International Commission on Trichinellosis: Recommendations on the use of serological tests for the detection of *Trichinella* infection in animals and man

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Introduction

Serological methods are widely used for detection/diagnosis of infection and/or disease in man and animals. The use and interpretation of serological tests varies based on many factors. The recommendations provided here take into account the best current methods for serological detection of *Trichinella* infection in man and animals and provide guidance on the appropriate use of these serological tools.

The International Commission on Trichinellosis (ICT) does not recommend use of indirect (serological) methods for testing individual carcasses of food animals at slaughter for the purpose of assuring food safety (Gamble *et al.*, 2000). This recommendation is consistent with practical legislation of many governmental bodies, under which meat inspection programs for *Trichinella* in pork, horse and game meats are performed using a direct method such as artificial digestion (see European Commission, 2005, 2014; OIE 2013). Legislation for inspection of meat and meat products for the presence of *Trichinella* includes direct tests only, which if performed properly, have the sensitivity and specificity sufficient to detect all carcasses with infections > 1-3 larvae per gram of meat, the minimum level of infection considered to be of public health concern. Currently available serological methods do not meet this standard for protection of public health due to a low rate of false negative results.

Although serological methods for detection of *Trichinella* infection are not considered to be suitable for the purpose of meat inspection, there are important uses for these tests including: 1) surveillance of infection prevalence and epidemiological investigations in animal populations, (for example, serology could be used to rule out infection in a population or for monitoring

animals introduced into a herd that is established as having no risk for *Trichinella*); and, 2) presumptive or retrospective diagnosis and surveillance of human infection. Due to the various methods that have been employed for serological detection of *Trichinella* infection, the ICT has compiled a series of recommendations for the use and interpretation of these tests.

Serological Methods

Types of assays

Many types of serological assays have been, and continue to be, used for the detection of *Trichinella* infections in animals and man. For animals, serological assays include, but are not limited to: 1) indirect immunofluorescence assay (IFA) employing cryostat sections of infected rodent muscle, frozen sections of free muscle larvae or formalin fixed whole larvae; 2) immuno-electrotransfer blot assay (IETB), or western blot using crude extract (CWE) antigens or muscle larvae excretory-secretory (ES) products (Frey et al. 2009; Noeckler et al. 2009, Gómez-Morales et al., 2012; 2014); 3) enzyme immunohistochemical technique (EIH) employing cryostat sections of infected rodent muscles (e.g., diaphragm) or frozen sections of free muscle larvae; 4) enzyme-linked immunosorbent assay (ELISA) employing the ES products of the muscle larvae (Gómez-Morales et al. 2009) or synthetic tyvelose as antigen (Gamble et al., 2004) and 5) immunochromatographic strip using ES antigens (Zhang et al., 2009; Fu et al., 2013)

For detection of human infection, indirect hemagglutination (IHA), bentonite flocculation, and latex agglutination do not have acceptable levels of sensitivity to be considered useful (Sandoval et al., 1990; 1995). IFA and the ELISA are the more commonly used screening tests, the last being the most sensitive, especially when confirmed by western blot (see below; Dupouy-Camet and Bruschi, 2007; Gomez Morales et al., 2012).

The ELISA is the most commonly used method for the detection of *Trichinella* infection in both animals and man. Its main advantages are low cost, reliability, ready standardization, and an acceptable balance of sensitivity and specificity. It is the only serological method recommended by the Office International des Epizooties (OIE, 2013) for testing domestic pigs. For these reasons, the ELISA will be the primary focus of these recommendations. Other types of serological tests can have practical applications; therefore, the principles for use of the ELISA (requirements for performance, suitability for particular species, etc.) should be points for consideration in selecting any serological test for detection of *Trichinella* infection.

In the 1970's, somatic antigens prepared from whole body extracts of muscle larvae (CWE) were used in the ELISA for detection of *Trichinella* antibodies in pigs. It is now recognized that CWE, produced from first stage muscle larvae collected by artificial digestion of muscle from an infected animal, should not be used for serological tests of either animals or humans due to a high probability of cross-reactions with other pathogens. For example, cross-reactions occur with serum samples from humans infected with *Loa loa*, *Toxocara* sp., and *Anisakis* (J. Dupouy-Camet, personal communication; E. Pozio, unpublished data). Despite these drawbacks, somatic antigens are still used in western blot because their production is less time consuming and cheaper than production of ES antigens (Frey et al., 2009; Noeckler et al. 2009).

