Detection of viable metacestodes of Taenia spp. in human, porcine and bovine serum samples with the use of a monoclonal antibody-based sandwich ELISA

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Table of contents
General information
Definitions and abbreviations
Method
Quality control
Reporting and archiving
References
Revision
Approval

1 General information

- Background information

Monoclonal antibody-based antigen detection uses two monoclonal antibodies to "trap" the antigen circulating in blood, CSF or urine\(^1\) (Correa et al., 1989; Harrison et al., 1989; Choromanski et al., 1990; Brandt et al., 1992; Wang et al., 1992; Garcia et al., 1998, 2000; Erhart et al., 2002; Castillo et al., 2009). These assays give a positive result when viable, well-established cysts are present.

The monoclonal antibodies used in the test developed by Brandt et al. (1992) and modified by Van Kerckhoven et al. (1998) and Dorny et al. (2000) were originally prepared against antigens from T. saginata, but can not only detect viable cysticerci in bovines, but also cysticerci of T. solium in pigs and humans. The cross-reactions between antigens produced by T. solium and T. saginata metacestodes are a welcome advantage here. There are, unfortunately, also cross-reactions with antigens from T. hydatigena metacestodes (Dorny et al., 2004) and T. s. asiatica metacestodes (Fall et al., 1996; Geerts et al., 1992) in pigs. Therefore, in regions where these parasites are endemic, the detection of T. solium cysticercosis is restricted (Dorny et al., 2001) and accurate data on the prevalence of porcine cysticercosis are not easily available or are of questionable reliability (Rajshekhar et al., 2003).

\(^1\) The test used for antigen detection in urine is a slightly modified test!

- General principle of the test

To use this assay (see Figure), a purified antibody (the "capture" antibody) is bound to a solid phase (a polystyrene plate). Antigen is then added and allowed to complex with the bound antibody. Unbound products are then washed away; a labeled second antibody (the "detection" antibody) is allowed to bind to the antigen, thus completing the "sandwich". The assay is then quantified by measuring the amount of labeled antibody. The intensity of the color can be measured (OD). The obtained OD's are then processed statistically to
determine whether a sample is positive or not. This interpretation is based on a set of known positive and negative reference samples.

- First (capturing) antibody  B158C11A10
- Blocking
- Serum (with Ag)
- Second (detecting) antibody  B60H8A4
- Streptavidine labelled peroxidase
- OPD substrate

Figure 1: The general principle of the sandwich ELISA

1.1 Reagents

- Reference codes and suppliers

Table 1 gives you a list of the products used in the ELISA assay. For accurate pricing and availability, please contact the company/supplier.

Please note that the information in this table can change without prior notice!

Table 1: Product name, quantity, company/supplier name and product reference code of the products needed to do the ELISA.

| Product                   | Quantity   | Company or supplier name | Reference code    |
|---------------------------|------------|--------------------------|-------------------|
| Biotin Protein Labeling Kit | 5 reactions | Roche                    | 1418165           |
| Carbonate-Bicarbonate Buffer tablets | 50 capsules | Sigma-Aldrich            | C3041             |
| H2O2 30% P.A. ISO         | 250 ml     | VWR                      | 1.072.090.250     |
| Immunoplate Maxisorp F96  | 60 plates   | VWR                      | NUNC439454        |
| Monoclonal antibodies     | for 10 plates | please contact VHU      |                   |
| Na2CO3 powder             | 1 kg       | VWR                      | 1.063.921.000     |
| NAHCO3 powder             | 1 kg       | VWR                      | 1.063.291.000     |
**Monoclonal antibodies**

The monoclonal antibodies are developed, produced and labeled by the Veterinary Helminthology Unit (VHU). The production procedure is described in general by Harlow and Lane (1988).

- **The capturing antibody**
  
  The capturing antibody (B158C11A10) is used at 5 µg/ml coating buffer (pH 9.6). The quantity of monoclonal you must take to have 5 µg depends on the batch of monoclonal you are using. We recommend that the capturing antibody be stored at -20°C.

