CHAPTER 2.1.16.

TRICHINELLOSIS

SUMMARY

Trichinellosis in humans is caused by eating raw or undercooked meat from Trichinella-*infected domestic animals, or game. Animals become infected by feeding on* Trichinella-*infected muscles. Ingested infective larvae mature and mate in the small intestine of host species including humans, pigs, rats, bears, walruses, horses (occasionally) and many other flesh-eating mammals, and birds and reptiles. The adult worms survive less than 2 months. The larvae produced migrate and persist in the muscles of their hosts. Susceptible new hosts become infected by ingestion of muscle tissue that contains these larvae.*

***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 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 preferred muscle sites, particularly in low level infections, and these sites may vary by host species. It is important that preferred sites be sampled to maximise test sensitivity. For example, in pigs, the diaphragm (crus) and tongue muscles are the two most preferred sites, whereas in horses, the tongue harbours the most worms, followed by the masseter, diaphragm and 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 larvae that are released from muscle 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, uneven distribution of larvae within tissues is a limiting factor. This is 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 carcass 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 disease surveillance.

***Serological tests:** Serological assays are the most common tests used for indirect detection. The sensitivity and specificity of serological methods are mainly dependent upon the type and quality of antigen used. Most serological test performance (validation) data are from pigs. False negative serological results may occur 3 weeks or longer after muscle larvae become infective in pigs with light or moderate infections. A low rate of false-positive results has also been reported for serological tests. For surveillance or verification of* Trichinella-*free herds or regions, serological methods are acceptable. For the purposes of 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). The specificity of ELISA for* Trichinella *infection is directly linked to the type and quality of the antigen employed in the test. Secretory antigens collected by short-term (18–20 hours) maintenance of* T. spiralis *muscle larvae in vitro and synthetic carbohydrate antigens currently provide the most specific and economical source. It is critical that appropriate positive and negative control sera be used to ensure that ELISAs are performing at a minimum acceptable level of sensitivity and specificity. The digestion of 100 g or*

more of tissue is recommended as a confirmatory test for serologically positive animals. There is a critical need for an international bank of validated reference sera to provide a common standard for Trichinella *serological assays.*

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

A. INTRODUCTION

Clinical signs of trichinellosis are not generally recognised in animals, and its main importance is as a zoonosis. Trichinellosis in humans is caused by eating raw or undercooked meat from *Trichinella*-infected food animals or game (Gajadhar *et al.*, 2006). The short-lived adult worms live in the small intestine of host species including humans, pigs, rats, bears, walruses, horses, many other flesh-eating mammals, and some birds and reptiles. The parasite has a direct life cycle. Within hours following consumption of infected muscle by a suitable host, first stage muscle larvae (L1) are released by digestion and burrow into the villi of the small intestine. They develop rapidly into adults (males up to 1.8 mm long, females up to 3.7 mm long) and survive for less than 2 months. During this time, copulation takes place and the ovo-viviparous females release new-born larvae (NBL), which migrate via venules and lymphatics into the general circulation. NBL are distributed throughout the body where they invade striated muscles, showing predilection for specific muscle groups. For example, in pigs, the diaphragm pillar and tongue usually contain the highest concentration of larvae, followed by the masseter, and in horses, the tongue followed by masseter, diaphragm and neck muscles. Predilection sites vary by host species, but in general, tongue, masseter and diaphragm are optimal sites for sampling. Current knowledge on predilection sites is available for several host species (Nockler *et al.*, 2000). In cases of severe infection most voluntary muscles contain high numbers of larvae. The larvae of most *Trichinella* species become encapsulated in collagen in host musculature where they remain infective for years.

