



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

Bruschi F.<sup>1</sup>, Gomez-Morales M.A.<sup>2</sup>, Hill D. E.<sup>3</sup>

<sup>1</sup>Department of Translational Research, N.T.M.S. Università di Pisa, Via Roma 55 56126 Pisa, Italy, <sup>2</sup>Unit of Gastroenteric and Tissue Parasitic Diseases, Istituto Superiore di Sanità, viale Regina Elena 299, 00161 Rome, Italy,

<sup>3</sup>USDA, ARS, ANRI, APDL, Building 1040, Room 103, BARC-East, Beltsville, MD 20705

### **Introduction**

Serological methods are widely used for detection of infection in man and animals. The use and interpretation of serological tests vary 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 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. 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.

### **Assays**

Many types of serological assays have been, and continue to be, used for the detection of *Trichinella* infections in animals and man. Serological assays include, but are not limited to:

- 1) enzyme-linked immunosorbent assay (ELISA) employing excretory-secretory (ES) antigens of the muscle larvae (ML);
- 2) immuno-electrotransfer blot assay (IETB), also named western blot (WB), using crude worm extract (CWE) or ES antigens;

- 3) indirect immunofluorescence assay (IFA), using formalin- fixed whole larval preparations, cryostat sections of infected rodent muscle or frozen sections of free ML;
- 4) enzyme immunohistochemical (EIH) technique, employing cryostat sections of infected rodent muscle or frozen sections of free ML;
- 5) lateral flow (LF) methods, using immunochromatographic strips and ES antigens.

For detection of human and swine infections, ELISA is the most commonly used screening test; positive results should be confirmed by WB.

The main advantages of ELISA are high throughput potential, low cost, reliability, standardization, and an acceptable balance between sensitivity and specificity. It is the only serological method in animals recommended by the World Organization for Animal Health (OIE). 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 considered in selecting any serological test for detection of *Trichinella* infection.

### Antigens

For serological testing by ELISA, the ICT recommends the use of ES antigens obtained from the *in vitro* maintenance of *Trichinella* ML. This antigen preparation contains a group of immunodominant, structurally related glycoproteins that are recognized by animals and humans infected with *Trichinella spiralis*, or any of the other species of *Trichinella* currently known. When compared with somatic worm extracts, these antigens have limited cross-reactivity with sera from animals infected with other parasites. Numerous methods have been published for the preparation of ES antigens; however, for consistency among preparations and reproducibility of ELISA data, the ICT recommends the method published in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals

([http://www.oie.int/fileadmin/Home/eng/Health\\_standards/tahm/2.01.20\\_TRICHINELLOSIS.pdf](http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.20_TRICHINELLOSIS.pdf)).

*Trichinella* ES antigens are routinely prepared from *T. spiralis* ML because this species is readily maintained in laboratory animals, and ES antigens collected from *T. spiralis* interact with infection sera from all *Trichinella* species and genotypes identified thus far.

### Reagents

To maximize test sensitivity and specificity, it is recommended that a species-specific anti-IgG conjugate rather than a Protein A conjugate be used in the ELISA or WB.

### Sample collection

Serum is the preferred sample for indirect detection of *Trichinella* infection. After collection, blood samples should be clotted, sera collected, and, if not used for testing immediately, frozen at  $-20^{\circ}\text{C}$ . Samples frozen at  $-20^{\circ}\text{C}$  may be used for several months; however, it is recommended that repeated freezing and thawing of samples should be avoided in order to prevent a decline in antibody titers and an increase in non-specific reactivity. If samples are to be used frequently they should be stored in aliquots. For periods of storage greater than 3 months, serum samples should be frozen at  $-80^{\circ}\text{C}$  or lyophilized. If freezing is not possible, 1% merthiolate (used at 1:10,000 dilution) or another suitable preservative should be added to each serum sample.

For tests performed on animal carcasses, where blood or serum is not available, tissue fluids can be used as alternative sources of antibody. Usually, samples of tissue fluids are used at a lower dilution (higher concentration) in serological assays as antibody concentration in tissue fluids may be 10-fold lower than that found in serum. When 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.

Another alternative to blood samples is blood spots on filter paper. 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 should be 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 checked. Furthermore, the test should be conducted within a laboratory quality system. In particular, each batch or lot of antigens should be evaluated by checkerboard titration against a standardized positive control serum.

