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

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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 can be used for the serological diagnosis of human trichinellosis.

1. Principle of the method

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

2. 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).

3. Acronyms

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

4. 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)

Scale 0.001÷310g ; 0.001/0.01g resolution

Automatic plate washer (strongly recommended)

ELISA plate microtiter reader, 450 nm

Freezer ≤ -15°C

Freezer ≤ - 50°C

Ice maker

Incubator 37 ± 1°C

Magnetic stirrer

Adjustable volume dispenser (e.g. Multipette Eppendorf®) alternatively pipettes could be used

pH meter
Refrigerator 1÷8°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±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 then bring the solution to the final volume; refrigerate. The solution must be used within 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 then bring the solution to the final volume; store at room temperature. If needed, clear the solution by filtration. The solution must be used within 6 months.

6.4 Washing solution

Tween 20	1 mL
Analytical grade water	up to 2000 mL

The solution shall be prepared 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, 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 shall 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 diluter

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

The solution shall 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. The solution must be used within 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 (ES Ag) (see ANNEX 1)

The antigens shall be reconstituted using analytical grade water to 25-50 µg/vial. Reconstituted E/S Ag, frozen at <-50°C is stable for 5 years. The reconstituted antigens shall be diluted, 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

Before use, 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, the working dilutions recommended by the supplier for use in ELISA should be tested. If none of the above dilutions is optimal, further dilutions of the reagent are prepared until identify the optimal one (i.e. the dilution of the conjugate in which the differences in optical density, O.D., between positive and negative controls are maximum, maintaining the minimum background, as can be seen from the O.D. value of the blank). The conjugate must be distributed in aliquots and stored frozen at <-50°C. Under these conditions it remains stable for at least 5 years. After the expiration date its suitability will be verified, through the O.D. values detected in the positive and negative controls, in the analytical sessions in which it is used. Before execution of the test, a conjugate aliquot shall be diluted to the optimum concentration with the dilution solution referred to in point 6.6. Once diluted, store the conjugate refrigerated (1÷8°C) and use it within 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).

6.14 Sera to be tested

Each serum shall be tested at a 1/200 dilution in duplicate, using the appropriate diluter. The dilution shall be performed on ice immediately before use.

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 for up to 24 h.

7.2. *Procedure*

Fill the microtiter plate with 100µL per well of ES Ag in carbonate buffered saline; incubate for 1h at 37°C.

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

Add 200 µL blocking solution per well; incubate for 1 h at 37°C.

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

Diluted each serum 1/200

Add 100µL of each diluted sample per well and incubate for 30 min at 37°C.

Perform in duplicate each serum dilution.

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

Add 100µL of the diluted anti–human IgG peroxidase labelled antibodies per well and incubate for 1h at 37°C.

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

Add 100µL TMB substrate per well; incubate for 10 min at room temperature.

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

8. Interpretation of the results

The test results can be considered as 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 (i.e., 0.230);

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.

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:

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

$I_e \geq 11.8\%$, *Trichinella* **positive** serum

$I_e < 11.8\%$, *Trichinella* **negative** serum

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

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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

96 wells plates

5. Reagents and chemicals

5.1 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. If stored refrigerated, the solution must be used within 6 months.

5.2 PBS, pH 7.3±0.2 with antibiotics 5 X

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

Store refrigerated and use within 2 months.

5.3 RPMI 1640 with antibiotics 5 X

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

Store refrigerated and use within 2 months.

5.4 Complete RPMI medium

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

Store refrigerated and use within 2 months.

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

5.6 RPMI 1640 culture medium.

5.7 HEPES-1M Buffer solution.

5.8 L-Glutamine 0.2M solution.

5.9 Sterile 0.22 μm syringe filters.

5.10 Device for concentrating by ultrafiltration with cellulose membrane filter, 5000 MWCO cut off (e.g. Amicon Ultra 15).

5.11 Coomassie Protein Assay Reagent (e.g. Pierce ®, 1856209).

5.12 Slide-A-Lyzer Dialysis Cassettes, 3.5K MWCO 3-12 ml Capacity (e.g. Pierce ®, 66110).

5.13 Cocktail of protease inhibitors.

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

5.15 Sterile conical tubes 15, 50 mL.

5.16 Tissue culture flasks T-75.

6 Procedure

a) Place the solutions 5.1, 5.2 and 5.3 into the 37°C incubator for at least 1 h.

b) Count MSL (5.14) under the inverted microscope.

c) Wash $5x10^5$ MSL (5.14) three times (20 min each wash) by sedimentation in a sterile 50 ml conical tube with 45 mL of warm sterile PBS/Penicillin/Streptomycin solution (5.2). 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 (5.14) under a laminar flow hood and wash them 5 additional times by sedimentation in a sterile 50 mL conical tube with 45 mL of warm RPMI 1640/Penicillin/Streptomycin solution (5.2).

e) Resuspend the worms (5.14) in warm maintenance media (5.4) at a concentration of 5,000 larvae/mL and place them into T-75 tissue culture flasks.

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

g) Check MSL (5.14) viability and the absence of bacterial contamination by microscopy.

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

i) Filter the media (E/S Ag) through a 0.22 μm filter (5.9). Discard the MSL.

j) Keep the E/S Ag at +4°C until concentration, if stored more than 24 hours, the E/S Ag should be frozen at -15°C.

k) Fill in the concentration tube (5.10) with 15 mL of E/S Ag (point i).

l) Centrifuge 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 until point “p”.

n) Repeat point “k” to “m” until the E/S Ag has been properly concentrated.
o) If the E/S Ag has been concentrated 100 times or more, proceed to point “p”, otherwise centrifuge the tube again.
p) Determine the protein concentration by the Bradford method (5.11).
q) Check the O.D. by the spectrophotometer at 280nm/260nm. The ratio should be $\geq$ 1.
r) Dialyze (5.12) the concentrated E/S Ag versus PBS at +4°C for at least 4 hours.
s) Add to the E/S Ag 1µL/mg of the cocktail of protease inhibitors (5.13).
t) Aliquot the antigen in 1 mL vials at a total protein concentration of 0.5 mg/vial and store at ≤-15°C.
u) The antigen can be lyophilized and stored refrigerated for 5 years.

7. Quality Control

The batch is considered suitable for serology 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.