Within the genus *Trichinella* twelve genotypes have been identified, eight of which have been designated as species (Gajadhar *et al.*, 2006; Krivokapich *et al.*, 2008; Murrell *et al.*, 2000; Pozio & Zarlenga, 2005). *Trichinella spiralis* (T-1) is distributed in temperate regions world-wide and is commonly associated with domestic pigs. It is highly infective for domestic and sylvatic swine, mice and rats, but it can also be detected in other mammalian carnivores and horses. *Trichinella nativa* (T-2) occurs in mammalian carnivores of arctic and sub-arctic regions of North America, Europe and Asia. *Trichinella britovi* (T-3) is found predominantly in wild animals and pigs, and occasionally in horses. It occurs in temperate regions of Europe, Asia, and in Northern and Western Africa. *Trichinella pseudospiralis* (T-4) is cosmopolitan in distribution and has been recovered from raptorial birds, wild carnivores and omnivores, including rats and marsupials in Asia, North America, Europe and Australia. Unlike most other *Trichinella* genotypes, T-4 is not enclosed within a collagen capsule in muscle. *Trichinella murrelli* (T-5) is found in mammalian carnivores of North America. It has low infectivity for domestic pigs, but poses a risk to humans who eat game meats and has been reported in a horse. *Trichinella* T-6 is cold-climate-adapted and appears to be closely associated with *T. nativa* in northern North America (Pozio & Zarlenga, 2005). Both *T. nativa* and T-6 are highly resistant to freezing. They have not been found in pigs or horses and experimentally shown to have limited infectivity for these host species. *Trichinella nelsoni* (T-7) has been isolated from mammalian carnivores and sporadically from wild pigs in Eastern Africa. *Trichinella* T-8 has been detected in mammalian carnivores in Namibia and South Africa and *Trichinella* T-9 in mammalian carnivores in Japan (Pozio & Zarlenga, 2005). T-8 and T-9 have some intermediate characteristics with *T. britovi* and *T. murrelli*, respectively. Like *T. pseudospiralis*, *T. papuae* (T-10) and *T. zimbabwensis* (T-11) are non-encapsulated muscle parasites. *Trichinella papuae* has been reported from wild and domestic pigs and farmed crocodiles in Papua, New Guinea. *Trichinella zimbabwensis* has been described in farmed and wild crocodiles in Zimbabwe, Ethiopia and Mozambique and in monitor lizards in Zimbabwe. Experimentally, it shows a high infectivity for a wide spectrum of mammalian hosts including pigs and rats (Pozio & Zarlenga, 2005). The putative twelfth genotype is based on larvae from a single mountain lion from Argentina (Krivokapich *et al.*, 2008). All species and genotypes of *Trichinella* cause disease in humans, but not all naturally infect pigs. Risk of establishing *Trichinella* infection in pig herds is presented by *T. spiralis, T. britovi, T. pseudospiralis,* and *T. papuae*, 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, but in animals the infection is clinically inapparent. The short-lived adult worms in the intestine can cause transient gastroenteritis, but the most severe signs and symptoms result from the migration and presence of the larvae in voluntary muscle. The disease is transmitted by eating infected meat, primarily from swine or wildlife sources, that has not been sufficiently cooked (or otherwise made safe).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 processing (cooking, freezing, or curing of meat), and by preventing the exposure of food animals to meat harbouring *Trichinella* larvae including uncooked food waste, rodents and other wildlife (Gajadhar *et al.*, 2006; Gamble, 1997; Gamble *et al.*, 2000). Game meats should always be considered a potential source of infection, and should be tested or properly cooked. *Trichinella* found in game

meats (mainly *T. nativa*, T-6 and to a lesser degree *T. britovi*) may be resistant to freezing and therefore untested, frozen game poses a public health risk.

Testing methods for the detection of *Trichinella* infection in pigs and other species either: (a) directly demonstrate the parasite in tissue samples; or (b) indirectly demonstrate the parasite by using immunological methods to detect specific antibodies to *Trichinella* spp. in blood, serum or tissue fluid samples (Gajadhar *et al.*, 2009).