Requirements for the development and validation of a serological test in animal populations are specified in the OIE Manual ([http://www.oie.int/fileadmin/Home/eng/Health\\_standards/tahm/1.01.06\\_VALIDATION.pdf](http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/1.01.06_VALIDATION.pdf)).

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. It is important that the user of any test conducts an independent evaluation of test performance using panels of defined positive and negative sera, representative of the population to be tested.

### **Use of Serology in Animals**

Animal hosts can harbor infective ML as early as 18 days post infection, in some cases before detectable antibodies are present; further, infection with low numbers of larvae can result in an extended period of seronegativity before anti-*Trichinella* antibody is detectable in serum. It has been reported that the correlation between seropositivity and the presence of *Trichinella* ML decreases at low infection rates. For these reasons, serological methods should not be used for the detection of *Trichinella* infection in individual food animal carcasses for the purpose of protecting human health.

#### *ELISA for detection of Trichinella infection in domestic swine populations*

*Suitability of test* - The ELISA, due to its ease of use, low cost, rapidity in obtaining results, and potential for standardization and automation for large numbers of samples, is the test of choice for surveillance in domestic pigs. The ELISA, using ES antigens, has been shown to have greater sensitivity than digestion of 1g samples in animals with low (i.e. <3 larvae per gram (lpg)) worm burdens. However, this increased sensitivity, as compared with direct testing methods, is offset by the reduced ability to detect antibodies in recently infected animals, even when infective larvae are found in the muscle. Thus, the test not advised for individual carcass control.

*Validation of ELISA* - 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). Prior to using ELISA for detection of antibodies to *Trichinella*, the test should be fully validated with an appropriate number of positive and negative samples from the test population. Validation should take into account that 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 developed. False positives can occur from non-specific serological reactivity to components in a complex antigen preparation, or to cross-reacting antibodies generated from a different helminth infection. This is particularly evident in free-ranging and backyard pigs which are also at higher risk for *Trichinella* sp. infection. Therefore, positive results should be confirmed by WB.

*Antigen preparation* - The quality of ES antigens used in the ELISA is of primary importance, and depends upon adherence to proper methods for the cultivation of *Trichinella* ML and proper purification and storage of the antigen. The method for the preparation of ES antigens has been published in the OIE Manual.

*Methodologies* - A general method for conducting an ELISA test in pigs is described in the OIE Manual ([http://www.oie.int/fileadmin/Home/fr/Health\\_standards/tahm/2.01.16\\_TRICHINELLOSIS.pdf](http://www.oie.int/fileadmin/Home/fr/Health_standards/tahm/2.01.16_TRICHINELLOSIS.pdf)). Standard antigens, reference sera and scientific consultation can be obtained from subject matter authorities, such as ICT members' laboratories ([www.trichinellosis.org](http://www.trichinellosis.org)) and the OIE Reference Laboratories for *Trichinella* ([www.oie.int/eng/oie/organisation/en\\_listeLR.htm](http://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 anti-*Trichinella* antibodies to appear in the blood. For low-grade infections (< 1 lpg), antibodies may not be detected by ELISA for 4-7 weeks or longer following exposure, while antibodies might be detected from 2.5-3 weeks in pigs with higher numbers of ML. 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. 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 would result from declining antibody titer.

Reference swine sera positive for anti-*Trichinella* antibodies are not available on the international market; however, swine sera from experimentally infected animals have been collected and their validity and stability tested. These reference sera are available upon request at the European Union Reference Laboratory for Parasites (<http://www.iss.it/crlp/>).

#### *Indirect detection of Trichinella infection in other animals, including wildlife*

Several ELISAs to monitor wildlife populations, such as wild boar, have been described. Among these, the competitive ELISA allows for the detection of specific antibodies irrespective of their isotype or host origin and has a potential value as a multispecies surveillance tool. 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. Besides the compromised quality of the blood samples, the validation of serological assays is also hampered by a lack of reliable reference sera. Any serology test used to detect *Trichinella* infection in animal species other than pigs should likewise be fully validated. Examples of ELISA performance in animal species other than swine are presented in Table 1.

#### *Interpretation of results*

It is imperative to determine the positive cut-off value and associated sensitivity and specificity on the basis of a panel of serum samples (at least 100-200 sera representative of the animal population for which the test will be used). Alternative methods such as a binary mixed model analysis, which was shown effective for other animal parasitic diseases, are not feasible at a low expected prevalence of the infection. The animal genotype, feeding habits, pathogen exposure, and environmental characteristics can influence the background of a serological test. All these factors are relevant in wildlife and other animal species that are not raised under controlled conditions.