During the 1980s, the specificity of the ELISA was improved by utilizing ES antigens obtained from the *in vitro* maintenance of *Trichinella* muscle larvae (Gamble et al., 2004). These antigens, originating from larval secretions, consist of a group of structurally related glycoproteins. Information regarding *Trichinella* antigens has been summarized by Ortega-Pierres et al. (1996). The predominant antigens recognized by animals and humans infected with *Trichinella spiralis*, or any of the other species of *Trichinella* currently known, are the so-called TSL-1 group. TSL-1 antigens are found in the stichocyte cells and on the surface of the parasite's cuticle; they are actively secreted by first-stage larvae in the muscle. These antigens are produced for diagnostic use by in vitro maintenance of isolated muscle larvae or by biochemical methods of recovery from this same parasite stage. Methods for the preparation of these antigens have been published (OIE, 2013). As noted, the TSL-1 antigen epitopes recognized by antibodies in *Trichinella*-infected animals and humans are common to all encapsulated and non-encapsulated species (Appleton et al., 1991). Consequently, *Trichinella* antigens can be routinely prepared from *T. spiralis* muscle larvae, because this species is readily maintained in laboratory rodents, and used for ELISA to detect infection with any *Trichinella* species. TSL-1 antigens share a common carbohydrate epitope (tyvelose), which has been synthesized (Reason et al., 1994).

In fact, ELISA using ES antigens or the carbohydrate tyvelose antigen, is considered the most suitable diagnostic test available (Forbes et al., 2004; Tattiyapong et al., 2011), but the use of these antigens is not exempt from problems due to cross-reactivity, which yields false positive results (FP), or low sensitivity in detecting anti-*Trichinella* IgG (Nöckler et al., , 2004; Gamble et al., 2004; Møller et al. 2005; Nöckler and Kapel, 2007; Davidson et al., 2009; Gómez-Morales et al., 2009; 2012; Szell et al., 2012).   For these reasons, different efforts to increase the specificity of

ELISA have been attempted, for example TSL-1 antigens have been purified by affinity chromatography using monoclonal antibodies in either an indirect or in a capture ELISA, obtaining a better specificity in comparison with the larval crude extract (Escalante et al., 2004). Attempts have been made to identify new *Trichinella* candidate antigens for diagnosis (Wang et al. 2009; Nuamtanog et al. 2012; Liu et al., 2013), but the identified antigens have not been tested with large panels of sera from hosts naturally or experimentally infected with *Trichinella*, so their usefulness has not been validated.

Reagents

Better specificity is obtained in the ELISA and IETB using a species-specific anti-IgG reagent as compared with a Protein A conjugate. For these reasons, it is strongly recommended that a species-specific anti-IgG reagent be used although it may be necessary to have it custom made.

Sample collection

Serum is the preferred sample for indirect detection of *Trichinella* infection. After collection, blood samples should be clotted and the sera frozen at –20°C as soon as possible; samples frozen at –20°C may be used for several months, avoiding repeated freezing and thawing of samples, which can cause a decline in antibody titers; therefore, samples that will be used frequently should be made into aliquots. For periods of storage greater than 3 months, serum samples should be frozen at –80°C or lyophilized. If freezing is not possible, 1% merthiolate or another suitable preservative, should be added to each serum sample at a dilution of 1:10,000.

For tests performed using animal carcasses, where blood or serum is not available, tissue fluids or meat juice are alternative sample sources (Gamble, 2004). Generally samples of tissue fluids are used at a lower dilution (higher concentration) in serological assays; antibody concentration in tissue fluids may be 10-fold lower than that found in serum (Kapel et al., 1998; Møller et al., 2005). If meat samples are used for the extraction of tissue fluids it is recommended to wash the tissue, cut it into small pieces, freeze and thaw it and use these extracts instead of muscle fluids found in an original sampling, which is often mixed with blood and/or other surface contaminants.