The risk of laboratory acquired infection for analysts is minimal if good laboratory practices are followed. Infection is acquired 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 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 critical when testing proficiency samples containing live larvae in a non-endemic region.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The only recommended procedures for the detection of *Trichinella* larvae in muscle tissues are digestion assays. A number of digestion assays are officially recognised in various countries for trade purposes. The International Commission on Trichinellosis (ICT) recommends several of these assays, which are documented standards in the European Union (EU), Canada and elsewhere (Canadian Food Inspection Agency, 2010; European Commission, 2005). 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. Although there is general consensus that the digestion assay is the best procedure, a universally accepted digestion test protocol for trade and food safety purposes is not yet available. The digestion assay recommended here is based on desirable innovations inherent in some digestion assays that are accepted for international trade purposes.

a) **Recommended direct procedure for testing muscle tissue (the prescribed test for international trade)**

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 will identify infected pigs, horses or other animals infected with *T. spiralis* as early as 17days 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. Samples tested for food safety related purposes should be stored at 4°C and tested as soon as possible, and certainly before putrefaction occurs. 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. 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/g of tissue, and testing of a 5 g sample increases sensitivity to 1 larva/g of tissue(Gamble *et al.*, 2000; Nockler *et al.*, 2000). Where large amounts of tissue (up to 100 g) are available for digestion, the sensitivity of this test is further increased.

Sampling: tests are usually conducted on carcass samples collected post-mortem. Muscle samples are taken from predilection sites, usually the diaphragm pillars or tongue of pigs, or tongue or masseter muscles of horses. For wildlife in which predilection sites are unknown, tongue (preferred), diaphragm or masseter should be taken. The sample sizes for carcass inspection testing are based on reliable detection of animals harbouring 1 lpg in tissue, but for surveillance purposes, a higher sensitivity is required to provide better disease prevalence data and to overcome sampling limitations such as those seen with wildlife. Surveillance samples should be ≥10 g and taken from predilection sites (if known). Samples of 100 g would allow detection of 0.01 lpg in the source tissue and if the sample was a predilection site, a low or negative result would indicate a low larval load in the rest of the carcass, with an associated low risk for transmission. For food safety testing or carcass inspection, individual samples of 100 g may be taken from one 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 that make up the pool will determine the sensitivity of the method. The ICT recommends 5-g samples per pig for testing in endemic areas (Gamble *et al.*, 2000). For testing horsemeat, a minimum of 5 g per carcass is required. (Gamble *et al.*, 2000 For horses originating from endemic areas, or if horsemeat is consumed raw, a 10 g sample is recommended (Gamble *et al.*, 2000).

Confirmatory testing of pooled digestion samples and serologically positive animals: When a pool of samples from different animals is digested and has a positive result, additional digestion tests should be used to retest animals individually 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 genotype identification.

- **Digestion and detection**

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

ii) Digestive solution: Prepare the appropriate volume of HCl/water solution by combining the HCl with tap water (e.g. for 2.0 litres use 11 ml of 37% HCl or 16 ml of 25% HCl). Do not add pepsin to the solution at this time. This solution should be preheated to 45°C before use.

iii) Remove as much fat and fascia as possible from each sample of meat.

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. Add 50–100 ml of the water/HCl solution for a 100 g sample pool.

vi) Chop the meat in a blender until it is homogeneous (no chunks of meat should be present; the sample should be the consistency of pureed baby food). This is usually achieved with several 1–3-second pulses. Add approximately 100 ml of the prepared water/HCl solution and blend until the mixture is uniformly liquid. This may take 5–10 seconds (additional solution may be needed).

vii) Sprinkle 10 g of pepsin (1:10,000 NF/1:12500 BP/2000 FIP; granular or equivalent amount of liquid pepsin) onto the homogenate, add about 200 ml of water/HCl solution, and blend for about 5 seconds.

viii) Transfer the homogenised sample to a 3-litre beaker containing a stir bar. Add the remainder of the 2 litres of water/HCl solution by pouring the water/HCl into the blender and rinsing all residual homogenate into the 3-litre beaker. Rinse any adhering material from the blender lid into the beaker using 10–20 ml of digestive solution from a squirt bottle.

ix) 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 not 45±2°C, the sample should be allowed to warm to this temperature before the timing of the digestion is started.

x) Allow the digestion to proceed for 30 minutes. 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 should be continued for an additional 30 minutes or until the pieces are digested. Care should be taken to ensure that the digestion temperature range is not exceeded. Alternatively, the digestion may be performed at 37°C for a longer period of time.

xi) Within 5 minutes of removal from the magnetic stirrer hot plate pour the digestion fluid through a 177–180-µm sieve and into a 2-litre separatory funnel. Rinse the beaker with room temperature tap water from a squirt bottle and pour this through the sieve into the 2-litre separatory funnel.

