



In vitro diagnostic kit

Cysticercosis Ag ELISA



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IVD

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The apDia Cysticercosis Antigen (Ag) ELISA is an Enzyme Immunoassay for the qualitative determination of viable metacestodes (cysticerci) of *Taenia* spp. in human and porcine serum samples.

BACKGROUND INFORMATION

General information

Taenia solium cysticercosis is an infection of humans and pigs with the metacestode larvae (cysticercus) of *Taenia solium*. Circulating antigen detection in serum is an important diagnostic method that indicates the presence of viable parasites. The monoclonal antibodies (IgG isotype) used in this assay are produced against excretory secretory products (ESP) of viable *T. saginata* cysticerci (Brandt *et al.*, 1992; Dorny *et al.*, 2004). The glycoprotein antigens detected by these monoclonal antibodies are present on the tegument and in the excretory-secretory-products of metacestodes (Draelants *et al.*, 1995).

The assay only demonstrates the presence of viable cysticerci, it does not detect degenerated or calcified cysticerci. In this respect, unlike antibody detection, measurement of circulating antigen levels allows differentiation of cysticercosis cases with viable parasites, with antigen levels correlating to the numbers and size of lesions. It can as such also provide a tool for serological monitoring of antiparasitic therapy in human or pigs: antigen levels drop rapidly after successful anthelmintic treatment (Deckers & Dorny, 2010).

Porcine cysticercosis

The assay is genus specific, not species specific. The assay does not allow the differentiation between infections of different *Taenia* species in pigs (*T. solium*, *T. hydatigena*, *T. asiatica*). This should be taken in consideration when using the cysticercosis Ag ELISA for diagnosis of *T. solium* in countries where *T. hydatigena* and *T. asiatica* are common in pigs (e.g. SE Asia) (Dorny *et al.*, 2004).

In experimentally infected pigs, circulating antigens were first detected between 2 and 6 weeks post infection and remained present generally throughout an observation period of 6 months, even in pigs carrying only five to eight living cysts. The minimum number of living cysts, that could be detected using the cysticercosis Ag ELISA, was one (Dorny *et al.*, 2004; Nguetkam *et al.*, 2003).

Human cysticercosis

Because *T. solium* is the only *Taenia* spp. causing cysticercosis in man, the test is specific. No cross-reactions were observed with sera from patients with parasitologically and/or serologically confirmed infections with *Schistosoma* (n = 3), hydatid cysts (n = 10), *Ascaris* (n = 38), *Trichuris* (n = 38), filaria (n = 3), *Entamoeba* (n = 3), *Plasmodium* (n = 7) and *Trypanosoma* (n = 8) (Erhart *et al.*, 2002).

The sensitivity of the assay decreases when the number of viable cysts is low; infections with one viable cyst are often not detectable.

Antigen levels are generally higher in extraparenchymal neurocysticercosis (NCC) (particularly subarachnoid NCC) than in intraparenchymal NCC; therefore, high antigen levels should lead one to suspect the presence of extraparenchymal NCC (Rodriguez *et al.*, 2009).

PRINCIPLE OF THE CYSTICERCOSIS AG ELISA

The pretreated controls and samples are added to the B158C11A10 monoclonal antibody-coated wells. During this incubation step circulating antigens from viable cysticerci are bound specifically to the wells. After removal of the unbound serum proteins by a washing procedure, the antigen-antibody complex in each well is detected with specific peroxidase-conjugated B60H8A4 monoclonal antibodies. After removal of the unbound conjugate, the strips are incubated with a chromogen solution containing tetramethylbenzidine and hydrogen peroxide: a blue colour develops in proportion to the amount of immune complex bound to the wells of the strips. The enzymatic reaction is stopped by the addition of 0.5M H₂SO₄ and the absorbance values at 450 nm are determined.

REAGENTS

1. Coated Microtiterstrips – **MTP**

10 microtiter plates, each with 12 x 8-well strips coated with B158C11A10 monoclonal antibodies.

2. Negative Control – **CTLNEG**

3 vials, each containing 2 ml animal serum negative for the presence of *Taenia* spp. Contains 0,09 % NaN₃ and 0.05 % Proclin 300.

3. Positive Control – **CILPOS**

3 vials, each containing 2 ml animal serum with *Taenia* spp. cysticercus antigen. Contains 0.09 % NaN₃ and 0.05 % Proclin 300.

4. Conjugate – **CONJ**

2 vials, each containing 60 ml peroxidase conjugated monoclonal B60H8A4 antibodies. Contains antimicrobial agents and an inert red dye.

5. Washing Solution – **WASH 20x**

4 vials, each containing 125 ml 20x concentrated phosphate buffered washing solution. Contains 0.05 % Proclin 300.

6. Chromogen Solution – **CHROM**

4 vials, each containing 30 ml of a solution containing H₂O₂ and tetramethylbenzidine.

7. Stopping Solution – **STOP**

1 vial, containing 60 ml of 0.5M H₂SO₄.

