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

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1. Aim and field of application

To determine the presence of anti-*Trichinella* antibodies by an enzyme linked immunosorbent assay (ELISA) in swine sera.

The method is used to screen pig populations during seroepidemiological surveys on the exposure of swine to *Trichinella*.

2. Principle of the method

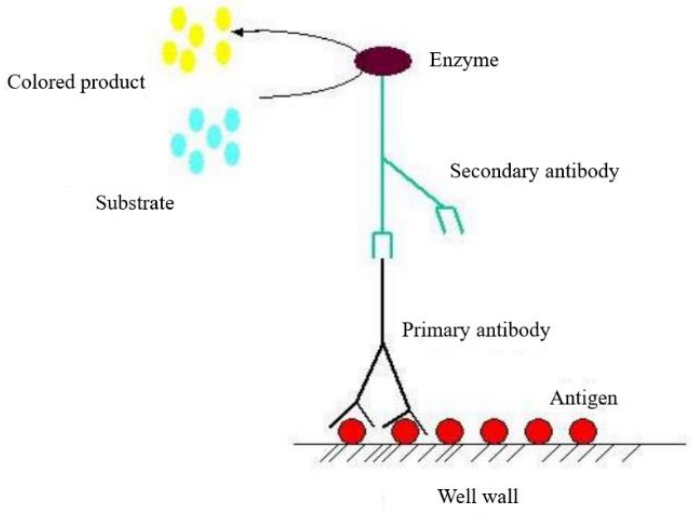
A 96-well microtiter polystyrene plate is coated with *Trichinella spiralis* excretory/secretory antigens (E/S Ag) partially purified in conditions able of maintaining the antigens' native form.

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 antigen are removed by washing; peroxydase conjugated anti-swine IgG secondary antibody is then added to each well. This second incubation allows the conjugate to bind to the swine 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 swine 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 by comparing the color intensity of the wells containing the test sera with those containing the controls.



3. References

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

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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:

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

Analytical balance

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

Water filtration system for analytical-grade water; if not available, use commercially available analytical-grade water.

6. Reagents and chemicals

6.1 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

Completely dissolve compounds in 750 mL of 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.

6.2 Sera and conjugate diluter

BSA	0.50 g
Tween 20	0.025 mL
PBS (6.1)	up to 50 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 complete solubilization of BSA. Add 0.025 mL Tween 20 (visually check for the presence of any air bubbles inside the pipette); mix by vortexing and bring it to volume. If refrigerated (1÷8°C), stability 24 h.

6.3 Washing solution

Tween 20	1 mL
Analytical grade water	up to 2000 mL

Prepare the solution immediately before use, as follows: add 1.999 L of water and 1 mL of Tween 20 to a 2 L flask (visually check for the presence of any air bubbles inside the pipette). Mix by magnetic stirring until the solution is clear. If refrigerated (1÷8°C), stability 24 h.

6.4 Blocking solution

BSA	0.25 g
Tween 20	0.025 mL

PBS (6.1)	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 complete solubilization of BSA. Add 0.025 mL Tween 20 (visually check for the presence of any air bubbles inside the pipette); mix by vortexing and bring to volume. If refrigerated (1÷8°C), stability 24 h.

6.5 Stop solution

HCl (36,46 g/Mol; d: 1,19)	8.3 mL
Analytical grade water	up to 100 mL

Prepare the solution under a chemical hood, store at room temperature. Stability 6 months.

6.6 Excretory/secretory antigens (E/S Ag) (see ANNEX 1)

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

Prepare Carbonate buffered saline, pH 9.6±0.2 as following

Na_2CO_3	1.21 g
$NaHCO_3$	2.92 g
Analytical grade water	up to 1000 mL

Completely 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.

6.7 Anti-*Trichinella* sp. positive control swine sera

Serum coming from pig experimentally infected with *Trichinella* sp. larvae (positive control). Positive sera are considered as reference material of the European Union Reference Laboratory for Parasites (EURL-P).

6.8 Anti-*Trichinella* sp. negative control swine sera

Serum coming from domestic pig tested negative for the presence of *Trichinella* sp. by chloride-peptic digestion.

6.9 TMB (3, 3', 5, 5' tetramethylbenzidine) peroxidase substrate
This substrate is recommended; if not available, any other peroxidase substrate can be used.

6.10 96-well flat-bottomed microtiter plate

6.11 Peroxidase labelled anti–swine IgG secondary antibodies

The reagent may be supplied either in liquid formulation or lyophilized and can be stored frozen (temperature < -50°C) until the package is opened.

Upon opening the package, the lyophilized reagent material must be rehydrated with analytical-grade water, vortexing until its complete solubilization.

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, in liquid formulation or once resuspended and aliquoted, if stored frozen (temperature < -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 6.2. Once diluted, store the conjugate refrigerated (1÷8°C). Stability 24 hours.

7. Procedure

7.1 Test and control samples preparation

Thaw the test samples 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. Before use, mix them by vortexing

Dilute 1:50 the test and control sera as follows: in a 1-2 mL conical bottom tube add 10 µL of serum and 490 µL diluting solution (6.2). Diluted sera can be stored refrigerated (1÷8°C), stability 24 h.

7.2. *Procedure*

Take the required microplates from the refrigerator (temperature 1–8°C).

Fill, each well of the microtiter plate, with 100µL of E/S Ag (6.6) in carbonate buffered saline; incubate for 1h at 37±1°C.