xii) Rinse the sieve into the 2-litre separatory funnel by squirting a small volume of room temperature tap water through the top of the sieve. There should be no undigested pieces of muscle remaining on the sieve, although small remnants of fat, fascia and other tissues may be present. Allow the fluid in the separatory funnel to settle undisturbed for 30 minutes.

xiii) 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.

xiv) 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 (do not pour off the upper 30 ml, as this will disturb the sediment).

xv) Gently swirl the remaining 10 ml of fluid and quickly transfer it into a gridded Petri dish or larval-counting basin. Rinse the tube or cylinder into the Petri dish twice using 5 ml of tap water each time. The layer of fluid in the petri dish should not be more than a few millimetres deep.

xvi) Wait a minimum of 1 minute to allow larvae to settle to the bottom, then use a stereomicroscope at ×10–16 magnification to systematically examine each grid of the Petri dish for the presence of *Trichinella* larvae. The detection of any suspect larvae on the systematic examination must be confirmed by the identification of morphological details at a higher magnification such as ×40. If the sediment is cloudy or otherwise difficult to examine, it will require further clarification as described below.

xvii) Digests should be examined soon after they are ready. Under no circumstance should examination of digests be postponed until the following day.

xviii) If digests are not examined within 30 minutes of their preparation they may require clarification as described below.

xix) 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. Add additional tap water to bring the volume to 45 ml. Let 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 (do not pour off the supernatant, as this will disturb the sediment). Save the removed fluid for disposal or decontamination after the sample has been read.

Repeat steps xv and xvi.

xx) In the event of a positive or doubtful result, a further sample should be collected from each carcass making up the pooled sample. These should be tested individually or in successive smaller pools until the individual infected animals are identified.

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 tissues should be examined. If the counts are high, appropriate dilutions must first be made.

Larvae recovered from muscle digestion may be stored in 70–75% ethanol (or 95% for long-term storage) for subsequent genotyping by polymerase chain reaction (PCR) (see Section B.1.c).

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 by the ICT (Gamble *et al.*, 2000) and elsewhere (Gajadhar & Forbes, 2001; Gajadhar *et al.*, 2009) and should include regular use of proficiency testing (Forbes *et al.*, 1998; 2005, Gajadhar *et al.*, 2009).

b) Other direct detection methods

i) *The double separatory funnel method:* this assay is recommended as an alternative to the commonly used digestion procedure described above, and is approved by the EU for export use. 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 horse meat (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 125 ml is drained into a 500-ml separatory funnel. The volume is increased to 500 ml by adding 375 ml of tap water, and the resultant suspension is allowed to settle for an additional 10 minutes. Finally, 22–27 ml of sediment is drained into a Petri dish and observed for larvae as previously described.

ii) *The mechanically assisted pooled sample digestion method/sedimentation technique (Equivalent method A, Regulation [EC] No. 2075/2005):* this method uses a heated Stomacher blender for the digestion phase, and a separatory funnel for sedimentation of the larvae (European Commission, 2005).

iii) *Automatic digestion method* for pooled samples of up to 35 g (Trichomatic 35): this method involves an automated digestion chamber and a membrane filter for the recovery and examination of larvae (*Equivalent method C, Regulation [EC] No 2075/2005, European Commission, 2005*). Critical steps in digestion and larval recovery are difficult to control in the automatic method and it is not recommended by the ICT.

c) Other tests

i) *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 the infection to the farm of origin. Specific primers have been developed that allow the identification of single larva collected from muscle tissues at the species and genotype level by PCR (Pozio & La Rosa, 2003). Requests for speciation or genotyping of *Trichinella* larvae can be made through the OIE Reference Laboratories in Rome, Italy or Saskatoon, Canada (see Table given in Part 3 of this *Terrestrial Manual;* and www.iss.it/Trichinella/index/asp).

ii) *Trichinoscopy:* This method involves the compression of multiple 2 ×10 mm pieces of muscle tissue between two glass plates (compressorium) until they become translucent, followed by examination using a microscopic technique. There are good comparative data available indicating that trichinoscopy is not as sensitive as digestion assays, so it is **not recommended** by the ICT or EU for the routine examination of carcasses (European Commission, 2005; Gajadhar *et al.*, 2009).