Another alternative method for sample collection and storage is blood spots on filter paper (Vu et al., 2010). This method is useful when there are no facilities to store frozen samples; blood spots may be stored at room temperature in closed plastic bags to prevent rehydration.

Validation and Quality Control

An acceptable serological assay is one that is properly standardized and validated for its intended purpose. All components of the test that are critical for maintaining suitable performance (critical control points) should be identified and appropriately controlled, and the test should be conducted within a laboratory quality system (OIE, 2013). In particular, each batch or lot of antigen should be checked by checkerboard titration against a standardized positive control serum. Independent validation should be performed using sera from infected and *Trichinella*-free animals or humans representative of the local population where the test is being used. The cut-off value of the ELISA, for example, can be different depending on host genetic background and exposure to antigens in the local environment and in food. On every ELISA plate control positive and negative sera are required to assure the test is performing properly (OIE, 2013). The performance of the test relative to specificity and sensitivity should meet the purpose of the test. When intended for use in epidemiological studies, the sensitivity and specificity of the ELISA, and other serological tests, must be considered when interpreting results. Estimates of sensitivity and specificity can be obtained by testing adequate numbers of known positive and known negative animals or humans. The negative control group should be representative of the local population and positives should represent various stages and levels of infection. For humans, blood donors often represent a good source of reference serum samples. The Office International des Epizooties (OIE, 2013) recommends a minimum of 300 known positive and 1000 known negative samples to establish sensitivity and specificity.

For a comprehensive discussion of requirements for the validation of a serological test, the reader is referred to the OIE (2013) (www.oie.int/eng/normes/mmanual/A_00013.htm) Commercially available serological tests such as the ELISA often must meet specific standards of sensitivity and specificity established by an individual country's licensing authority. Users of commercial tests should verify that the test has been adequately evaluated using international reference standards and has received the approval of any relevant regulatory authorities. The user of any test should always conduct an independent evaluation of test performance using panels of defined positive and negative sera that are representative of the population to be tested.

Use of Serological Methods in Animals

Animals can harbor infective muscle larvae as early as 18 days post infection (Despommier, 1998), in some cases before detectable antibody is present; further, infection with low numbers of

larvae can result in an extended period of seronegativity before anti-*Trichinella* antibody is detectable in serum (Nöckler et al., 2005). Consequently, serological methods, including the ELISA, should not be used for the detection of *Trichinella* infection in individual food animal carcasses at slaughter for the purpose of protecting human health. Serology tests, including the ELISA, are suitable for the purpose of surveillance programs to estimate the prevalence of infection in an animal population.

ELISA for detection of Trichinella *infection in swine*

Suitability of test **-** The ELISA, due to its ease of use, economy, rapid test results, and potential for standardization and automation for large numbers of samples, is the test of choice for surveillance purposes in domestic pigs. The ELISA, using ES or tyvelose as the antigen, has been shown to have greater sensitivity than digestion of 1g samples in animals with low (<3 lpg) worm burdens; infections of as few as 1 larva per 100 g of tissue can be detected by ELISA (Gamble *et al.*, 1983; Kapel and Gamble, 2000). It is advisable, when possible, to examine animals found serologically positive in the ELISA by artificial digestion to confirm infection, to estimate worm burdens, and to identify the species of *Trichinella* by molecular genotyping (Zarlenga *et al.,* 1999). Artificial digestion using an appropriate amount of tissue (i.e., 100 grams or more), and from the preferred anatomical site in the animal species in question, should be performed according to ICT Recommendations (Gamble *et al.*, 2004).