- **The detecting antibody**
  
  The detecting antibody (B60H8A4) is labeled to biotin and is used at 1.25 µg/ml blocking buffer. The quantity of monoclonal you must take to have 1.25 µg depends on the batch of monoclonal you are using. We recommend that you store the antibody at +4°C. Following the manufacturer’s instructions, we added 1% bovine serum albumin.

**Buffers and product preparations**

1. **Phosphate Buffered Saline (PBS)**
   
   The PBS buffer is prepared using tablets. One tablet in 100 ml of RO-DI water yields a 100 ml PBS buffer, pH 7.3.

2. **Trichloroacetic acid (TCA)**
   
   The solution used for the "pretreatment" of the serum samples is a 5% (W/V) solution in RO-DI water. Example: dissolve 0.5 g of TCA crystals in 10 ml RO-DI water.

3. **Washing buffer**
   
   The washing buffer consists of PBS with 0.05% (V/V) Tween 20. Example 1: 1 liter of PBS + 0.5 ml of Tween 20. Example 2: 1 liter of PBS + 0.555 g of Tween 20 (density is 1.11)
4. Blocking buffer
The blocking buffer consists of washing buffer + 1% (V/V) of Newborn Calf Serum (NBCS).
Example: 49.5 ml of PBS-Tween 20 + 0.5 ml of NBCS
Note that the NBCS has to be heat inactivated before use. To do this, you must put the serum at 56°C for 30 minutes (in a waterbath for example).

5. Coating buffer
The coating buffer is prepared using powder-filled capsules.
One tablet in 100 ml of RO-DI water yields a 0.05 M carbonate/bicarbonate buffer, pH 9.6.

Alternative:
Stock solution A: Na2CO3 (0.06 M) = 0.159 g/25 ml
Stock solution B: NaHCO3 (0.06 M) = 0.504 g/100 ml
10 ml A + 50 ml B + 175 ml RO-DI water.
Carefully add HCl or NaOH until pH to 9.6 is reached.
Adjust volume to 250 ml with RO-DI water

6. Neutralisation buffer (Carbonate/Bicarbonate buffer, 0.156 M, pH 10)
Stock solution A: Na2CO3 (0.61 M) = 6.466 g/100 ml
Stock solution B: NaHCO3 (0.61 M) = 5.124 g/100 ml
72 ml A + 55 ml B + 300 ml RO-DI water.
Adjust pH to 10 by adding either HCl or NaOH.
Once the pH is set, adjust the volume to 500 ml with RO-DI water.

7. Phosphate citrate buffer
The Phosphate-citrate buffer is prepared using tablets.
1 tablet in 100 ml of RO-DI water to obtain a 0.05M Phosphate citrate buffer, pH 5.0.

8. Sulfuric acid (H2SO4)
The acid we use comes in cartridges. Take the contents of a cartridge and add RO-DI water up to 250 ml. This gives you 250 ml of H2SO4 4N.
• Recommended storage temperature and storage time for buffers and products

Table 2 shows the recommended storage time and storage temperature for the products and buffers used in the test. Please note that storage times of 3-4 months are indications.

Always check the state of your products. If you see a contamination inside a 1 month old buffer, you should discard it immediately. On the other hand, an unopened autoclaved bottle of PBS can still be used after 6-7 months. Check your Tween 20 each time you use it to see if it is still clear. Should it become cloudy, the Tween 20 should be replaced.

The storage time of Streptavidin-HRP depends on the storage temperature.

