Detection of anti-\textit{Trichinella} antibodies in human serum by indirect ELISA

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ANNEX 1 Production of excretory/secretory antigens from muscle larvae of \textit{Trichinella spiralis}
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 partially purified in conditions capable of maintaining the antigens’ native form.
   
   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**
   
   
   

4. **Definitions**
   
<table>
<thead>
<tr>
<th>L</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
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<tr>
<td>Ag</td>
<td>Antigen</td>
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<tr>
<td>Ab</td>
<td>Antibodies</td>
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<td>Ag E/S</td>
<td>Excretory/Secretory antigens</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>H</td>
<td>Hours</td>
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<tr>
<td>Min</td>
<td>Minutes</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
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</tbody>
</table>

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., Multipette Eppendorf®)
pH meter
Pipette aid
Refrigerator +4°C ± 2°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

\[
\begin{align*}
\text{KH}_2\text{PO}_4 & : 0.34 \text{ g} \\
\text{Na}_2\text{HPO}_4 & : 1.21 \text{ g} \\
\text{NaCl} & : 8.0 \text{ g} \\
\text{Analytical grade water} & : \text{ up to 1000 mL}
\end{align*}
\]

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.

6.3 Carbonate buffered saline, pH 9.6 ± 0.2

\[
\begin{align*}
\text{Na}_2\text{CO}_3 & : 1.12 \text{ g} \\
\text{NaHCO}_3 & : 2.92 \text{ g} \\
\text{Analytical grade water} & : \text{ up to 1000 mL}
\end{align*}
\]

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.

6.4 Washing solution

\[
\begin{align*}
\text{Tween 20} & : 1 \text{ mL} \\
\text{Analytical grade water} & : \text{ up to 2000 mL}
\end{align*}
\]

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

\[
\begin{align*}
\text{BSA} & : 0.25 \text{ g} \\
\text{Tween 20} & : 0.05 \text{ mL} \\
\text{PBS} & : \text{ up to 50.00 mL}
\end{align*}
\]

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 diluter

\[
\begin{align*}
\text{BSA} & : 1.00 \text{ g} \\
\text{Tween 20} & : 0.05 \text{ mL}
\end{align*}
\]
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 ANNEX 1)
The antigens should be reconstituted using 60 µL of analytical grade water. The reconstituted antigens should then be brought to a final volume of 12mL with carbonate buffer saline pH 9.6. The reconstitution and the subsequent dilution steps 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. The dilution should be prepared on ice immediately before use.

6.12 Anti-Trichinella sp. seropositive control sera
100 µL of sera from Trichinella sp. infected persons (positive controls). Each positive control serum should be used at a 1/200 dilution in duplicate, 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 sera from Trichinella sp. free persons that resulted suitable for blood donation according to the Italian low (negative controls). Each negative control serum should be used at a 1/200 dilution in duplicate, 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 a 1/200 dilution in duplicate, using the appropriate diluter. 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 100µL per well of ES Ag in carbonate buffered saline; incubate for 1 h 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 100µL 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 1/200.
7.2.9 Wash 3 times in the automatic plate washer with the washing solution.
7.2.10 Add 100μL of the diluted anti-human IgG peroxidase labelled antibodies per well and incubate for 1h at 37°C.
7.2.11 Wash 3 times in the automatic plate washer with the washing solution.
7.2.12 Add 100μL TMB substrate per well; incubate for 10 min at room temperature.
7.2.13 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

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 by the validation method (i.e., 0.230);
8.1.2 The OD value of the positive control sera has to be >1.0 of the unit absorbance;
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 < 0.15 unit absorbance, and on the same negative control sample it has to be < 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:

\[I_e \geq 11.8\%, \ \text{Trichinella positive serum}\]
\[I_e < 11.8\%, \ \text{Trichinella negative serum}\]

9. Characteristics of the method

This method was studied 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 personal. 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