Sensitivity and specificity - The ELISA for detection of *Trichinella* infection in pigs, as with all serological tests, has imperfect diagnostic sensitivity (Se) and specificity (Sp); however, most studies report estimates of 93-99% Se and Sp in reference populations (Frey et al., 2009; Gómez-Morales et al., 2009; Nöckler et al., 2009). Serological detection of *Trichinella* infection in pigs is impacted by both technical (laboratory proficiency, quality of the antigen used in the assay) and biological factors (initial infecting dose, days post infection of serum collection). The technical variables can be particularly troublesome in ELISAs which are not properly validated and controlled, while variability in biological factors will always occur in naturally infected pig populations. False negatives can occur during a period of prolonged seroconversion due to a low infectious dose or low larval density in muscle tissue, or from collection of serum before a detectable antibody response has been generated. False positives occasionally occur as a result of non-specific serological reactivity to components in a complex antigen preparation, or cross reacting antibodies generated as a result of a different helminth infection.

Use of a crude somatic antigen extract of *Trichinella* (e.g., Melcher's antigen) in the ELISA results in cross-reactions (false-positive reactions) in pigs infected with other parasites and/or microbes (Gamble *et al.*, 1983). Several studies have demonstrated that both ES and tyvelose antigens reduce the cross-reactions (Gamble *et al.*, 1983, 1988, 1997; Tattiyapong *et al.*, 2011). Despite these issues, the sensitivity of the ELISA in standardized, validated commercially available kits using an ES antigen has been reported to range from 94.4 – 98.4%, provided sufficient time has elapsed for infected animals to develop an antibody response (see Interpretation of Results, below), while specificity ranges from 93.8 – 99.6%.

The quality of ES antigen used in the ELISA is of primary importance, and depends upon adhering to proper methods for the cultivation of *Trichinella* muscle larvae and proper purification and storage of the antigen (Gamble *et al.*, 1988). Hill *et al.* (2013, unpublished) conducted a ring trial comparing *Trichinella* ES antigens from 5 different laboratories, each of which prepared ES antigens following a standardized method and a method normally used in their laboratory. Significant differences were seen between ES products produced by the five laboratories, but also between ES produced in the same lab by the standardized method vs the home laboratory method.

Tyvelose has been used in a number of studies of *Trichinella* seroprevalence in pigs and has been shown to be useful for surveillance activities (Forbes et al., (2004); Chávez-Larrea et al., 2005; Møller *et al*., 2005). The sensitivity of the ELISA using tyvelose has been reported to be lower than that using ES antigens (Gamble *et al.*, 1997; Møller *et al*., 2005). However, Forbes et al., (2004) described a Reciever Operater Characteristic (ROC) optimized tyvelose ELISA which performed equal to or better than a ROC-optimized ES ELISA (Se and Sp tyvelose ELISA-94.3 and 96.7%, versus ES-ELISA-84.9 and 96.0%).

Methodologies - A general method for conducting an ELISA test in pigs is described in the OIE Manual of Standards for Diagnostic Tests and Vaccines (Chapter 2.1.1.6) (Gamble, 2012) (http://www.oie.int/fileadmin/Home/fr/Health_standards/tahm/2.01.16_TRICHINELLOSIS.pdf)). By special arrangements, standard antigens, reference sera and scientific consultation can be obtained from ICT members' laboratories (www.trichinellosis.org) and the OIE Reference Laboratories (www.oie.int/eng/oie/organisation/en_listeLR.htm).

Interpretation of results - The level of infection of pigs with *Trichinella* larvae (worm burden) is directly correlated with the time required for antibodies to appear in the blood. For light infections (< 1 larva per gram of tissue), antibodies may not be detected by ELISA for 4-7

weeks or longer following exposure (Gamble *et al.*, 1983; Gamble, 1996, 1998), while antibodies might be detected from 2.5-3 weeks in pigs with higher numbers of muscle larvae (Smith and Snowdon, 1989; Kapel and Gamble, 2000). Thus, there is a risk of obtaining false-negative results during the early stages of infection, and for this reason, the ELISA and other tests measuring a serological response cannot be used for individual carcass testing. Studies comparing these false-negative serology results with the ability to detect larvae by direct methods in these low worm burden animals have not been performed.

There is no correlation between the ultimate worm burden (larvae per gram of tissue) and the resulting optical density (OD) in the ELISA in serologically positive pigs once seroconversion has taken place. Therefore, artificial digestion of tissue is an important adjunct to ELISA to determine the public health risk associated with infected animals. *Trichinella* antibodies may persist in pigs for extended periods of time (Nöckler *et al.*, 1995; Kapel and Gamble, 2000). It can be assumed that in slaughter pigs, which have a live weight of 90 to 100 kg at an age of 25 to 30 weeks, it is unlikely that a false-negative finding will result from declining antibody titer (Nöckler *et al.*, 2000).

