Detection of anti-*Opisthorchis* antibodies in human serum by indirect ELISA

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1. Aim and field of application

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

The method can be used for the serological diagnosis of human opisthorchiasis.

2. Principle of the method

Opisthorchiasis is a zoonotic disease caused by liver flukes of the genus *Opisthorchis* (*Ophisthorchis viverrini* and *O. felineus*) which are transmitted by the ingestion of raw or undercooked fish parasitized by the larval stage (metacercaria). Each species have a particular geographical distribution: *O. felineus* has been documented in humans and/or animals in 13 countries of the European Union as well as in the Russian Federation and Siberia, *O. viverrini* is present in South Asia (Laos, Cambodia and Thailand). A wide range of species of freshwater fish of the family Cyprinidae can be naturally infected by these trematodes. Carnivore mammals such as cats, dogs, and foxes act as definitive hosts where the parasite develops into adults in the intra- and extra-hepatic bile ducts and in the gallbladder. Humans are an accidental host.
Most people with opistorchiasis have unspecific symptoms or no symptoms at all, whereas heavy and long lasting infections are linked to hepatobiliary diseases including hepatomegaly, cholangitis, fibrosis of the periportal system, cholecystitis, and gallstones, and are strongly associated with cholangiocarcinoma (CCA). *O. viverrini* is classified as group 1 carcinogens by the International Agency for Research on Cancer. A specific and early diagnosis of opisthorchiasis in humans is crucial for an appropriate and timely treatment. Even if detection of fluke eggs in stools represents the best way to reach a definitive diagnosis of opisthorchiasis, it has become increasingly unreliable in cases of low worm burden. Therefore, the detection of specific antibodies by a serological test, as ELISA, has been considered as a complementary tool to establish the definitive diagnosis of these infections. In addition, serology is an excellent tool to monitor the success of the treatment during the follow up.

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

Control and test serum sample, properly diluted, are distributed in the wells, allowing any anti-*Opisthorchis* 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

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4. Definitions

ELISA	Enzyme Linked Immunosorbent Assay
Ag	Antigen
Ab	Antibodies
Ag E/S	Excretory/Secretory antigens
BSA	Bovine Serum Albumin

5. Devices/instruments

The following instruments are needed to prepare the reagents to perform the ELISA procedure:

Adjustable pipettes (volumes: 1÷200 µL)

Balance (0.01÷100gr)

Automatic plate washer (strongly recommended)

ELISA plate microtiter reader, 450 nm

Freezer ≤ -15°C

Freezer ≤ -50°C

Ice maker

Incubator 37 ± 1°C

Magnetic stirrer

Adjustable volume dispenser (e.g., Multipette Eppendorf®)

pH meter

Pipette aid

Refrigerator 1÷8°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

KH_2PO_4	0.34 g
Na_2HPO_4	1.21 g
NaCl	8.0 g
Analytical grade water	up to 1000 mL

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; keep the solution refrigerate 1÷8°C. Stability: 6 months.

6.3 Carbonate buffered saline, pH 9.6 ± 0.2

Na_2CO_3	1.12 g
$NaHCO_3$	2.92 g
Analytical grade water	up to 1000 mL

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. Stability: 6 months.

6.4 Washing solution

Tween 20	1 mL
Analytical grade water	up to 2000 mL

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, 1÷8°C, the solution should be used within 24 h.

6.5 Blocking solution

BSA	0.25 g
Tween 20	0.025 mL
PBS	up to 50 mL

The solution shall be prepared immediately before use, as follows: place 0.25 g BSA directly in a 50 mL tube; add 40 ml of PBS buffer and mix by vortexing until the BSA is completely dissolved. Add 0.025 mL Tween 20; mix by vortexing and bring to volume. If refrigerated, 1÷8°C, the solution must be used within 24 h.

6.6 Sera and conjugate diluter

BSA	1.00g
Tween 20	0.05mL
PBS	up to 100 mL

The solution shall 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 to volume. If refrigerated, 1÷8°C, 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. Stability: 6 months.

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 A)

The antigens shall be reconstituted using of analytical grade water. The reconstituted antigens shall then be brought to a final concentration of 2 µg/µL with carbonate buffer saline pH 9.6. The reconstitution and the following dilution steps should be performed on ice immediately before use.

6.11 Peroxidase labelled anti–human IgG goat antibodies

The conjugate shall be used at the optimal dilution calculated by checking board titration. The dilution shall be prepared on ice immediately before use.

6.12 Anti-*Opisthorchis* sp. positive control sera

100 µL of sera from *Opisthorchis* sp. infected persons (positive controls). Each positive control serum shall 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-*Opisthorchis* sp. negative control sera

100 µL of sera from *Opisthorchis* sp. free people that resulted suitable for blood donation according to the Italian low (negative controls). Each negative control serum shall be used at a 1/200 dilution in duplicate, using the appropriate diluter. The dilution shall be performed on ice immediately before use.

6.14 Sera to be tested

Each serum shall be tested at a 1/200 dilution in duplicate, using the appropriate diluter. The dilution shall be performed on ice immediately before use.

7. Procedure

7.1 Test and control samples preparation

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 (1÷8°C) for up to 24 h.

