# 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 (ELISA) in human sera.

The method is 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 antigens (E/S Ag) partially purified in conditions able of maintaining the antigens' native form.

Control and test serum samples, 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 antigens are removed 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 removed 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

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. 5:1723-9

OIE/World Organisation for Animal Health, 2018. Trichinellosis (infection with *Trichinella* spp.), Chapter 3.1.20. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. World Organization for Animal Health www.oie.int/eng/normes/mmanual/A_00013.htm.

Bruschi F, Gómez-Morales MA, Hill D. 2019. International Commission on Trichinellosis: Recommendations on the use of serological tests for the detection of *Trichinella* infection in animals and humans. Food and Waterborne Parasitology, https://doi.org/10.1016/j.fawpar.2018.e00032.DM March 3, 2005 (Italian Official Journal. April 13, 2005 n. 85).

## 4. Acronyms

| | |
|---|---|
| ELISA | Enzyme Linked Immunosorbent Assay |
| Ag | Antigen |
| Ab | Antibodies |
| E/S Ag | Excretory/Secretory antigens |
| BSA | Bovine Serum Albumin |

## 5. Devices/instruments

The following instruments are needed to perform the ELISA procedure.

Adjustable pipettes (volumes: 0.5-10 µL, 15-300 µL, 5-1000 µL)

Technical scale; 0.01g resolution

Automatic plate washer (strongly recommended)

Microtiter plate spectophotometer, 450 nm

Freezer, ≤ -15°C

Freezer, ≤ - 50°C

Refrigerator, 1÷8°C

Incubator 37±1°C

Magnetic stirrer

Adjustable volume dispenser, alternatively pipettes could be used

pH meter

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±0.2

| | |
|---|---|
| $KH_2PO_4$ | 0.34 g |
| $Na_2HPO_4$ | 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±0.2) and bring the solution to the final volume. Store at 1÷8°C. Stability 6 months.

6.3 Carbonate buffered saline, pH 9.6±0.2

| | |
|---|---|
| $Na_2CO_3$ | 1.12g |
| $NaHCO_3$ | 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±0.2) and bring the solution to the final volume. Store at room temperature. Stability 6 months.

6.4 Washing solution

| | |
|---|---|
| Tween 20 | 1 mL |
| Analytical grade water | up to 2000 mL |

Prepare the solution immediately before use, as follows: add 1 mL of Tween 20 into 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 (1÷8°C), stability 24 h.

6.5 Blocking solution

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

Prepare the solution 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 (1÷8°C), stability 24 h.

6.6 Sera and conjugate diluter solution

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

Prepare the solution 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 (1÷8°C), stability 24 h.

6.7 Stop solution

HCl 1N in analytical grade water. Prepare the solution under a chemical hood; store at room temperature. Stability 6 months.

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 (E/S Ag) (see ANNEX 1)

Reconstitute the antigens using analytical grade water to 25-50 µg/vial. Reconstituted E/S Ag, frozen at <-50°C is stable for 10 years. Diluted the reconstituted antigens with carbonate buffer saline pH 9.6, to a final concentration of 5 µg/mL just before use.

6.11 Peroxidase labelled anti–human IgG goat antibodies

On opening the packaging, rehydrate the freeze-dried material with analitycal grade water shaking on vortex until its complete dissolution.

The solution can be stored refrigerated (1÷8°C) for a week.

To determine the optimal working dilution of the conjugate, a titration curve is performed by testing working dilutions higher, lower and equal than those recommended by the supplier for use in ELISA. The working dilution of the conjugate is the dilution in which the differences in optical density, O.D., between positive and negative controls are maximum maintaining the minimum background, set by the O.D. value of the blank.

The conjugate, once rehydrate, if distributed in aliquots and stored frozen at <-50°C remains stable for at least 20 years. After the expiration date, its suitability is verified, through the O.D. values detected in the positive and negative controls, in the analytical sessions in which it is used.

To perform the test, dilute the conjugate aliquot to the optimum concentration with the dilution solution referred to in point 6.6. Once diluted, store the conjugate refrigerated (1÷8°C). Stability 24 hours.

6.12 Anti-*Trichinella* sp. positive control sera

Sera positive for antibodies anti-*Trichinella* coming from individuals affected by trichinellosis confirmed by muscular biopsy.

6.13 Anti-*Trichinella* sp. negative control sera

Sera from *Trichinella* sp. free individuals that resulted suitable for blood donation according to the Italian low (negative controls).

## 7. Procedure

*7.1 Test and control samples preparation*

Thaw the test sera and the positive and negative control sera by storing them at 1÷8°C for at least 5 h.

Once thawed, keep them in an ice bath and stir them by vortexing before use.

Dilute 1:200 the test and control sera as follows: in a 1-2 mL conical bottom tube add 5 µL of serum and 995 µL diluting solution. Diluted sera can be stored refrigerated (1÷8°C), stability 24 h.

