Identification of *Trichinella* spp. proteins recognized by specific IgG in serum of infected pigs by western blotting

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1 AIM AND FIELD OF APPLICATION

This document defines an immune-enzymatic method, western blotting, for the qualitative detection of anti-*Trichinella* spp. IgG in swine sera.

The method can be used as confirmatory test to screen pig populations on the exposure of swine to *Trichinella* spp. during sero-epidemiological surveys.

2 PRINCIPLE OF THE METHOD

Partially purified *Trichinella spiralis* excretory/secretory antigens are separated by SDS–PAGE electrophoresis. After running, proteins are transferred to a nitrocellulose membrane, which is then cut into 20 strips. Each strip is blocked with skimmed milk to saturate the sites free from antigens and singularly located in plastic trays.

Then strips are incubated with diluted control and test pig serum samples, allowing anti-*Trichinella* IgG that may be present in sera, to bind to the antigens on the nitrocellulose membranes.

The antibodies not binding to the antigens are eliminated by washing; goat anti-swine IgG conjugated with horseradish peroxidase (HRP) is then added to each strip. This second incubation allows the conjugate to bind to the swine antibodies that were bound to the antigens onto the strip surface.
The excess conjugate is eliminated by washing, and the activity of the enzyme HRP bound to the swine antibodies, is measured by adding a chromogen substrate. This allows to display a pattern of stained bands corresponding to those proteins that reacted specifically with anti-IgG antibodies to *Trichinella* spp., present in the pig serum (figure 1).

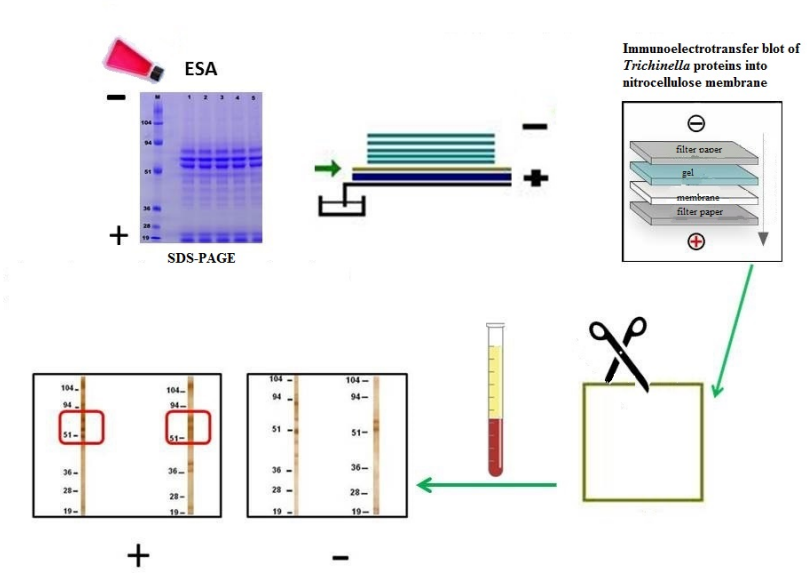

Figure 1. Schematic representation of the method

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4 ACRONYMS

Ag	Antigen
Ab	Antibody
Ag E/S	Excretory/secretory antigens
BSA	Bovine serum albumin
Wb	Western blotting

5 DEVICES/INSTRUMENTS

Power supplies
Thermomixer
pH meter
Analytical or technical balance
Refrigerator, 1÷8°C
Freezer, ≤- 50°C
Freezer, ≤-15°C
Magnetic stirrer
Vortex mixer
Micropipettes (0.5-10µL, 5-100µL, 15-300µL, 50-1000µL)
Water analytical grade
Tray X Cell SureLock Mini-cell for vertical electrophoresis
Mini Trans-Blot Module
Scalpel
Pipette aid
Serological pipettes

6 REAGENTS AND CHEMICALS

Electrophoresis

6.1 Preparation of the test and control sample for electrophoretic run

Ag E/S	100 µg
NuPAGE LDS Sample Buffer (4X)	50 µL
NuPAGE Reducing Agent(10X)	20 µL
Analytical Grade water	up to 200 µL

Stir using a magnetic stirrer until completely dissolved.