2. Serological tests

A variety of immunological assays have been described for the diagnosis of trichinellosis in domestic and wild animals (Gamble et al., 2004). Methods include immunofluorescence assay (IFA), immuno-electrotransfer blot (IEBT), western blot, enzyme immunohistochemical assays, and enzyme-linked immunosorbent assays (ELISA). Except for the ELISA, these tests have not been standardised, and reagents are not available for routine use. Nevertheless, the ICT has provided a uniform set of recommendations for the development and use of serological tests for the detection of circulating antibodies (Gamble et al., 2004). The ELISA is the only immunological assay endorsed 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.

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. Nevertheless, for reasons given below, the ELISA is not recommended for the testing of individual pigs, horses or other animals for food safety purposes.

a) Enzyme-linked immunosorbent assay (ELISA)

• **Sensitivity and specificity**

Infection levels as low as one larva/100 g of tissue are detectable by ELISA in pigs (Gamble *et al.*, 2004). This high level of sensitivity makes serological testing by ELISA a useful method for detecting ongoing transmission of *Trichinella* infection at the farm or for more broadly based surveillance programmes. A disadvantage of serology for the detection of *trichinellosis* is the low rate of false-negative results observed in infected animals. This is primarily due to the lag time of the immune response following the ingestion of infective larvae. Detectable levels of antibody are not usually present in pigs until 3–5 weeks or more following exposure (Gamble, 1996; Gamble *et al.*, 1996). For this reason, serological methods are not recommended for individual carcass testing. Serological responses in pigs persist for a long time after infection with no decline in titre; however, antibody has been reported to decline in horses within a few months following infection (Nockler *et al.*, 2000). Serological tests may be of little practical use in horses as antibody titres eventually drop below diagnostic 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. Currently, no validated serological assay is available for non-pig host species.

• **Samples**

The use of ELISA to detect the presence of parasite-specific antibodies provides a rapid method that can be performed on serum, blood or tissue fluid collected before or after slaughter (Gamble & Patrascu, 1996).The dilution used is different for serum than for tissue fluid (Nockler *et al.*, 2005).

• **Antigens**

The specificity and sensitivity of ELISA is largely dependent on the quality of the antigen used in the test. Antigens that are specifically secreted from the stichocyte cells of living L1 larvae and bear the TSL-1 carbohydrate epitope are recognised by *Trichinella*-infected animals. The antigens recognised in worm ES products consist of a group of structurally related glycoproteins with molecular weights of 45–55 kDa (Ortega-Pierres *et al.*, 1996). A synthetic carbohydrate antigen (Tyvelose) has also been used in ELISA. Studies in swine indicate that Tyvelose may be as good as ES antigen for surveillance testing in pigs however, the sensitivity of the ELISA using this synthetic antigen is lower than that using ES antigens (Forbes *et al.*, 2004; Gamble *et al.*, 1997).Antigen preparations have been developed that provide a high degree of specificity for *Trichinella* infection in pigs (Gamble *et al.*, 1988). The *T. spiralis* ES antigens used in

the ELISA are conserved in all species and genotypes of *Trichinella* (Ortega-Pierres *et al.*, 1996), and therefore infection may be detected in pigs or other animals harbouring any of the twelve genotypes.

- **Antigen production**

Diagnosis of *Trichinella* infection by ELISA can be accomplished by using stichosome antigens collected from the 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, it has been demonstrated that antigen prepared from any of the *Trichinella* species can be used for detection of antibodies in infected animals regardless of the infecting species (Kapel & Gamble, 2000). Parasites to be used for antigen preparation may be maintained by serial passage in mice, rats or guinea pigs.

