection in pigs (Bruschi *et al.*, 2019; Gamble *et al.*, 1988).

2.1.4. Antigen production

Detection of anti-*Trichinella* antibodies by ELISA can be accomplished by using ES products of *Trichinella* larvae in culture (Gamble *et al.*, 1988). For purposes of standardisation, it is recommended that *T. spiralis* be used for antigen production for food animal testing. However, as indicated above, it has been demonstrated that antigens prepared from any of the *Trichinella* species can be used for detection of antibodies in infected animals regardless of the

infecting species (Bruschi *et al.*, 2019). Parasites to be used for antigen preparation may be maintained by serial passage in mice, rats or guinea-pigs.

To prepare antigens for use in the ELISA (Gamble *et al.*, 1988), *T. spiralis* first-stage larvae are recovered from skinned, eviscerated, ground mouse or rat carcasses by digestion in 1% pepsin with 1% HCl for 30 minutes at 37°C (as otherwise described in Section B.1.1.4). These larvae are washed (three times for 20 minutes each) in Dulbecco's modified Eagle's medium (DMEM) with penicillin (500 units/ml) and streptomycin (500 units/ml), and then placed (at a concentration of 5000 L1 per ml) into DMEM supplemented with HEPES (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid) (10 mM), glutamine (2 mM), pyruvate (1 mM), and penicillin (250 units/ml)/streptomycin (250 µg/ml) (complete DMEM) at 37°C in 10% CO_2 in air. Culture medium is recovered after not more than 18 hours, worms are removed by filtration, and the fluid is concentrated under pressure with a 5000 Da molecular weight retention membrane or by a centrifugal filter device using a similar molecular weight cut-off. ES antigens should be supplemented with protease inhibitors to preserve quality and may be stored frozen for short periods at −20°C or for longer at −70°C; they consist of approximately 25 protein components as determined by SDS/PAGE (sodium dodecyl sulphate/polyacrylamide gel electrophoresis), many of which bear the diagnostic TSL-1 carbohydrate antigen epitope.

Antigen purity is critical to the specificity of the ELISA. Steps should be taken to monitor growth of bacteria visually, either by phase microscopy or by plating a sample of media. Cultures showing any bacterial growth should be discarded. Larvae should not be maintained in culture longer than 18 hours; worm deterioration after this time contributes to leaking of somatic antigens that reduce test specificity. Antigens, produced as described, should have a 280:260 nm absorbance ratio of >1.0. The antigens obtained from *in-vitro* culture of *Trichinella* larvae should be tested against a panel of known negative and positive sera before use.

2.1.5. Test procedure

An example of an ELISA for detecting *Trichinella* infection in pigs is given below. It is essential that all reagents used in the assay be standardised for optimal concentration to obtain reliable results. Typical values are indicated in the example.

i) Coat 96-well microtitre plates with 100 µl/well of *T. spiralis* ES antigens diluted to 5 µg/ml in coating buffer (50 mM carbonate/bicarbonate buffer, pH 9.6). Coating is performed for 60 minutes at 37°C or overnight at 4°C.

ii) Wash antigen-coated wells three times with wash buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 5.0% non-fat milk powder and 1.0% Triton X-100. Following each washing, plates are blotted dry.

iii) Dilute pig sera 1/50 or 1/100 in wash buffer. Alternative sources of antibodies that may be used in place of sera include whole blood or tissue fluids at a dilution of 1/5 or 1/10 (Nockler *et al.,* 2005). Add 100 µl of diluted sera to antigen-coated wells. A known positive and negative serum sample should be used on each plate at the same dilution as the test sera. Incubate at room temperature for 30 minutes.

iv) Wash wells three times as in step ii.

v) Add 100 µl/well of an affinity-purified rabbit anti-swine IgG–peroxidase conjugate at an appropriate dilution in wash buffer. Following the addition of the secondary antibody, incubate the plates for 30 minutes at room temperature.

vi) Wash wells three times as in step ii. Rinse once with distilled water.

vii) Add 100 µl of a suitable peroxidase substrate (e.g. 5'-aminosalicylic acid 0.8 mg/ml with 0.005% hydrogen peroxide, pH 5.6–6.0).

viii) After 5–15 minutes, read plates for colour density at 450 nm on an automated microplate reader. Values obtained in the ELISA above the established cut-off value are considered to be positive (Jacobson, 1998).

Commercial adaptations of the ELISA are available. The manufacturer must validate the kit prior to licensure and the user should also evaluate the performance of the kit, prior to use, by using selected negative and positive reference samples.

The test should be conducted within an environment in which internationally accepted standards of quality management, such as ISO 17025, have been implemented.

In addition to the use of standard reference sera, all commercial and in-house ELISAs should be evaluated against a bank of negative control sera that represents the population under test, and a group of positive animals that represents different stages of infection, as per ICT guidelines.

C. REQUIREMENTS FOR VACCINES

There are no vaccines available for prevention of *Trichinella* infections.

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NB: There are WOAH Reference Laboratories for trichinellosis (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests and reagents for trichinellosis

NB: FIRST ADOPTED IN 1989 AS SWINE TRICHINELLOSIS. MOST RECENT UPDATES ADOPTED IN 2023.