**Table 2: Recommended storage temperature and storage time.**

| Product/buffer               | Storage T     | Storage time                                      |
|------------------------------|---------------|---------------------------------------------------|
| Biotin Protein Labeling Kit  | + 4°C         | See expiration date                               |
| Neutralisation buffer        | + 4°C         | 3-4 months                                        |
| Carbonate-Bicarbonate Buffer | Ambient temp  | No indication                                     |
| PBS tablets                  | Ambient temp  | See expiration date                               |
| PBS buffer                   | + 4°C         | 3-4 months                                        |
| Tween 20                     | Ambient temp  | No indication                                     |
| Washing buffer               | + 4°C         | No more than 1 week, but it is recommended to prepare new buffer before each test |
| Newborn Bovine serum         | - 20°C        | See expiration date                               |
| Blocking buffer              | NA            | NA                                                |
| H2O2                         | + 4°C         | No indication                                     |
| B158C11A10                   | - 20°C        | 6-7 months                                        |
| B60H8A4 (biotinylated)       | + 4°C         | 3-4 months                                        |
| OPD tablets                  | + 4°C         | See expiration date                               |
| Streptavidin-HPR             | Follow manufacturer’s instructions | Follow manufacturer’s instructions |
| TCA ≥ 99.0% (titration)      | + 4°C         | No indication                                     |
| TCA solution                 | NA            | NA                                                |
| Phosphate citrate tablets    | Ambient temp  | No indication                                     |
| Phosphate citrate buffer     | + 4°C         | 3-4 months                                        |
| TITRISOL H2SO4               | Ambient temp  | See expiration date                               |
| H2SO4 4N solution            | Ambient temp  | 6 months                                          |

NA = Not applicable; prepare fresh buffer/solution before each test.
1.2 Apparatus, equipment and materials

- Centrifuge for eppendorf tubes
- Micro pipettes (0.5 µl - 10 µl, 50 µl - 200 µl, 100 µl - 1000 µl) + tips (When available: an Eppendorf Multipette 4780 + 5 ml cartridge tips and/or multichannel pipettes)
- ELISA plates
- ELISA reader with 492 (and if available 655 nm) filters
- Shaker incubator (37°C)
- Incubator at 30°C
- Vortex and magnetic stirrer
- Balance (reach: 0.001 g) + spatula
- Dark recipient for making the substrate or aluminum wrapping foil.
- Eppendorf tubes (1.5 ml) + rack
- Disposable syringe of 20 ml that is filled with washing buffer, a handheld dispenser connected to a container filled with washing buffer or a fully automated ELISA washer.
- Measuring flasks and beakers
- Falcon tubes (15 hand 50 ml)

1.3 Sample for analysis

- "Pretreatment" of the samples

Aim of the pretreatment:

1. Break down immune complexes to obtain free circulating antigen.
2. Reduce cross-reactions with sera of i.a. individuals infected with Trypanosoma.

To do this, the samples are mixed with an equal volume of trichloroacetic acid (TCA).

This breaks down the immune complexes. The samples are then neutralised (= bring pH from low the neutral) with a neutralisation buffer.

- Negative control sera (only 1 well required): 75 µl TCA (5%) solution + 75 µl serum sample.
- Positive control and unknown sera (2 wells per sample): 150 µl TCA (5%) solution + 150 µl serum sample.
- Mix immediately by vortexing.
• Incubate for 20 min. at ambient temperature.
• Mix again by vortexing.
• Centrifuge for 9 min. at 12000g.
• While centrifuging, prepare eppendorfs with 75 µl (negative control sera) or 150 µl (positive and unknown sera) neutralisation buffer (pH 10).
• Neutralise mixture by adding 75 or 150 µl (for negative controls or other samples, respectively) of the supernatant into the eppendorfs with the same amount of neutralisation buffer. This results in a final dilution of 1/4 of your sample(s).

Important note: When using positive control sample K504, you must "pre-dilute" the sample before doing the TCA treatment.

• Add 5 µl K504 serum to 795 µl PBS
• Vortex well
• From this 800 µl you take 150 µl and add it to 150 µl of TCA
Then you continue with a normal TCA treatment.
This gives the sample a final dilution of 1/640 as opposed to a 1/4 dilution for a normal sample.

• Controls
Please adopt the plate layout as it is shown in the table.
Note that positive controls (+1 & +2) and the unknown samples (? 1 to ? 40) are 2wells/sample, while the negative controls (-1 to -8) are 1 well/sample.