6.2 Gel for electrophoresis:

NuPAGE Novex 10% Bis-Tris Mini Gels 1.0mm X2D well Invitrogen

6.3 Prestained molecular weight markers:

Prestained SDS-PAGE Standards Low range Biorad	10 µL

6.4 Antioxidant: NuPAGE 500 µL

6.5 Running Buffer:

Mops SDS Running Buffer (20X)	50mL
Analytical grade water	up to 1000mL

Use the solution within 1 month.

6.6 Transfer Buffer:

Tris-Glycine (25X)	40mL
Metanol	200mL
Analytical grade water	up to 1000mL

Use the solution within 1 month

6.7 Nitrocellulose membrane 0.2 µm

6.8 Ponceau S Solution 20 mL

6.9 Mini-Incubation Trays

<u>Western blotting</u>

6.10 TBS Buffer

Tris 0.2 M	2.4 g
NaCl 3M	17.5 g
Analytical grade water	up to 2000mL

Use the solution within 6 months

Dissolve the compounds in about 1000 mL of analytical grade water under magnetic stirring. Check the pH (7.8±0.2) and then bring the solution to the final volume. Store at 1÷ 8°C.

6.11 TTBS Buffer

Tween 20	1 mL
Tris 0.2 M	2.4 g
NaCl 3M	17.5 g
Analytical grade water	up to 2000mL

Use the solution within 6 months

Dissolve the compounds in about 1000 mL of analytical grade water under magnetic stirring. Check the pH (7.8±0.2) and then bring the solution to the final volume. Store at 1÷ 8°C.

6.12 Sera and conjugate diluter

Skimmed milk powder	1.5 g
TTBS Buffer (pH 7.4)	up to 50 mL

Prepare the solution immediately before use, as follows: place 1.5g skimmed milk directly in a 50mL tube; add 40mL of TTBS buffer and mix by vortexing until milk is completely dissolved and bring it to volume. If refrigerated, use the solution within 24 h.

6.13 Blocking solution

Skimmed milk powder	2.5 g
TTBS Buffer	50 mL

Prepare the solution immediately before use, as follows: place 2.5g skimmed milk directly in a 50mL tube; add 40mL of TTBS buffer and mix by vortexing until the milk is completely dissolved and bring to volume. If refrigerated, use the solution within 24 h.

6.14 Serum samples from *Trichinella* free pigs (negative controls)

6.15 Anti-*Trichinella* spp. IgG positive control pig serum samples (positive controls).

Sera collected at day 60 p.i. from pigs experimentally infected with *Trichinella* spp.

6.16 Conjugate

Anti–swine IgG goat antibodies peroxidase labeled (horseradish peroxidase).

If frozen, store at temperature ≤-50°C until titration (determination of the optimal working dilution).
If the reagent is freeze-dried, it shall be rehydrated before using, by vortexing with analytical grade water until completely dissolved. Once rehydrated, determine the optimal working dilution, distribute the conjugate in aliquots and stored frozen at temperature ≤-50°C. In these conditions it remains stable for 20 years.

To determine the optimal working dilution of the conjugate (that is, the dilution which displays the specific bands in positive controls with a minimum background), the working dilutions recommended by the manufacturer are tested. If none of these dilutions results optimal, the further dilutions of the reagent shall be tested until the optimal one is obtained.

Before testing, dilute an aliquot of conjugate to the optimal concentration with the dilution solution. Once diluted, store the conjugate refrigerated at 1÷8 ° C and use within 24 hours.

6.17 Chromogen DAB (3, 3'diaminobenzidine).

Different kinds of chromogen are commercially available, liquid or in tablets. For preparation and use follow the instructions by the manufacturer.

Fo the liquid formula DAB (3,3'diaminobenzidine)/Enanched Liquid Substrate System, add a drop of DAB Liquid Chromogen Solution B in a 1.5mL tube. Dilute with 1mL of DAB Liquid Chromogen Solution A, mix and deposite on nitrocellulose membrane. Let for 5-30 minutes monitoring the bands appearance.