To prepare antigen for use in the ELISA (Gamble *et al.*, 1988), *T. spiralis* (T-1) muscle-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 described above). 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 density of 5000 L1/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 18–20 hours, worms are removed by filtration, and the fluid is concentrated under pressure with a 5000 Da molecular weight retention membrane. ES antigens thus recovered 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 either visually, by phase microscopy, or by plating a sample of media. Cultures showing any bacterial growth should be discarded. Larvae should not be maintained longer than 18 hours; worm deterioration after this time contributes to leaking of somatic antigens that reduce test specificity. Antigen, produced as described, should have a 280:260 nm absorbance ratio of >1.0. The antigens obtained from *in-vitro* maintenance of *Trichinella* larvae, should be tested against a panel of known negative and positive sera before use.

- **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 the 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 known 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 second 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 four times that of normal serum pool controls are considered to be positive. Values three times higher than normal are classified as suspect.

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.

There are currently no international standard reference sera to provide a common benchmark for *Trichinella* ELISAs. In addition to the use of international standard sera (when available), 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 for *trichinellosis* in food animals or game.

REFERENCES

BOIREAU P., VALLEE I., ROMAN T., PERRET C., MINGYUAN L., GAMBLE H.R. & GAJADHAR A. (2000). *Trichinella* in horses: a low frequency infection with high human risk. *Vet. Parasitol.,* **93**, 309–320.

CANADIAN FOOD INSPECTION AGENCY (2010). Meat Hygiene Manual of Procedures, Chapter 5, Sampling and Testing, Section 5.5.2.7.6, Double Separatory Funnel.

EUROPEAN COMMISSION (2005). Commission Regulation (EC) No. 2075/2005 of 5 December 2005 laying down specific rules on official controls for *Trichinella* in meat. *Off. J. European Union*, L**338**, 60–82(Regulation as last amended by Commission Regulation (EC) No 1245/2007: *Off. J. European Union,* L **281**, 19–20).

FORBES L.B., APPLEYARD G.D. & GADJADHAR A.A. (2004). Comparison of synthetic tyvelose antigen with excretory-secretory antigen for the detection of trichinellosis in swine using enzyme-linked immunosorbent assay. *J. Parasitol.,* **90**, 835–840.

FORBES L.B., HILL D.E., PARKER S., TESSARO S.V., GAMBLE H.R. & GAJADHAR A.A. (2008). Complete validation of a unique digestion assay to detect *Trichinella* larvae in horse meat demonstrates its reliability for meeting food safety and trade requirements. *J. Food. Prot.*, **71**, 558–563.

FORBES L.B. & GAJADHAR A.A. (1999). A validated *Trichinella* digestion assay and an associated sampling and quality assurance system for use in testing pork and horse meat. *J. Food Prot.*, **62**, 1308–1313.

FORBES L.B., RAJIC A. & GAJADHAR A.A. (1998). Proficiency samples for quality assurance in *Trichinella* digestion tests. *J. Food Prot.*, **61**, 1396–1399.

FORBES L.B., SCANDRETT W.B. & GAJADHAR A.A. (2005). A program to accredit laboratories for reliable testing of pork and horsemeat for *Trichinella*. *Vet. Parasitol.*, **132**, 173–177.

GAJADHAR A.A. & FORBES L.B. (2001). An internationally recognized quality assurance system for diagnostic parasitology in animal health and food safety with example data on trichinellosis. *Vet. Parasitol.*, **103**, 133–140.

GAJADHAR A.A., POZIO E., GAMBLE H.R., NOCKLER K., MADDOX-HYTTEL C., FORBES L.B., VALLEE I., ROSSI P., MARINCULIC A. & BOIREAU P. (2009). *Trichinella* diagnostics and control: Mandatory and best practices for ensuring food safety. *Vet. Parasitol.*, **159**, 197–205.

GAJADHAR A.A., SCANDRETT W.B. & FORBES L.B. (2006). Overview of food- and water-borne zoonotic parasites at the farm level. *Rev. sci. tech. Off. int. Epiz.*, **25** (2), 595–606.

GAMBLE H.R. (1996). Detection of trichinellosis in pigs by artificial digestion and enzyme immunoassay. *J. Food Prot.,* **59**, 295–298.

GAMBLE H.R. (1997). Parasites associated with pork and pork products. *Rev. sci. tech. Off. Int. Epiz.*, **16**, 496–506.