Table 3: Plate layout

|    | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A  | SC  | -1  | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   |
| B  | SC  | -2  | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   |
| C  | CC  | -3  | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   |
| D  | CC  | -4  | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   |
| E  | +1  | -5  | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   |
| F  | +1  | -6  | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   |
| G  | +2  | -7  | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   |
| H  | +2  | -8  | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   | ?   |

1.4 (Bio-)Safety and environment

• Always wear gloves when handling samples, especially human samples, as there might be HIV, ... danger
• TCA and H2SO4 may cause severe burns, so wear protective gloves and avoid release to the environment. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
• OPD may cause discomfort after skin contact and there is limited evidence of a carcinogenic effect, so wear protective gloves and avoid release to the environment.

2 Definitions and abbreviations

- B158C11A10 first -capturing- antibody
- B60H8A4 second -detecting- antibody
- CC conjugate control
- CSF cerebrospinal fluid
- ELISA enzyme linked immunosorbent assay
- g RCF - Rotational centrifugal force
- M molar
- N normal
- NBCS newborn calf serum
- nm nanometer
- OD optical density
- OPD ortho phenylenediamine
- PBS phosphate buffered saline
- RO-DI water reverse osmotic de-ionised water
- SD standard deviation
- SC substrate control
- TCA trichloroacetic acid
- VHU veterinary helminthology

3 Method

Check to make sure all reagents and products are prepared and/or available. If you have a lot of samples to test, start with the pretreatment of the samples (as described in section 1.3). If not, you can start with the coating of the plate and do the treatment during the incubation time of the coating and the blocking step.

Note: The below procedure should be done quickly. A multichannel pipet can be used to expedite the process.
| Coating                                                                 | • Capturing antibody (B158C11A10) (see tube for concentration) in coating buffer, @ concentration of 5 µg/ml | 15min 37°C shaking or overnight at 4°C |
|-------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|---------------------------------------|
|                                                                        | • 100µl in all wells except SC: only coating buffer                                             |                                       |
| Wash 1X                                                                 | • Washing buffer (PBS -Tw20 0.05%)                                                               |                                       |
|                                                                        | • Individual wells are completely filled with washing buffer, but spill-over from one well into another must be avoided. |                                       |
| Blocking                                                                | • Blocking buffer (PBS-Tw 20 0.05% + 1% NBCS)                                                  | 15min 37°C, shaking                   |
|                                                                        | • 150 µl in each well                                                                           |                                       |
| Sample                                                                  | • Empty the plate, but do not wash                                                               | 15min 37°C, shaking                   |
|                                                                        | • 100 µl of pretreated samples in designated wells                                             |                                       |
|                                                                        | • Positive controls and unknown samples: 2 wells/sample                                           |                                       |
|                                                                        | • Negative controls: 1 well/sample.                                                              |                                       |
|                                                                        | • In SC and CC: blocking buffer                                                                  |                                       |
| Wash 5X                                                                 | Washing buffer                                                                                   |                                       |
| Detecting                                                               | • Detecting antibody (B60H8A4-biot.) (see tube for concentration) in blocking buffer, @ concentration of 1.25 µg/ml | 15min 37°C, shaking                   |
|                                                                        | • 100µl in each well except SC: only blocking buffer                                            |                                       |
| Wash 5X                                                                 | Washing buffer                                                                                   |                                       |
| Conjugate                                                               | • Dilute peroxidase labelled streptavidin 1/10 000 in blocking buffer                            | 15min 37°C, shaking                   |
|                                                                        | • 100µl in each well except SC only blocking buffer                                             |                                       |
|                                                                        | • Take the OPD out of the fridge (20 minutes before use)                                        |                                       |
| Wash 5X                                                                 | Washing buffer                                                                                   |                                       |
| Substrate                                                               | • OPD: 1 tablet in 10ml phosphate citrate buffer. Prepare in a dark recipient (e.g. falcon tube wrapped in aluminium foil). | 15 minutes 30°C; dark, not shaking      |
|                                                                        | • Add the 10µl H₂O₂ just before just before putting the substrate on the plate.                 |                                       |
|                                                                        | (If you need more than 10 ml of OPD, you must take 2 tablets in 20 ml and add 20 µl of H₂O₂ and so on...) |                                       |
|                                                                        | • 100 µl in each well                                                                            |                                       |
| Stopping                                                                | • H₂SO₄ (4N)                                                                                    |                                       |
|                                                                        | • 50µl in all wells                                                                             |                                       |
| Read                                                                    | 492 and 655nm (The 492 nm filter is the most important one, as it is used to measure the maximum absorption. 655 nm is used to measure the background.) |                                       |