7 PROCEDURE

7.1 Samples and marker preparation

Use 100µg of Ag E/S for a gel and add all reagents. Stir using a magnetic stirrer until complete dissolution. Incubate 10 minutes at 70°C in the Thermomixer. Take a 10µl aliquot of the molecular weight markers (6.3) and incubate 1 minute at 40°C in the heating block.

7.2 Gel electrophoresis loading

Put one or two gels (6.2) in the electrophoresis tray for vertical electrophoresis (Figure 2). Fill half of the electrophoresis tray (about 500mL) with running buffer (6.5). Add 200mL of running buffer with 500µL of antioxidant in the central part of the tray.
Load the proteins in the gel by micropipette, connect the electrophoresis tray to the electrophoresis power supply and allow the separation of the proteins by applying 150 V for 1h at room temperature.

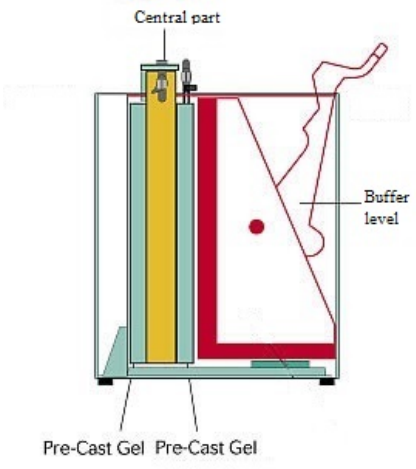


Figure 2. Representation of the vertical electrophoresis cassette.

7.3 Transfer to nitrocellulose membranes

At the end of the electrophoresis running, put the gel containing the proteins on a nitrocellulose membrane (6.7), according to the scheme of the Mini Trans-Blot Module shown in the figure 3.

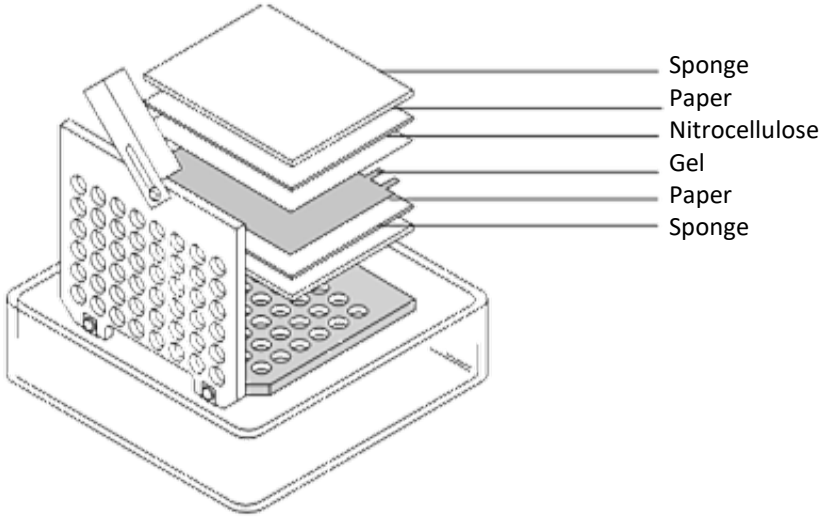


Figure 3. Scheme of the transfer from gel to nitrocellulose membrane protein.

Place the assembled system in the X cell SureLock (Figure 4) with transfer buffer (6.6) and ice block, connect the power supply unit for 1 h at + 4°C at 38 mA, so that proteins migrate from the gel to the nitrocellulose.

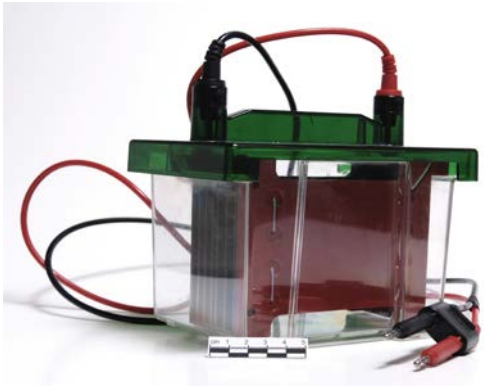


Figure 4. X cell SureLock protein transfer

7.4 Verification of proteins transfer

At the end of the transfer open the cassette, remove the nitrocellulose and place it in a tray with dye (6.8) to verify the effective transfer. Wash with tap water to remove excess dye and cut the filter into strips using a scalpel.