TABLE 1. The performance of ELISA with ES antigens in animal species other than swine.

Animal species	Notes	References
Horse	Antibody responses persisted in a dose dependent manner from 14 to 20 weeks post-infection (p.i) and then declined to undetectable levels, whereas, viable ML persisted in horse muscle for longer period of time	Hill et al., 2007 Nöckler et al. 2000
Dog	ELISA followed by a confirmatory Western blot using ES antigens have been developed and validated; no commercial kit is available	Gomez Morales et al., 2016
Wild boar	Results similar to those of domestic pigs but with a higher number of false positives	Cuttell et al., 2014; Gomez Morales et al., 2015

Bear	Lack of reference sera and validation studies	Rah et al., 2005; Asbakk et al., 2010; Mortenson et al., 2014
Fox	Lack of reference sera and validation studies	Davidson et al., 2009 ; Nöckler and Voigt, 1998
Crocodiles	Antibodies were not detectable after six weeks p.i. although live larvae were present in the muscles up to six months p.i.	Ludovisi et al., 2013

### **Use of Serological Methods in Humans**

Since there are no pathognomonic signs or symptoms for trichinellosis, clinical diagnosis in individuals 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. Because the collection of a muscle biopsy is invasive, painful, and does not always give the expected result even when the suspicion of trichinellosis is correct, serological findings, normally entailing the detection of specific IgG in serum, have practical diagnostic value.

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

### **Suitability of test**

Many serological tests are available for human diagnosis. However, the ICT recommends the use of an ELISA for screening and WB to confirm ELISA-positive sera. All tests should use ES



antigens. Serological diagnosis can be complicated by cross-reactivity, due to the presence of shared antigens of *Trichinella* spp. in other parasites and pathogens.

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 are not consistent, and consequently the diagnostic value of IgE antibodies without considering other laboratory findings is limited.

Generally, seroconversion in infected humans occurs between the third and fifth weeks of infection and antibody levels do not correlate with the severity or other aspects of the clinical course. IgG specific antibodies are detectable from 12 to 60 days post infection and may persist for more than 30 years after infection. The identification of IgG subclasses, although interesting for research purposes, does not contribute to the diagnosis. For interpreting human serology in the course of *Trichinella* infection the FAO/WHO/OIE guidelines for the surveillance, management, prevention and control of trichinellosis should be consulted.

An example of a detailed protocol for performing an ELISA with human sera is shown in ANNEX 1.

## **CONCLUSIONS**

These recommendations are based on current scientific information including unpublished data from laboratories with relevant expertise in this field. They represent the official position of the ICT regarding acceptable methods for the use and interpretation of serology testing for *Trichinella* infection in animals and humans. These recommendations are subject to change as new scientific information becomes available.

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# ANNEX 1

## Detection of anti-*Trichinella* antibodies in human serum by indirect ELISA

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## 1. Aim and field of application

To determine the presence of anti-*Trichinella* sp. antibodies by an enzyme linked immunosorbent assay in human sera.

The method can be used for the serological diagnosis of human trichinellosis.

## 2. Principle of the method

A 96-well microtiter polystyrene plate is coated with *Trichinella spiralis* excretory/secretory (E/S) antigens.

Control and test sera, properly diluted, are distributed in the wells, allowing any anti-*Trichinella* sp. antibodies that are present to bind to the adsorbed antigen.

The antibodies that do not bind to the antigen are eliminated by washing; peroxidase conjugated anti-human IgG goat antibody is then added to each well. This second incubation allows the conjugate to bind to the human antibodies that were bound to the antigens onto the well surface.

The excess conjugate is eliminated by washing, and the activity of the enzyme bound to the human antibodies is measured by adding a chromogen substrate. After incubation, the intensity of the developed color is determined by a spectrophotometer.

The result is interpreted comparing the color intensity of the wells containing the test sera with those containing the controls.

## 3. References

Gamble HR, Pozio E, Bruschi F, Nockler K, Kapel CM, Gajadhar AA. International Commission on Trichinellosis: recommendations on the use of serological tests for the detection of *Trichinella* infection in animals and man. *Parasite*. 2004 Mar;11(1):3-13.