3 Definitions

OD, optical density

4 Devices/Instruments

Incubator 36±38°C with 4-5% CO2
Spectrophotometer, MIPI 11.50
Freezer ≤-15°C
Refrigerator, 1°C ÷ 8°C
Freezer < -50°C
Amicon® pressure concentrating chamber
Adjustable micropipettes (up to 2 μL, 20 μL, 200 μL, 1000 μL)
Pipettes (1, 5, 10, 25 mL)
Inverted microscope
Magnetic stirrer

5. Reagents and chemicals

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

\[ \text{KH}_2\text{PO}_4 \] 0.34 g
\[ \text{Na}_2\text{HPO}_4 \] 1.21 g
\[ \text{NaCl} \] 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 7.2 ± 0.2) and then bring the solution to the final volume. Sterilize by filtration with a 0.22 μm filter. If stored refrigerated, it remains stable for 1 month.

5.2 PBS, pH 7.3 ± 0.2 with antibiotics (Penicillin and Streptomycin) 5 X
PBS 900 mL
Penicillin/Streptomycin solution 100 mL

Store refrigerated and use within 1 week.

5.3 RPMI 1640 with antibiotics (Penicillin and Streptomycin) 5 X
RPMI 1640 450 mL
Penicillin/Streptomycin solution 50 mL

Store refrigerated and use within 1 week.

5.4 Complete medium
RPMI 1640 285 mL
1M HEPES 1 mL
200mM Glutamine 1 mL
100mM Na-pyruvate 1 mL
Penicillin/Streptomycin 10 mL

Store refrigerated and use within 10 days.

5.5 Penicillin (5000 mg/mL)-Streptomycin (5000 units/mL) solution, Euroclone®, ECB 3055D
5.6 RPMI 1640 culture medium, Euroclone®, ECB 9006L
5.7 HEPES-1M Buffer solution, Euroclone®, EUMO 180D
5.8 L-Glutamine 0.2M solution,
5.9 Sterile 0.22 μm syringe filters
5.10 YM-3 filters Amicon ®
5.11 Coomassie Protein Assay Reagent®, Pierce®, 1856209
5.12 Slide-A-Lyzer Dialysis Cassettes, 3.5K MWCO, 3 - 12 ml Capacity Pierce®, 66110
5.13 Cocktail of protease inhibitors (Sigma P8465, Saint Louis, Mo USA)
5.14 *Trichinella spiralis* muscle larvae suspension (MLS)
5.15 Sterile conical tubes 15, 50 mL
5.16 Tissue culture flasks 75 mL

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 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 (5.2). At each change of the washing solution, gently shake the worms to dislodge adherent bacteria. After 20 min of sedimentation, remove the washing solution by aspirating with a vacuum pump.
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 (5.2).
e) Resuspend the worms in warm maintenance media (5.4) at a concentration of 5,000 larvae/mL and place them into 25 mL tissue culture flasks.
f) Incubate the flasks in 5% CO₂ at 37°C for 16-18 h.
g) Check MSL viability and bacterial contamination by microscopy.
h) Place the cultures under a laminar flow hood and remove worms from the media by sedimentation in 50 mL conical tubes.
i) Filter the media through a 0.2 μm filter. Discard the MSL.
j) Concentrate the filtered media 100 X in the Amicon® pressure concentrating chamber using a YM-3 filter at +4°C. The obtained solution may be clarified by washing with PBS (5.1) in the Amicon® chamber or by dialysing for at least 4 h in PBS. Keep the excretory/secretory antigen solution (E/S Ag) on ice until freezing.

k) Check the optical density by the spectrophotometer at 280nm/260nm ratio. The ratio should be ≥ 1.

l) Determine the protein concentration by the Bradford method (5.11).

m) Add to the E/S Ag 1µL/mg of the cocktail of protease inhibitors (5.13).

n) Aliquot the antigen in 1 mL vials at a total protein concentration of 0.1÷2.5 mg and store at ≤ -15°C.

o) 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 5 ‘g’;
   - MSL viability, controlled as described in point 5 ‘g’;
   - Optical density ratio of 280nm/260nm of the final antigen solution ≥ 1, as specified in point 5 ‘k’.

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