ELISA for detection of Trichinella *infection in horses*

Suitability of the test - Studies in horses have shown that serological responses to *Trichinella* infection in this species are less consistent than responses observed in pigs, rodents and humans (Smith and Snowdon, 1987; van Knapen *et al.*, 1987; Polidori *et al.*, 1989; Soule *et al.*, 1989; Gamble *et al.*, 1996; Voigt *et al.*, 1997; Yepez-Mulia *et al.*, 1999; Sofronic- Milosavjevic *et al.*, 2001; Pozio *et al.*, 2002a; Hill et al., 2007). Studies in horses experimentally infected with 5,000 *Trichinella spiralis* larvae reported a decline in antibody titer at week 15 post-infection, at which time infective larvae were still present in the muscle tissue (Voigt *et al.*, 1997). Pozio et al (2002a) experimentally infected horses with *T. spiralis* and *T. murrelli*; antibody responses in the ELISA using ES, tyvelose, or a crude worm extract were not detectable 5-6 months after infection, though infectious muscle larvae were still present. Hill et al., (2007) infected horses experimentally with 1000, 5000, or 10,000 *T. spiralis* muscle larvae and followed antibody responses biweekly for 1 year. Antibody responses persisted in a dose dependent manner for 5 months and then declined to undetectable levels. Viable muscle larvae persisted in horse muscle for the duration of the experiment.

Low specificity with ES and tyvelose antigen, and low sensitivity due to a decreasing antibody titer, even in the presence of high numbers of larvae in the musculature, have been

reported using ELISA and IETB (Soule *et al.*, 1989; Pozio *et al.*, 1997, 2002a). However, Sofronic-Milosavljevic et al.,(2005) reported better success in detecting persistent antibody responses (32 weeks post infection (PI)) in experimentally infected horses using the Western blot and IFA versus the ELISA (18 and 20 weeks PI using ES or tyvelose, respectively).

Considering the present state of knowledge, the ICT cannot recommend serological methods for use in horses either for detection of single infections or for reliable surveillance. However, further studies are needed, especially in endemic regions, to determine if serological methods might have some value.

Game and other animals intended for human consumption

Suitability of the test - Relatively few studies have used serological methods for estimating *Trichinell*a infection in game animals intended for human consumption. Consequently, insufficient information is available on the performance of the ELISA in game animals to recommend this method for individual carcass inspection or for epidemiological studies for the purpose of estimating true prevalence. The use of serological methods for determining prevalence of *Trichinella* infection in wildlife must be preceded by a thorough evaluation of test sensitivity and specificity in the species and population being tested. The ICT discourages the use of serological methods for the detection and surveillance of *Trichinella* infection in wildlife without such an evaluation. Any evaluation of serological methods should include digestion of a minimum of 100 grams of tissue for comparative purposes. This caution on the use of serological methods for testing wild animals is suggested by studies that reported a high serological prevalence for *Trichinella* in animals without larvae in muscles, from regions where *Trichinella* infection was never observed (Vercammen *et al.*, 2002), or the prevalence rate detected by artificial digestion was 100 times lower than that recorded using serological methods (Nöckler and Voigt, 1997a; Wacker *et al.*, 1999). Due to the high number of parasitic, bacterial, fungal, and viral infections which can be present in wildlife, the risk of cross-reactions with non-specific antibodies is very high, and it is likely that some reports of serologically positive animals are the result of cross-reactions. The greater sensitivity of ELISA might also be a factor when testing wildlife populations where worm burdens may be very low (only a few larvae per kilogram of tissue; Gamble et al., 1996). Further, hydrolyzed serum may be all that is available from wildlife for testing due to suboptimal storage conditions during sampling; these sera may give erroneous results.