Centers for Disease Control, Office of Health and Safety, [www.cdc.gov/od/ohs/biosfty/bmb14/b4af.htm](http://www.cdc.gov/od/ohs/biosfty/bmb14/b4af.htm)

Gómez-Morales MA, Ludovisi A, Amati M, Cherchi S, Pezzotti P, Pozio E. 2008. Validation of an enzyme-linked immunosorbent assay for diagnosis of human trichinellosis. *Clin Vaccine Immunol*. 15:1723-9.

## 4. Definitions

ELISA	Enzyme Linked Immunosorbent Assay
Ag	Antigen
Ab	Antibodies
Ag E/S	Excretory/Secretory antigens
BSA	Bovine Serum Albumin
PBS	Phosphate Buffered Saline
H	Hours
Min	Minutes
RT	Room temperature

## 5. Devices/instruments

The following instruments are needed to prepare the reagents to perform the ELISA procedure.

Adjustable pipettes (volumes: 1 - 1000 µL)

Balance (0.01-100gr)

Automatic plate washer (strongly recommended)

ELISA plate microtiter reader

Freezer -20/-30°C

Ice maker

Incubator 37°C

Magnetic stirrer  
Adjustable volume dispenser (e.g., Multipipette Eppendorf®)  
pH meter  
Pipette aid  
Refrigerator  $+4^{\circ}\text{C} \pm 2^{\circ}\text{C}$   
Vortex

## 6. Reagents and chemicals

The step-by-step procedure for preparing the reagents is described below.

### 6.1 Analytical grade water

### 6.2 Phosphate buffered saline (PBS), pH $7.3 \pm 0.2$

$\text{KH}_2\text{PO}_4$	0.34 g
$\text{Na}_2\text{HPO}_4$	1.21 g
NaCl	8.0 g
Analytical grade water	up to 1000 mL

Dissolve compounds in 750 mL of analytical grade water under magnetic stirring. Check the pH ( $7.3 \pm 0.2$ ) and then bring the solution to the final volume; refrigerate.

### 6.3 Carbonate buffered saline, pH $9.6 \pm 0.2$

$\text{Na}_2\text{CO}_3$	1.12g
$\text{NaHCO}_3$	2.92g
Analytical grade water	up to 1000 mL

Dissolve the compounds in 750 mL of analytical grade water under magnetic stirring. Check the pH ( $9.6 \pm 0.2$ ) and then bring the solution to the final volume; store at room temperature. If needed, clear the solution by filtration.

### 6.4 Washing solution

Tween 20	1 mL
Analytical grade water	up to 2000 mL

The solution should be prepared immediately before use, as follows: add 1 mL of Tween 20 to a 2 L flask; bring the solution to the final volume by adding analytical grade water and mix by magnetic stirring until the solution is clear. If refrigerated, the solution should be used within 24 h.

### 6.5 Blocking solution

BSA	0.25 g
Tween 20	0.05 mL
PBS	up to 50.00 mL

The solution should be prepared immediately before use, as follows: place 0.25 g BSA (bovine serum albumin) directly in a 50 mL tube; add 40 ml of PBS buffer and mix by vortexing until the BSA is completely dissolved. Add 0.05 mL Tween 20; mix by vortexing and bring to volume. If refrigerated, the solution must be used within 24 h.

### 6.6 Sera and conjugate diluent

BSA	1.00g
Tween 20	0.05 mL
PBS	up to 100 mL

The solution should be prepared immediately before use, as follows: place 0.50 g BSA directly in a 50 mL tube; add 40 ml of PBS buffer and mix by vortexing until BSA is completely dissolved. Add 0.025 mL Tween 20; stir by vortexing and bring it to volume. If refrigerated, the solution must be used within 24 h.