8. TCA Solution – **TCA**

1 vial, containing 100 ml of a 5 % trichloroacetic acid (TCA) (w/v) solution.

9. Neutralisation Buffer – **NB**

1 vial, containing 100 ml of a 0.156M carbonate/bicarbonate buffered solution.

MATERIALS REQUIRED BUT NOT SUPPLIED

1. Precision micropipettes and standard laboratory pipettes.
2. Clean standard laboratory volumetric glassware.
3. Eppendorf Tubes® or similar.
4. A centrifuge for Eppendorf Tubes® or similar (speed 12000g).
5. A calibrated thermostatic incubator for incubation at 37°C.
6. A microtiterplate reader capable of measuring absorbencies at 450 nm.
7. A microtiterplate shaker.

WARNINGS AND PRECAUTIONS FOR USERS

1. For *in vitro* diagnostic use only.
2. Do not mix reagents or coated microtiterstrips from kits with different lot numbers.
3. Chromogen Solution contains the hazardous ingredient N-Methyl-2-pyrrolidone at a concentration > 0,3 %. It is classified as a Reproductive Toxicant Category 1B. Following hazard statements are applicable:
H360D: May damage the unborn child.
Following precautionary statements are applicable:
P280: Wear protective gloves/protective clothing/eye protection/face protection.
P308+P313: IF exposed or concerned: Get medical advice/attention.
4. Some kit components contain sodium azide as a preservative. In order to prevent the formation of potentially explosive metal azides in laboratory plumbing, flush drains thoroughly after disposal of these solutions.



STORAGE CONDITIONS

1. Store the microtiterstrips in their original package with the desiccant until all the strips have been used.
2. Never use any kit components beyond the expiration date.

SPECIMEN COLLECTION AND PREPARATION

Human and porcine serum and plasma may be used in this assay. Remove serum from clot as soon as possible to avoid haemolysis. Lipemic and/or strongly haemolysed samples can cause false results. Transfer the serum to a clean storage tube. Specimens may be stored at 2-8 °C for a few days, or they can be stored frozen for a longer period of time. Avoid repeated freezing and thawing.

Human cerebrospinal fluid (CSF) may be used in the assay. TCA pre-treatment for CSF samples may be skipped (see chapter: Pre-treatment of samples/controls). When setting up the assay, use negative CSF as negative control (not included in the kit) instead of the negative serum control provided in the kit.

ASSAY PROCEDURE

General Remarks:

1. Use a separate disposable tip for each sample transfer to avoid cross-contamination.
2. All reagents must be allowed to come to room temperature before use. All reagents must be mixed without foaming.
3. Once the assay has been started, all steps should be completed without interruption.

Reconstitution of the Reagents

Washing Solution: dilute 125 ml of concentrated Washing Solution (5) to 2500 ml with distilled water. Reconstituted solution can be stored at least 1 month, store at 2 – 8 °C.

At higher temperatures, the concentrated Washing Solution (5) may appear cloudy without affecting its performance. Upon dilution, the solution will be clear.

Washing procedure

1. One cycle is done as follows:
 - Add 300 µl of diluted Washing Solution to all wells of the microtiterplate.
 - Remove Washing Solution immediately from the wells by inverting the microtiterplate and tapping dry on absorbent paper.
2. An automatic microtiterplate washer may be used but this will require adaptation of the washing procedure. If necessary, increase the number of cycles and include a soak time after each wash cycle based on the OD values of the negative and positive controls.

Pre-treatment of samples/controls for in duplo determination

For CSF specimens the pre-treatment step described in this chapter may be skipped. The positive control however must be pre-treated with TCA.

1. Add 150 µl TCA Solution (8) and 150 µl sample/control in an Eppendorf Tube®.
2. Mix immediately by vortexing.
3. Incubate for 5 to 20 minutes at room temperature.
4. Mix again by vortexing.
5. Centrifuge the tubes for 5 to 9 minutes at 12000g.
6. While centrifuging, prepare for each sample an Eppendorf Tube® containing 150 µl Neutralisation Buffer (9).
7. Neutralise mixture by adding 150 µl of the supernatant into the Eppendorf Tubes® with the same volume of Neutralisation Buffer (9). Mix by vortexing

Remark: this results in a final dilution of 1/4 of the samples/controls.

Pre-treatment of samples/controls for single determination

For CSF specimens the pre-treatment step described in this chapter may be skipped. The positive control however must be pre-treated with TCA.

1. Add 60 µl TCA Solution (8) and 60 µl sample/control in an Eppendorf Tube®.
2. Mix immediately by vortexing.
3. Incubate for 5 to 20 minutes at room temperature.
4. Mix again by vortexing.
5. Centrifuge the tubes for 5 to 9 minutes at 12000g.
6. While centrifuging, prepare for each sample an Eppendorf Tube® containing 60 µl Neutralisation Buffer (9).
7. Neutralise mixture by adding 60 µl of the supernatant into the Eppendorf Tubes® with the same volume of Neutralisation Buffer (9). Mix by vortexing.

Remark: this results in a final dilution of 1/4 of the samples/controls.