More studies are required to determine the influence of cross-reactions and quality of blood samples on the specificity of the ELISA and also to determine whether very low level infections may be responsible for some of the positive serological results obtained. To assure prevention of human disease, any meat-eating game animals intended for human consumption should be examined by artificial digestion, according to approved methods (Gamble *et al.*, 2000).

Sensitivity and specificity- Sensitivity and specificity should be determined by testing panels of sera from a sufficient number of known positive and known negative animals representing the species and population in question, prior to using a test. Since this is often not possible with wildlife species, the limitations should be clearly understood and explained when reporting or interpreting results.

Methodologies

Wild boar - A few epidemiological studies have used ES antigens for the evaluation of prevalence in naturally infected wild boar populations. From the Netherlands, 31 of 458 samples (6.8%) were positive (van der Giessen *et al.*, 2001) and from Germany, 234 of 16,888 samples were positive, corresponding to a seroprevalence of 1.4% (Nöckler *et al.*, 1999). This seroprevalence is much higher (up to 100 times) when compared with the prevalence of *Trichinella* in wild boar as determined by artificial digestion (between 0.001 and 0.01 % in Germany). Similar results were found by Cuttell et al. (2014), who surveyed 673 Australian wild boar by ELISA, Western blot, or artificial digestion, and found 3.5 and 2.3% seroprevalence by 2 ELISA tests coupled with Western blot, while all artificial digests were negative. In another study, the combined use of ELISA and a highly sensitive and specific Western blot had a sensitivity 31.4 times higher than that of the digestion (Gómez-Morales et al 2014). Hill et al., (2014) found an overall prevalence of 3.0% in 4231 feral swine/wild boar in a nationwide study in the U.S., while artificial digestion of 300 swine tongues from this same study recovered very low numbers of muscle larvae (<2 lpg) from 1.0% of the tongues. These findings suggest a higher sensitivity of the ELISA versus artificial digestion due to low worm burdens in naturally infected animals. Such differences may also be related to cross reactions resulting from infection with other tissue-migrating nematodes (*Toxocara*, *Ascaris*, *Spirura*), but no studies have been conducted to evaluate this phenomenon in wild boar.

In contrast, the experimental studies of Kapel (2001) have shown a good correlation between larval burden and antibody response. In this study, an antibody response was detectable by ELISA 3 to 4 weeks after experimental infection of wild boars with different encapsulated

Trichinella species, however, seroconversion was delayed in infections with non-encapsulated species. The antibody level remained stable in wild boars infected with *T. spiralis, T. britovi* and *T. nelsoni*, but the rapid decrease of antibodies directed against *T. nativa* and *Trichinella* T6 was apparently associated with the rapid disappearance of larvae.

Bear - Several studies have reported the use of ELISA to detect infection in bears (*Ursus arctos, Ursus americanus; Ursus maritimus*) (Zarnke *et al.*, 1997; Nutter *et al.*, 1998; Rah et al., 2005; Asbakk et al., 2010; Mortenson *et al.*, 2014). Better specificity in the ELISA was obtained using a customized anti-bear IgG reagent as compared with the Protein A conjugate in the ELISA test (Gamble H.R., unpublished data). Dubey et al., (2013) evaluated matched serum and tissues from 300 hunted black bear (*Ursus americanus*) in the U.S. Only 2 bears were seropositive in the E/S ELISA using an anti-bear IgG conjugate; *T. murrelli* was isolated from tongues of both bears. The other 298 bears were both tissue and seronegative.