7.2. *Procedure*

Fill, each well of the microtiter plate, with 100µL of E/S Ag in carbonate buffered saline; incubate for 1h at 37±1°C.

Wash 3 times in the automatic plate washer with the washing solution.

Dispense, in each well, 200 µL blocking solution per well, incubate for 1 h at 37±1°C.

Wash 3 times in the automatic plate washer with the washing solution.

Dispense, in duplicate, 100µL per well of each serum (negative, positive controls and testing samples).

Dispense 100µL of sera and conjugate diluter solution (6.6) in the blank wells.

Incubate per 30 minutes at 37±1°C

Wash 3 times in the automatic plate washer with the washing solution.

Dispense, in each well, 100µL of the diluted anti–human IgG peroxidase labelled antibodies and incubate for 1h at 37±1°C.

Wash 3 times in the automatic plate washer with the washing solution.

Dispense, in each well, 100µL TMB substrate; incubate 10 minutes at room temperature.

Stop the reaction by adding 50µL of the stop solution per well and read the reaction in the microtiter plate spectophotometer at 450nm.

## 8. Interpretation of the results

The test results is valid if all of the following criteria are fulfilled:

The O.D. value of the negative control sera is lower than the *cut off* value determined by the validation method;

The O.D. value of the positive control sera is >1.0 of the unit absorbance;

The difference, in O.D., between the two measures made on the same positive control serum in strict conditions of repeatability is $\leq$ 0.15 unit absorbance; the difference, in O.D., between the two measures made on the same negative control serum is $\leq$ 0.05 unit absorbance in at least six out of eight controls of the analytical session.

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.

To evaluate the obtained results:

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

Subtract from each mean value the mean O.D. value of the blanks ($OD_b$).

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

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

Test result is POSITIVE (presence of anti- *Trichinella* antibodies) if the extinction value $I_e \geq 11.8\%$.

Test result is NEGATIVE (absence of anti- *Trichinella* antibodies) if the extinction value $I_e < 11.8\%$.

## 9. Characteristics of the method

This method was validated in terms of diagnostic sensitivity, specificity and repeatability. The results of the validation procedure confirmed that the method is suitable for the expected aim and field of application.

## 10. Safety measures

This method has to be carried out only by authorized personnel. The operator should wear individual protection devices during the test performance. For the general safety measures, refer to the guidelines of CDC.

ANNEX 1

# Production of excretory/secretory antigens from muscle larvae of *Trichinella spiralis*

## 1 Aim and field of application

To describe the production of excretory/secretory antigens from muscle larvae of *Trichinella spiralis*.

The product can be used as antigens for serology to detect anti-*Trichinella* spp. antibodies.

## 2 References

Gamble HR, Anderson WR, Grahan CE, Murrell KD. 1983. Diagnosis of swine trichinellosis by enzyme-linked immunosorbent assay (ELISA) using an excretory-secretory antigen. Vet. Parasitol. 13, 349-361

Gamble HR, Rapic D, Marinculic A, Murrell KD. 1988. Evaluation of excretory-secretory antigens for the serodiagnosis of swine trichinellosis. Vet. Parasitol. 30, 131-137

OIE/World Organisation for Animal Health, 2018. Trichinellosis (infection with *Trichinella* spp.), Chapter 3.1.20. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. World Organization for Animal Health www.oie.int/eng/normes/mmanual/A_00013.htm.

## 3 Acronyms

**OD**, optical density

**E/S Ag**, Excretory/Secretory antigens

## 4 Devices/Instruments

Incubator 37±2°C with 4-5% CO2

Spectrophotometer UV/VIS

Freezer ≤-15°C

Refrigerator, 1°C ÷ 8°C

Freezer ≤ -50°C

Laminar flow hood

Adjustable micropipettes ( up to 2 µL, 20 µL, 200 µL, 1000 µL)

Pipettes (1, 5, 10, 25 mL)

Inverted microscope

Magnetic stirrer

Refrigerate centrifuge

## 5. Reagents and chemicals

5.1 *Trichinella spiralis* muscle larvae suspension (MLS).

5.2 Phosphate Buffered Saline (PBS), pH 7.3±0.2

| | |
|---|---|
| $KH_2PO_4$ | 0.34 g |
| $Na_2HPO_4$ | 1.21 g |
| NaCl | 8.0 g |
| Analytical grade water | up to 1000 mL |

Dissolve the compounds in 750 mL of analytical grade water under magnetic stirring. Control the pH (it must be 7.3±0.2) and then bring the solution to the final volume. Sterilize by filtration with a 0.22 µm filter. Stored refrigerate (1÷ 8°C). Stability 6 months.

5.3 PBS, pH 7.3±0.2 with antibiotics 5 X

| | |
|---|---|
| PBS | 950 mL |
| Penicillin/Streptomycin or Antibiotic/Antimycotic solution | 50 mL |

Store refrigerated (1÷ 8°C). Stability 2 months.