### 6.7 Stop solution

- HCl 1N in analytical grade water. Prepare the solution under a chemical hood; store at room temperature.
- 6.8 TMB (3, 3', 5, 5' tetramethylbenzidine) peroxidase substrate  
This substrate is recommended; if not available, any other peroxidase substrate can be used.
- 6.9 96-well flat bottomed microtiter plate
- 6.10 Excretory/secretory antigens (ES Ag) (see OIE Manual,  
[http://www.oie.int/fileadmin/Home/eng/Health\\_standards/tahm/2.01.20\\_TRICHINELLOSIS.pdf](http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.20_TRICHINELLOSIS.pdf))  
The antigens at the appropriate concentration (for example 5µg/mL) should be brought to a final volume of 12 mL with carbonate buffer saline pH 9.6. The dilution should be performed on ice immediately before use.
- 6.11 Peroxidase labelled anti-human IgG goat antibodies  
The conjugate should be used at the optimal dilution calculated by checking board titration versus a standardised positive control serum. The dilution should be prepared on ice immediately before use.
- 6.12 Anti-*Trichinella* sp. seropositive control sera  
100 µL of diluted sera from *Trichinella* sp. infected persons (positive controls). Each positive control serum should be properly diluted (e.g., 1/200), using the appropriate diluter. The dilution should be performed on ice immediately before use.
- 6.13 Anti-*Trichinella* sp. negative control sera  
100 µL of diluted sera from *Trichinella* sp. free persons (negative controls). Each negative control serum should be properly diluted (f e.g., 1/200), using the appropriate diluter. The dilution should be performed on ice immediately before use.
- 6.14 Sera to be tested  
Each serum should be tested at the same dilution that control sera, using the appropriate diluent. The dilution should be performed on ice immediately before use.

## 7. Procedure

### 7.1 Preparing test and control samples

- 7.1.1 Thaw the test sera and the positive and negative control sera by storing them at +1-8°C for at least 5 h.
- 7.1.2 Once thawed, keep them in an ice bath and stir them by vortexing before use.
- 7.1.3 Dilute 1:200 the test and control sera as follows: in a 1-2 mL conical bottom tube, add 5 µL of serum and 990 µL diluting solution. Diluted sera can be stored refrigerated for up to 24 h.

### 7.2. Analytical procedure.

- 7.2.1 Fill the microtiter plate with 100 µL per well of ES Ag in carbonate buffered saline; incubate for 1h at 37°C.
- 7.2.3 Wash 3 times in the automatic plate washer with the washing solution.
- 7.2.4 Add 200 µL blocking solution per well; incubate for 1 h at 37° C.
- 7.2.5 Wash 3 times in the automatic plate washer with the washing solution.
- 7.2.6 Add 100 µL of each diluted sample per well and incubate for 30 min at 37°C.
- 7.2.7 Each serum dilution should be performed in duplicate.
- 7.2.8 Sera should be diluted (e.g., 1/200).
- 7.2.9 Wash 3 times in the automatic plate washer with the washing solution.
- 7.2.10 Add 100 µL of the diluted anti-human IgG peroxidase labelled antibodies per well and incubate for 1 h at 37°C.
- 7.2.11 Wash 3 times in the automatic plate washer with the washing solution.

- 7.2.12 Add 100  $\mu$ L TMB substrate per well; incubate for 10 min at room temperature.
- 7.2.13 Stop the reaction by adding 50  $\mu$ L of the stop solution per well and read the reaction in the ELISA plate microtiter reader at 450 nm.

## 8. Interpretation of the results

8.1 The test results can be considered as valid if all of the following criteria are fulfilled:

- 8.1.1 The OD value of the negative control sera should be lower than the *cut off* value determined during the validation process of the method
- 8.1.2 The OD value of the positive control sera has to be higher than the *cut off* value determined during the validation process of the method;
- 8.1.3 The difference in OD between the 2 measures made on the same positive control sample in strict conditions of repeatability has to be  $\leq 0.15$  unit absorbance, and on the same negative control sample it has to be  $\leq 0.05$  unit absorbance.

If even only one of the above-reported criteria is not met, the test has to be considered as non-valid and the sera should be tested again.

8.2 Calculate the mean of the 2 duplicates for each positive sera (PS) and for each test sera (TS).

8.3 Subtract from each mean value the mean OD value of the blanks ( $OD_b$ ).

8.4 Select the higher OD value among the positive control sera ( $PS_{max}$ ), and for each sample calculate the extinction value ( $I_e$ ) according to the following formula:

$$I_e (\%) = \frac{OD \text{ mean duplicates TS} - OD_b}{OD \text{ mean duplicates highest PS} - OD_b} \times 100\%$$

where:  $I_e \geq 11.8\%$ , *Trichinella* positive serum  
 $I_e < 11.8\%$ , *Trichinella* negative serum

## 9. Safety measures

This method should be carried out only by authorized personnel. The operator should wear personal protection equipment (PPE) during the test performance. For the general safety measures, refer to the guidelines of CDC.