Fox - Experimental studies in silver foxes (*Vulpes vulpes*) showed that use of an ES antigen in an ELISA is a suitable method for serological detection of *Trichinella* infection in this species (Kapel, 2000). Specific antibodies were detectable in all foxes infected with 500 or 2000 larvae between 2 and 6 weeks post infection, and these antibodies persisted throughout the experiment (30 and 76 weeks post-infection) with mean larval recovery rate at post mortem ranging from 4.3 to 11.3 larvae per gram of tissue (Nöckler and Voigt, 1997b). Anti-dog-IgG peroxidase conjugate is suitable as an alternative to the species-specific anti-fox antibody system since there is a close phylogenetic relationship between foxes and dogs. Davidson *et al.*(2009) tested ES and tyvelose antigen in ELISA against sera from 328 wild foxes and 16 experimentally infected farmed foxes in Norway. Both antigens performed well in the ELISA, however comparison with Western blot results using Melchers antigen proved less consistent. Results from field studies by Wacker *et al.*(1999)demonstrated a 100 times higher *Trichinella* seroprevalence in the red fox population (7.7%) compared to a prevalence of 0.07% in foxes examined by artificial digestion. As in wild boars, the huge difference between seroprevalence and actual detection of parasites may be due, in part, to the higher sensitivity of the ELISA (0.01 larvae per g) and the limited ability to detect muscle larvae in small samples of muscle tissue. It should be considered that the larval recovery rate in the majority of foxes might be very low, i.e. about or less than 0.1 larvae per g of musculature, as published by Enemark *et al.* (2000) and van der Giessen *et al.* (2001).

Special considerations

The variability of collection methods for game meat serum samples often creates problems in conducting serological tests. Samples are frequently contaminated by bacteria or fungi, or they may be hemolysed; these problems can cause false positive results. Filtration of contaminated samples with a 0.22 µm filter is useful to reduce the background, but is not always entirely effective.

Interpretation of results

False negative results have been observed in wild animals (e.g., foxes and wild boars) with long-term low-level infections with sylvatic species *of Trichinella*, (e.g., *T. britovi*) (Pozio E., unpublished data).

It is imperative to evaluate the cut-off value on the basis of a panel of serum samples (at least 100-200 sera), which are representative of the animal population for which the test will be used. The animal genotype, feeding habits, pathogen exposure, and environmental characteristics can influence the background of a serological test. This preliminary evaluation should be done for both commercial kits and laboratory developed tests. It is also important that the cut-off value be confirmed, every time the antigen, reagents or materials (e.g., ELISA plate) are modified or substituted.

Use of Serological Methods in Humans

Since there is no pathognomonic sign or symptom for trichinellosis, clinical diagnosis is often difficult. Consequently, diagnosis is based on three main criteria: anamnesis based on epidemiological data, clinical evaluation, and laboratory tests including serology and/or the detection of *Trichinella* larvae in a muscle biopsy (Dupouy-Camet & Bruschi, 2007). Because the collection of a muscle biopsy is invasive, painful, and does not always give the expected result even if the suspected diagnosis is correct, serological findings diagnosis, normally carried out by the detection of specific IgG in serum has a practical diagnostic value.

There are 3 objectives in the immunodiagnosis of human trichinellosis: (a) recognizing the acute infection to allow early anthelminthic treatment; (b) making a retrospective diagnosis; and (c) adding information to the epidemiology of the infection. (Ljungström 1983)

Suitability of test

Many serological tests are available for human diagnosis (Dupouy-Camet and Bruschi, 2007). As already stated, ELISA, IFA and IETB are the more frequently used. Diagnostic laboratories should use more than one test for screening: for example, ELISA to detect the response against a soluble antigen (preferably ES) and IFA to detect antibodies reacting with cuticular antigens of the larva.

IETB has shown promise for discriminating trichinellosis patients from patients with other helminth infection, although possible cross-reactions may occur with cases of anisakiasis (Yera et al., 2003) and schistosomiasis (Dupouy-Camet J., unpublished data). In fact, serological diagnosis for parasitic infections, especially searching for antibodies specific for nematodes, can be complicated by cross-reactivity, in particular when using parasite crude extracts and to a lesser extent ES antigens (Gomez-Morales et al., 2008). The presence of shared antigens of *Trichinella* spp. in other parasites and pathogens has been reported (Intapan et al., 2006, Bruschi & Gomez-Morales 2014).

Due to its high sensitivity, the ELISA is the most commonly used first screening test for the detection of IgG antibodies followed by IETB to confirm positive results as has been recently proposed (Gomez-Morales et al., 2008, 2012; 2014). In these studies, a distinctive pattern of 53–72 kDa for recognizing *Trichinella* spp. infections in humans by IETB has been defined, with an optimal sensitivity and specificity. Even sera with a high OD obtained in ELISA, but falsely positive (from individuals without trichinellosis), did not react with this pattern. It should be mentioned, however, that 84 % and 71 % of the sera from the same patients also reacted with 104–111 kDa and 38–42 kDa proteins, respectively, and with a lower frequency also with other proteins (Gomez-Morales et al. 2012). However, sometimes the IETB is used as the only test for humans (Bruschi & Dupouy-Camet 2014).