7.2 *Procedure*

7.2.1 Fill the microtiter plate with 100µL per well of 2µg/mL of *Opisthorchis* sp ES Ag in carbonate buffered saline; incubate for 1h 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 shall 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

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

- The OD value of the negative control sera shall be lower than the *cut off* value determined by the validation method (i.e., 17%);
- The OD value of the positive control sera has to be $\geq$ to the lowest OD value among reference sera used for the calculation of the diagnostic sensitivity;
- The difference in OD between the two measures, made on the same positive control sample in strict conditions of repeatability, has to be $\leq$ 0.2 absorbance unit, and on the same negative control sample it has to be $\leq$ 0.1 absorbance unit.

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.

8.1 Calculate the mean of the two duplicates for each positive serum (PS) and for each test serum (TS).

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

8.3 Select the higher OD value among the positive control sera (PS_{max}), and for each sample calculate the ELISA Index (E_I) according to the following formula:

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

where: $E_I \geq 17$ %, *Opisthorchis* positive serum

$E_I < 17\%$, *Opisthorchis* negative serum

9. Characteristics of the method

This method was validatedin 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 (Centers for Disease Control, Office of Health and Safety, www.cdc.gov/od/ohs/biosfty/bmbl4/b4af.htm)

ANNEX A

Production of excretory/secretory antigens from adult worms of *Opisthorchis felineus*

1 Aim and field of application

To describe the production of excretory/secretory antigens from adult worms of *Opisthorchis felineus*.

The product can be used as antigens for serology to detect anti- *Opisthorchis* spp. antibodies.

2 References

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3 Definitions

OD, optical density

4 Devices/Instruments

Incubator 37±1°C with 4-5% CO2

Spectrophotometer UV/VIS

Freezer ≤-15°C

Refrigerator, 1°C ÷ 8°C

Freezer ≤ -50°C

Laminar flow hood

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

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 (7.2 ± 0.2) and then bring the solution to the final volume. Sterilize by filtration with a 0.22 µm filter. Store the solution refrigerate (1÷8°C). Stability: 6 months.

5.2 PBS, pH 7.3 ± 0.2 with antibiotics 5X

PBS	950 mL
Penicillin/Streptomycin or Antibiotic/Antimycotic solution	50 mL

Store refrigerated (1÷8°C). Stability: 2 months.

5.3 Complete 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

Store refrigerated (1÷8°C). Stability: 2 months.

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

5.5 RPMI 1640 culture medium.

5.6 HEPES-1M Buffer solution.

5.7 L-Glutamine 0.2M solution.

5.8 Sterile 0.22 µm syringe filters.

5.9 Device for concentrating by ultrafiltration with cellulose membrane filter, 5000 MWCO cut off (e.g. Amicon Ultra 15).

5.10 Coomassie Protein Assay Reagent (e.g., Pierce ®, 1856209).

5.11 Slide-A-Lyzer Dialysis Cassettes, 3.5K MWCO, 3 - 12 ml Capacity (e.g., Pierce ®, 66110).

5.12 Cocktail of protease inhibitors.

5.13 *Opisthorchis felineus* adults worms.

5.14 Sterile conical tubes 15, 50 mL.

5.15 6 wells plates for cell cultures.

5.16 96 wells plate.

6 Procedure

a) Place the solutions 5.2 and 5.3 into the 37°C incubator for at least 1 h.

b) Wash the worms three times by sedimentation in a sterile 50 ml conical tube with 45 mL of warm sterile PBS with antibiotics. At each change of the washing solution, gently shake the worms to dislodge adherent bacteria. After sedimentation, remove the washing solution by aspirating with a pipette.

c) Determine the number of parasites under microscopic observation.

d) Suspend the worms in warm complete media in a sterile 50 mL tube. Work in a laminar flow hood.

e) Place 2-5 worms/5mL in 6 wells plates

f) Incubate the plates in 5% CO_2 at 37°C for 24 h.

g) After 24 hours, check worms viability and absence of bacterial and fungal contamination by microscopy.

h) Collect the media, discarding dead parasites. Add fresh complete media to the plates with living parasites. Repeat steps “g” and “h” every 24 hours whenever the parasites are still alive. Finally, discard the worms.

i) Filter the media through a 0.22 μm filter.

j) Keep the E/S Ag at +4°C until concentration, if stored more than 24 hours, the E/S Ag should be frozen at -15°C..

k) Fill in the concentration tube 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) If the E/S Ag has been concentrated 100 times or more, proceed to point "p", otherwise centrifuge the tube again.

p) Dialyze the concentrated E/S Ag versus PBS at +4°C for at least 4 hours.

q) Check the optical density by the spectrophotometer at 280nm/260nm. The ratio should be $\geq$ 1.

r) Determine the protein concentration by the Bradford method.

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

t) Aliquot the antigen in 1 mL vials at a total protein concentration of 0.5 mg/vial and store at ≤-15°C.

u) 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 6 'g';
- Worms viability, controlled as described in point 6 'g';
- Optical density ratio of 280nm/260nm of the final antigen solution $\geq$ 1, as specified in point 6 'q'.

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 (www.cdc.gov/od/ohs/biosfty/bmbl4/b4af.htm.)