GAMBLE H.R., BESSONOV A.S., CUPERLOVIC K., GAJADHAR A.A., VAN KNAPEN F., NOECKLER K., SCHENONE H. & ZHU X. (2000). International Commission on Trichinellosis: Recommendations on methods for the control of *Trichinella* in domestic and wild animals intended for human consumption. *Vet. Parasitol.*, **93**, 393–408.

GAMBLE H.R., GAJADHAR A.A. & SOLOMON M.B. (1996). Methods for the detection of trichinellosis in horses. *J. Food Prot.*, **59**, 420–425.

GAMBLE H.R. &PATRASCU I.V. (1996). Whole blood, serum, and tissue fluids in an enzyme immunoassay for swine trichinellosis. *J. Food Prot.*, **59**, 1213–1217.

GAMBLE H.R., RAPIC D., MARINCULIC A. & MURRELL K.D. (1988). Evaluation of excretory-secretory antigens for the serodiagnosis of swine trichinellosis. *Vet. Parasitol.*, **30**, 131–137.

GAMBLE H.R., POZIO E., BRUSCHI F., NÖCKLER K., KAPEL C.M.O. & GAJADHAR A.A. (2004). International Commission on Trichinellosis: Recommendations on the Use of Serological Tests for the Detection of *Trichinella* Infection in Animals and Man. *Parasite*, **11**, 3–13.

GAMBLE H.R., WISNEWSKI N., &WASSOM D. (1997). Detection of trichinellosis in swine by enzyme immunoassay using a synthetic glycan antigen. *Am. J. Vet. Res.*, **58**, 417–421.

HILL D.E., FORBES L.B., KRAMER M., GAJADHAR A.A. & GAMBLE H.R. (2007). Larval viability and serological response in horses with long-term infection of *Trichinella spiralis*. *Vet. Parasit.,* **146**, 107–116.

KAPEL C.M.O. & GAMBLE H.R. (2000). Infectivity, persistence, and antibody response to domestic and sylvatic *Trichinella* spp. in experimentally infected pigs. *Int. J. Parasitol.*, **30**, 215–221.

KRIVOKAPICH S.J., GONZALEZ PROUS C. L., GATTI G.M., CONFALONIERI V., MOLINA V., MATARASSO H. &GUARNERA E. (2008). Molecular evidence for a novel encapsulated genotype of *Trichinella* from Patagonia, Argentina. *Vet. Parasitol.*, **156**, 234–240.

MURRELL K.D., LICHTENFELS J.R., ZARLENGA D.S. & POZIO E. (2000). The systematics of the genus *Trichinella* with a key to species. *Vet. Parasitol.*, **93**, 293–307.

NOCKLER K., POZIO E., VOIGT W.P. & HEIDRICH J. (2000). Detection of *Trichinella* infection in food animals. *Vet. Parasitol.*, **93**, 335–350.

NOCKLER K., SERRANO F.J., BOIREAU P., KAPEL C.M. & POZIO E. (2005). Experimental studies in pigs on *Trichinella* detection in different diagnostic matrices. *Vet. Parasitol.,* **132**, 85–90.

ORTEGA-PIERRES M.G., YEPEZ-MULIA L., HOMAN W., GAMBLE H.R., LIM P., TAKAHASHI Y., WASSON D.L. & APPLETON J.A. (1996). Workshop on a detailed characterization of *Trichinella spiralis* antigens: A platform for future studies on antigens and antibodies to this parasite. *Parasite Immunol.*, **18**, 273–284.

POZIO E. & LA ROSA G. (2003). PCR-derived methods for the identification of *Trichinella* parasites from animal and human samples. *Methods Mol. Biol.,* **216**, 299–309.

POZIO E., SOFRONIC-MILOSAVLJEVIC L., GOMEZ MORALES M.A., BOIREAU P. &NÖCKLER K. (2002). Evaluation of ELISA and Western blot analyses using three antigens to detect anti-*Trichinella* IgG in horses. *Vet. Parasitol.,* **108**, 163–178.

POZIO E. & ZARLENGA D.S. (2005). Recent advances on the taxonomy, systematics and epidemiology of *Trichinella*. *Int. J. Parasitol.,* **35**, 1191–1204.

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