Recently, a glycan microarray approach has been used with the aim to select for synthetic glycan antigens possibly useful for serodiagnosis of trichinellosis. A glycan array which contained over 250 different glycan antigens was used. A GalNAcβ1-4(Fucα1-3) GlcNAc-R (LDNF) was identified as a glycan antigen recognized by sera derived from *Trichinella*-infected individuals. An ELISA based test using a glycan represented by 5 LDNF molecules coupled to bovine serum albumin gave 67% specificity (false positive results were mainly obtained with cysticercosis and strongyloidosis sera) and 96% sensitivity (Aranzamendi et al., 2011).

In most trichinellosis cases, increased parasite-specific IgG, IgA and IgM serum levels accompany the infection; however, increases in parasite-specific IgE antibody and total IgE is not consistent, consequently their diagnostic value without considering other laboratory findings is limited (Dupouy-Camet & Bruschi, 2007).

Generally, seroconversion occurs between the third and fifth week of infection and antibody levels do not correlate with the severity of the clinical course or with a particular clinical course (Bruschi & Murrell, 2002). IgG specific antibodies are detectable from 12 to 60 days post infection and may persist for more than 30 years after infection (Fröscheret al., 1988). The sequential appearance of specific antibodies of the various IgG subclasses during the early stage of a *T. spiralis* infection has been studied with a follow up lasting one year, with a resulting peak response of IgG1 occurring before IgG4 and in about half of the patients simultaneously with IgG3. After one year, a shift of specific antibodies to IgG4 was observed. (Ljungström et al., 1988). In other studies, *Trichinella*-specific IgM, IgG, and IgA antibodies measured in sera from symptomatic and asymptomatic patients remained positive up to 15 years after infection (Pinelli et al., 2007). The measured IgG4 antibody response to a 45 kDa *T. spiralis* antigen also persisted at least 18 months in *T. spiralis* infected patients (Pinelli et al., 2004) and up to 15 years after *T. britovi* infection (Pinelli et al., 2007).

An algorithm for serological diagnosis of human trichinellosis is under evaluation and shown in Fig. 1 (Bruschi and Gomez-Morales, in preparation).

ALGORITHM FOR SEROLOGICAL DIAGNOSIS OF HUMAN TRICHINELLOSIS
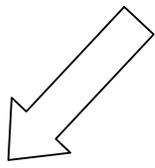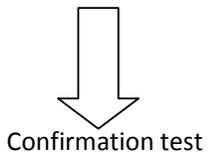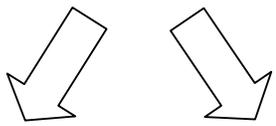
Screening tests
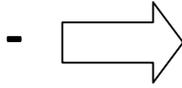
ELISA (E/S Ag)   If   **–** ⟹   with an R* < 1 no further analyses in

IFA (ML as antigen)   absence of epidemiological factors;

                                             with an R* > 1 and < 1.3 with an
                                             epidemiological risk (occurrence of an outbreak)
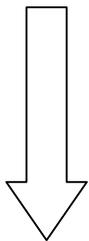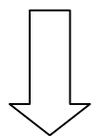
If   **+**

Confirmation test

WB (E/S antigen)

Bands of   Bands of different M.W. (over 72 kDa. And under 48 kDa.)

48-55 kDa**
and/or 59-63
and/or 64-72

Diagnosis confirmed   Evaluate during following weeks for possible seroconversion

*R= O.D. of the sample/cut off in ELISA tests
**In reducing conditions

CONCLUSIONS

This guideline is based on the best scientific published information and on the unpublished data from laboratories with a great expertise in this field and represent the official position of the International Commission on Trichinellosis regarding acceptable methods and the evaluation of the sensitivity and specificity. These recommendations are subject to change as new scientific information becomes available.

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