**4 Quality control**

There is a conjugate and substrate control on each plate (CC en SC, respectively). Non-specific reactions between the plate, coating/blocking and conjugate are intercepted by the conjugate control. The quality of the substrate (e.g.
by influence of light) can be traced by the substrate control. Both controls need to be negative (below cut-off value). The table below shows the contents of the SC and CC controls for each step of the ELISA assay.

**Table 4: SC and CC controls**

| ELISA step   | SC                      | CC                      |
|--------------|-------------------------|-------------------------|
| Coating      | Only coating buffer     | Capturing antibody      |
| Blocking     | Blocking buffer         | Blocking buffer         |
| Sample       | Blocking buffer         | Blocking buffer         |
| Detecting    | Blocking buffer         | Detecting antibody      |
| Conjugate    | Blocking buffer         | Streptavidin            |
| Substrate    | Substrate               | Substrate               |

Negative control samples (-1 to -8) are used to calculate the cut-off of the assay and should be matched to the species of the samples to be tested, to avoid any bias in the interpretation of the results.

Positive control samples (+1 & +2) are used to see if the assay itself was successful.

If desired, you can contact the VHU for a set of positive and negative reference samples to evaluate the assay in your lab.

- **Consistency between the wells**
  
  One of the most important aspects of any assay is consistency and standardization of conditions as this will affect the reproducibility and accuracy of your results.

  Therefore (automatic) multichannel pipets and plate washers provide more consistent and faster results, as well as higher throughput. Certainly in this ELISA the use of a multichannel pipet is very important if you work with full multiwell plates because of the short incubations. Further calibrations of all pipettors on a regular base are necessary, or there can be significant variation in the results.

- **Technical validation**

  - The eight negative reference samples can be checked by means of the following formula:

    $100 \times \frac{SD}{MEAN} < 100$

  - The unknown samples can be evaluated also with the above formula.

  - The positive reference samples are used to avoid mistakes during the test. The fact that they give a positive result suffices.

- **Interpretation of the results**

  - All positive and unknown samples are done in duplicate. Check if the 2 wells containing the same sample give roughly the same OD. If this is ok, you then calculate the average OD for every sample.
average OD = \[\frac{\text{OD}_{\text{well 1}} + \text{OD}_{\text{well 2}}}{2}\]

- The cut off is calculated based on the OD’s of the negative samples using a variation of the students test (Sokal and Rohlf, 1981). Once the cut off has been determined, it can be used to calculated a ratio.

**Ratio = average OD/cut off**

- When the ratio is > 1, the sample is considered positive with a certainty of 99.9 %.
- There is a Microsoft Office Excel file available to assist with these calculations.

Please contact the VHU for more information.

5 Reporting and Archiving
- Storage location of all raw data generated, L:Biomedical Science/Parasitology/ Vet helm/ labo/ ELISA
- Storage location of samples. -20°C Freezer VHU

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### Revision

| Changes with respect to the previous published version: | Version number: ..... |
|------------------------------------------------------|-----------------------|
| New format of WEBISO                                  |                       |
### Approval

| Created by (First Version)      | Name and function          |
|--------------------------------|-----------------------------|
| Anke Van Hul, lab technician   |                             |

| Updated by (Current Version):  | Name and function          |
|--------------------------------|-----------------------------|
| ELISA cysticercosis protocol.v1.5 |                             |

| Approved by:                   |                             |
|--------------------------------|-----------------------------|
| Pierre Dorny, Unit Head        |                             |