Assay Procedure

1. Take the necessary amount of test strips (1) and return non-used strips in the sachet. In each run 1 negative control (2) and 1 positive control (3) should be included.
2. Add 100 µl of the pre-treated samples/controls to each well. Determine the samples preferably in duplo. Seal strips securely with a microplate sealer.
3. Incubate for 15 minutes at 37°C while shaking at 700-800 rpm.
4. After incubation, wash the microtiterstrips with 300 µL of Washing Solution (5), apply 5 cycles. Change Washing Solution for each cycle. Finally empty the microtiterstrips and remove excess fluid by blotting the inverted strips on absorbent paper.
5. Add 100 µl of Conjugate Solution (4) to the wells. Seal strips securely with a microplate sealer.
6. Incubate for 15 minutes at 37°C while shaking at 700-800 rpm.
7. After incubation, wash the microtiterstrips 5 times with 300 µL of Washing Solution (5) as described in step 4.
8. Add 100 µl of Chromogen Solution (6) to each well. Seal strips securely with microplate sealer.
9. Incubate for 15 minutes at room temperature. Avoid light exposure during this step.
10. After incubation, add 50 µl of Stopping Solution (7) and read OD at 450 nm with reference wavelength 600-650 nm in a microplate reader within 15 minutes after stopping.

RESULTS

Cut-off calculation and calculation of Ag Index for human specimens

- Mean ODneg is used to calculate the cut-off by multiplying its value by 2:

$$\text{Cut-off value} = \text{mean ODneg} \times 2$$

- The antigen index (Ag Index) of each sample is calculated by dividing the OD value of the sample (ODsample) by the cut-off value:

$$\text{Ag Index} = \text{mean ODsample} / \text{Cut-off value}$$

Cut-off calculation and calculation of Ag Index for porcine specimens

- Mean ODneg is used to calculate the cut-off by multiplying its value by 3,5:

$$\text{Cut-off value} = \text{mean ODneg} \times 3,5$$

- The antigen index (Ag Index) of each sample is calculated by dividing the OD value of the sample (ODsample) by the cut-off value:

$$\text{Ag Index} = \text{mean ODsample} / \text{Cut-off value}$$

Interpretation of the results for detection of cysticercosis antigen in human and porcine specimens

A positive reaction corresponds to an Ag Index above or equal to 1.3

A negative reaction corresponds to an Ag Index below or equal to 0.8.

A grey-zone between Ag Index 0.8 and 1.3 has to be considered. Samples inside this "grey-zone" should be considered as doubtful and should be retested, preferably using a freshly drawn blood sample, alternatively, additional diagnostic methods should be used.

Negative result: Ag Index ≤ 0.8
Positive result: Ag Index ≥ 1.3
Doubtful result: 0.8 < Ag Index < 1.3

EXPECTED VALUES

Example of typical O.D. values for the Negative Control and Positive Control:

NC	0.027
PC	2.890

PERFORMANCE

Diagnostic and Analytical Sensitivity

Serum samples from 100 Peruvian patients diagnosed for cysticercosis, have been tested in the apDia Cysticercosis Ag ELISA. In this group, 94 patients tested positive. Based on imaging data and other clinical data, the 6 patients with the negative test result could be subdivided in 3 groups: 2 patients had no living cysts, 2 patients had 1 living cyst and 2 patients had 2 living cysts in the brain. All patients infected with at least 3 living cysts were tested positive.

In a study on infected animals, a panel of 31 animals infected with viable cysticerci of *Taenia* spp. were tested, all samples gave a positive result in the Cysticercosis Ag ELISA from apDia.

Specificity

Human serum samples of 20 patients negative for cysticercosis, originating from a non-endemic area in Peru, were tested in the apDia Cysticercosis Ag ELISA. In this area, other parasitosis cases are very prevalent. All samples were tested negative.

300 negative human samples from Belgian blood donors were analysed in the apDia Cysticercosis antigen ELISA. None of the 300 samples tested repeatedly positive while 2 samples gave a positive result in the first run resulting in a specificity of 99.3%.

300 negative porcine samples were analysed in the apDia Cysticercosis antigen ELISA. None of the 300 samples tested repeatedly positive while 1 sample gave a positive result in the first run resulting in a specificity of 99.6%.

TEST VALIDITY

- The individual absorbance value (optical density OD) of the positive control (ODpos) must be above 1.000.
- The average OD of the negative control (ODneg) must be lower than 0.100.
- If one of these specifications is not met, the results should be considered invalid and the series of tests should be repeated.

LIMITATIONS OF THE ASSAY

Strict adherence to the protocol and recommended equipment is necessary to obtain reliable test results. Accurate sample and reagent pipetting and timing of washing and incubation should be respected.

- As with all *in vitro* diagnostic tests, a definitive clinical diagnosis should not be made based only on the results of a single test. A complete evaluation by a physician is needed for a final diagnosis.
- The assay detects viable cysticerci, it does not detect degenerated or calcified cysticerci.
- The sensitivity of the assay decreases when the number of viable cysticerci is low.

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