Wash 3 times in the automatic plate washer with the washing solution (6.3).

Dispense, in each well, 200 µL blocking solution (6.4) per well; incubate for 1 h at 37° C.

Wash 3 times in the automatic plate washer with the washing solution (6.3).

Dispense, in duplicate, 100 µL of each of the diluted positive controls into wells PS1, PS2, PS3, and PS4, the diluted negative controls into wells NS1, NS2, NS3, and NS4, the diluted samples into wells SSxx, and 100 µL of the dilution solution (6.5) into wells BLANK 1 and BLANK 2.

Incubate per 30 minutes at 37±1°C

Microtiter plate scheme

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
A	PS1	PS1	SS1	SS5	SS9	SS13	SS17	SS21	SS25	SS29	SS33	SS37
B	PS2	PS2	SS1	SS5	SS9	SS13	SS17	SS21	SS25	SS29	SS33	SS37
C	PS3	PS3	SS2	SS6	SS10	SS14	SS18	SS22	SS26	SS30	SS34	SS38
D	PS4	PS4	SS2	SS6	SS10	SS14	SS18	SS22	SS26	SS30	SS34	SS38
E	NS1	NS1	SS3	SS7	SS11	SS15	SS19	SS23	SS27	SS31	SS35	SS39
F	NS2	NS2	SS3	SS7	SS11	SS15	SS19	SS23	SS27	SS31	SS35	SS39
G	NS3	NS3	SS4	SS8	SS12	SS16	SS20	SS24	SS28	SS32	SS36	BIANCO
H	NS4	NS4	SS4	SS8	SS12	SS16	SS20	SS24	SS28	SS32	SS36	BIANCO

Legend: PS1–PS4: positive control sera; NS1–NS4: negative control sera; SS1–SS39: test samples dispensed in duplicate; BLANK: serum dilution solution.

Wash 3 times in the automatic plate washer with the washing solution (6.2).

Add 100µL to each well of the diluted anti-swine IgG peroxidase labelled antibodies (6.11) and incubate for 1h at 37±1°C.

Wash 3 times in the automatic plate washer with the washing solution (6.2).

Add 100µL TMB substrate (6.9) to each well; incubate for 10 min at room temperature.

Stop the reaction by adding 50µL of the stop solution (6.5) to each well and read the reaction in the microtiter plate spectophotometer at 450nm.

8. Interpretation of the results

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

- The O.D. value of the negative control sera is lower than the cut off value, 0.338 O.D., determined by the validation method.
- The OD value of the positive control sera is more than 0.7 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 less or equal to 0.15 unit of absorbance; the difference, in O.D., between the two measures made on the same negative control serum is less or equal to 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 is 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 serum (TS).

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

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:

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

Test result is POSITIVE (presence of anti- *Trichinella* antibodies) if the extinction value $I_e \geq 18\%$.

Test result is NEGATIVE (absence of anti- *Trichinella* antibodies) if the extinction value $I_e < 18\%$.

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

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3 Acronyms

OD, optical density

E/S Ag, Excretory/Secretory antigens

4 Equipments

4.1 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 (0.5-10 µL, 15-300 µL, 5-1000 µL)

Pipettes (1, 5, 10, 25 mL)

Inverted microscope

Magnetic stirrer

Refrigerate centrifuge

96 wells plates

4.2 Reagents and chemicals

4.2.1 Muscle *Trichinella spiralis* larvae (MSL)

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

4.2.3 PBS, pH 7.3±0.2 with antibiotics 5 X

PBS	950 mL

	Penicillin/Streptomycin or Antibiotic/Antimycotic solution	50 mL

Sterilize by filtration with a 0.22 µm filter. Store refrigerated and use within 2 months.

4.2.4 RPMI 1640 with antibiotics 5 X

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

Sterilize by filtration with a 0.22 µm filter. Store refrigerated and use within 2 months.

4.2.5 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

Sterilize by filtration with a 0.22 µm filter. Store refrigerated and use within 2 months.

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

4.2.7 Sterile 0.22 µm syringe filters

4.2.8 Ultrafiltration concentration tubes, 5 kDa cut-off

4.2.9 Protein Assay Reagent

4.2.10 Slide-A-Lyzer Dialysis Cassettes, 3.5K MWCO

4.2.11 Cocktail of protease inhibitors

Tissue culture flasks T-75

Sterile conical tubes 15, 50 mL

96 wells plates.

5 Procedure

a) Place the solutions 4.2.3, 4.2.4 and 4.2.5 into the 37°C incubator for at least 1 h.

b) Count MSL under the inverted microscope.

c) Wash $5x10^5$ MSL 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. 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 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.

e) Resuspend the worms in warm maintenance media 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 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) Aspirate the supernatant (E/S Ag) and filter the media (E/S Ag) through a 0.22 µm filter. 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 kDa cut-off, 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) Centrifuge until the volume in the concentration chamber of the concentration tube is almost 10 mL.

p) Determine the protein concentration.

q) Check the O.D. by the spectrophotometer at 280nm/260nm. The ratio should be ≥ 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 (5.13).

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.

6 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 5 ‘g’;

MSL viability, controlled as described in point 5 ‘g’;

O.D. ratio of 280nm/260nm of the final antigen solution ≥ 1, as specified in point 5 ‘q.’

7 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.