5.4 RPMI 1640 with antibiotics 5 X

| | |
|---|---|
| RPMI 1640 | 475 mL |
| Penicillin/Streptomycin or Antibiotic/Antimycotic solution | 25 mL |

Store refrigerated (1÷ 8°C) and use within 2 months.

5.5 Complete RPMI medium

| | |
|---|---|
| RPMI 1640 | 480 mL |
| 1M HEPES | 5 mL |
| 200mM Glutamine | 5 mL |
| 100mM Sodium-pyruvate | 5 mL |
| Penicillin/Streptomycin or Antibiotic/Antimycotic | 5 mL |

Store refrigerated (1÷ 8°C) and use within 2 months.

5.6 Penicillin/Streptomycin or Antibiotic/Antimycotic solution (100x).

5.7 Sterile 0.22 μm syringe filters.

5.8 Device for concentrating by ultrafiltration, cut off 5kDa.

5.9 Protein assay kit.

5.10 Dialysis membrane, 3500 MWCO.

5.11 Cocktail of protease inhibitors.

Sterile conical tubes 15, 50 mL.

Tissue culture flasks.

96-well plates.

## 6 Procedure

a) Place the solutions 5.2, 5.3 and 5.4 into the 37°C incubator for at least 1 h.

b) Count MSL (5.1) under the inverted microscope.

c) Dispense $5x10^5$ MSL (5.1) in a sterile 50 ml conical tube, wash three times (20 minutes each wash) by sedimentation with 45 mL of warm sterile PBS/Penicillin/Streptomycin solution (5.3). At each change of the washing solution, gently shake the worms to dislodge adherent bacteria. After larvae sedimentation, remove the washing solution by aspirating with a pipette.

d) Place the tube containing the worms (5.1) under a laminar flow hood, wash five additional times by sedimentation with 45 mL of warm RPMI 1640/Penicillin/Streptomycin solution (5.4)

e) Resuspend the worms (5.1) in warm maintenance media (5.4) at a concentration of 5,000 larvae/mL and place them into a tissue culture flask.

f) Incubate the flasks in 5% $CO_2$ at 37±2°C for 16-18 h.

g) Check MSL (5.1) viability and the absence of bacterial contamination by microscopy.

h) Place the cultures under a laminar flow hood, transfer the culture media in 50 mL conical tubes and let the MSL sediment.

i) Aspirate the supernatant (E/S Ag) and filter the media through a 0.22 μm filter (5.7). Discard the MSL.

j) Keep the obtained E/S Ag refrigerated (1÷ 8°C) until concentration, if stored more than 24 hours, the E/S Ag should be frozen. Proceed with the concentration step only if the E/S Ag volume is at least 150mL.

k) Fill in the concentration tube (5.8) with 15 mL of E/S Ag (point i).

l) Concentrate by centrifuging 30 minutes at 3,000 g in a refrigerated centrifuge.

m) Retrieve the concentrated E/S Ag in a 50 mL tube and store refrigerated (1÷8°C) until point “p”.

n) Repeat point “k” to “m” until the E/S Ag has been properly concentrated.

o) Centrifuge until the volume in the concentration chamber of the concentration tube is almost 10 mL.

p) Determine the protein concentration by the protein assay kit (5.9) of both the E/S Ag and the eluate.

q) Check the O.D. by the spectrophotometer at 280nm/260nm. The ratio should be $\geq$ 1.

r) If the O.D. ratio 280nm/260nm is < 1, the E/S Ag is discharged because contaminated by DNA.

s) Dialyze (5.10) the concentrated E/S Ag versus PBS at +1÷ 8°C for at least 4 hours.

t) Add, to the E/S Ag, 1µL/mg of the cocktail of protease inhibitors (5.11).

u) Aliquot the antigen in 1 mL vials at a total protein concentration of 0.5 mg/vial and store at ≤-15°C. Stability 10 years.

v) The antigen can be lyophilized and stored refrigerated (1÷ 8°C). Stability 20 years if preserved dry.

w) When necessary, rehydrate the lyophilized E/S Ag using analytical grade water. Rehydrated E/S Ag has to be aliquoted and frozen. Stability 10 years.

## 7. Quality Control

The batch is considered suitable for serology test if all the following criteria are fulfilled:

Lack of microbial and fungal contamination in the culture flasks at the end of the incubation period, verified by direct microscopic observation at 400x magnifications, as specified in point 6 ‘g’;

MSL viability, controlled as described in point 6 ‘g’;

O.D. ratio of 280nm/260nm of the final antigen solution $\geq$ 1, as specified in point 6 ‘q.’

## 8. Safety

This method has to be carried out only by authorized personnel. The operator should wear individual protection devices during the test performance. For the general safety measures, refer to the guidelines of CDC.