7.5 Nitrocellulose blocking

Put the strips in the blocking solution (6.14) overnight at +4°C. Then, wash the strips with TTBS buffer (6.11). Once the strips are dried, they can be stored, wrapped in filter paper, at room temperature for up to two months.

7.6 Test and control samples preparation

Thaw the test sera, negative (6.14) and positive (6.15) control sera by storing in a refrigerator at +1÷8°C for at least 5 h. Once thawed, keep them in an ice bath and stir by vortexing before use.

Dilute 1:100 the test and control sera as follows: in a 1.5-2mL conical bottom tube dispense 1mL of diluting solution (6.12) with a serological pipette placed on a pipettor and discard 10µL through a micropipette. Alternatively, dispense 990µL diluting solution (6.12) with a micropipette and add 10µL of serum. Diluted sera can be stored refrigerated for up to 24 hours.

7.7 Analytical method.

a. Bring out the serum samples from the refrigerator.
b. Dispose the nitrocellulose strips in the appropriate Mini-Incubation Trays and wash them with TTBS solution (6.11) for 5 minutes at room temperature.
c. Incubate the control and test sera for 1 h at room temperature.
d. Wash 3 times for 5 minutes at room temperature with TTBS solution (6.11).
e. Incubate the diluted conjugate (6.16) 1h at room temperature
f. Wash 3 times for 5 minutes at room temperature with TTBS solution (6.11).
g. Wash 1 time for 5 minutes at room temperature with TBS solution (6.11).
h. Add the chromogen (6.17) until the bands are revealed.
i. Stop the reaction with tap water.

8 INTERPRETATION OF THE RESULTS

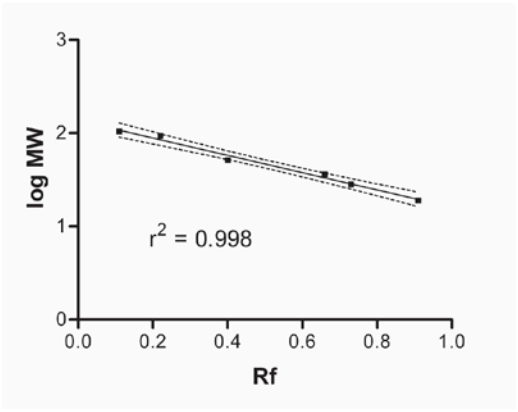
The electrophoretic separation of the proteins of *T. spiralis* and their subsequent transfer on nitrocellulose membrane are considered valid when all the markers (or standards) of molecular weight (MW) colored, used in each gel, were:

- electrophoretically separated**;**
- transferred to nitrocellulose membrane;
- for proteins standard of 104, 94, 51, 36, 28 and 19 kDa, the relative mobility is: 0.13, 0.24, 0.41, 0.66, 0.80, 0.91, respectively (S.D.±0.02). The relative mobility of each standard shall be in the range previously established by means of three independent experiments (Figure 5). If even one relative mobility is not within the specific range, the results shall be considered invalid, and the test has to be repeated.
  For the positive control, the relative mobility has been previously published (Gomez Morales et al., 2014)

The specific *T. spiralis* E/S proteins reacting with reference and test sera from pigs are identified by calculating their molecular weight (MW) as follows:

1. in a Microsoft Word Excel electronic sheet trace a PM log graph of pre-stained standards to their relative migration distance (Rf). Calculate the correlation coefficient (r^2), it has to be in the range 0.75-1 (Colton, 1988);
2. interpolate the Rf of *T. spiralis* specific E/S proteins which react with pig test and reference sera, to calculate their molecular weights;
3. the specific proteins that react with pig sera, both reference and test sera, must show a triple-band pattern in a region comprised between 48 and 72 kDa (the first band from 48 kDa to 55 kDa, the second band from 59 kDa to 62 kDa and the third band from 64 to 72 kDa).

	A	B	C	D	E	F	G	H	I
1	MW (kD) of the prestained molecular marker proteins	Migration distance (cm) of the prestained molecular marker proteins			Rf prestained molecular marker proteins			Rf Mean	Log MW
2		experiment 1	experiment 2	experiment 3	experiment 1	experiment 2	experiment 3	x	y
3	104	0,6	0,9	0,7	=PRODUCT(B3;1/B10)	=PRODUCT(C3;1/C10)	=PRODUCT(D3;1/D10)	=MEAN(E3:G3)	=LOG10(A3)
4	94	1,2	1,5	1,3	=PRODUCT(B4;1/B10)	=PRODUCT(C4;1/C10)	=PRODUCT(D4;1/D10)	=MEAN(E4:G4)	=LOG10(A4)
5	51	2,2	2,5	2,3	=PRODUCT(B5;1/B10)	=PRODUCT(C5;1/C10)	=PRODUCT(D5;1/D10)	=MEAN(E5:G5)	=LOG10(A5)
6	36	3,6	3,8	3,7	=PRODUCT(B6;1/B10)	=PRODUCT(C6;1/C10)	=PRODUCT(D6;1/D10)	=MEAN(E6:G6)	=LOG10(A6)
7	28	4,4	4,7	4,5	=PRODUCT(B7;1/B10)	=PRODUCT(C7;1/C10)	=PRODUCT(D7;1/D10)	=MEAN(E7:G7)	=LOG10(A7)
8	19	5	5,2	5,1	=PRODUCT(B8;1/B10)	=PRODUCT(C8;1/C10)	=PRODUCT(D8;1/D10)	=MEAN(E8:G8)	=LOG10(A8)
9									
10	Migration distance of the front	5,5	5,8	5,6					
11									
12									
13		Migration distance of the unknown proteins							
14					Rf unknown proteins			Rf Mean	Log MW
15		1,8	2,1	1,9	=PRODUCT(B15;1/B10)	=PRODUCT(C15;1/C10)	=PRODUCT(D15;1/D10)	=MEAN(E15:G15)	=TREND(I3:I8;H3:H8;H15;TRUE)
16		2,2	2,4	2,3	=PRODUCT(B16;1/B10)	=PRODUCT(C16;1/C10)	=PRODUCT(D16;1/D10)	=MEAN(E16:G16)	=TREND(I3:I8;H3:H8;H16;TRUE)
17		2,5	2,8	2,6	=PRODUCT(B17;1/B10)	=PRODUCT(C17;1/C10)	=PRODUCT(D17;1/D10)	=MEAN(E17:G17)	=TREND(I3:I8;H3:H8;H17;TRUE)
18									
19									
20									=POWER(10;I15)
21							Molecular weight of the unkown proteins		=POWER(10;I16)
22									=POWER(10;I17)



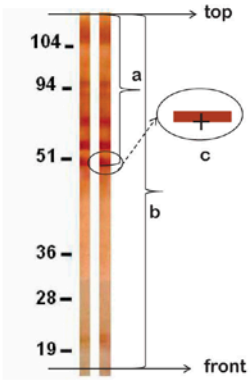


Figure 5. The above table shows the results of experiments carried out to determine the relative mobility of each molecular weight marker. The graph below on the left shows the regression line of the relative distance of the reference sera as a function of their molecular weight. The image below on the right shows the pattern of specific proteins and the E/S Ag of *T. spiralis*, which react with pig sera in Wb.

9 CHARACTERISTICS OF THE METHOD

This method has been characterized in terms of sensitivity, specificity and reproducibility. The results were used to confirm that the method is suitable for the intended purpose and are reported in the relevant validation file, to which reference is made.

10 SAFETY MEASURES

This test method can be performed only by experienced personnel. As potentially infected sera with zoonotic pathogens are handled, analysts are provided with personal protective equipment, such as disposable gloves and lab coats. For safety measures to be adopted during testing, refer to the WHO Laboratory Biosafety Manual – Third Edition (http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/).