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 antigens (Ag E/S) 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. Acronyms

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:

Automatic plate washer (strongly recommended)
Incubator 37 ± 1°C
ELISA plate microtiter spectrophotometer, 450 nm
Balance, resolution 0.01g
Refrigerator 1÷8°C
Freezer ≤ -15°C
Freezer ≤ -50°C
Magnetic stirrer
Vortex
Adjustable micropipettes e (0.5-10 µL, 15-300 µL, 5-1000 µL)
Analytic grade water production system or analytic water commercially available
Adjustable volume dispenser, alternatively pipettes could be used

6. Reagents and chemicals

The step-by-step procedure for preparing the reagents is described below.

6.1 Sera and conjugate diluter

BSA	0.50g
Tween 20	0.025mL
PBS	up to 50 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 dissolved completely,
- 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.

6.2 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
- mix by magnetic stirring until the solution is clear.

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

6.3 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.

6.4 Stop solution

HCl 1N in analytical grade water. Prepare the solution under a chemical hood; store at room temperature.

Stability: 6 months.

6.5 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.

6.6 Excretory/secretory antigens (ES Ag)

Store Ag E/S at a temperature ≤ -50°C, dilute immediately before use with carbonate buffer saline pH 9.6 ± 0.2 to reach a final concentration of 2 µg/µL with. To produce Ag E/S, see Annex A of the method.

Prepare carbonate buffered saline, pH 9.6±0.2 as follow:

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. Stability: 6 months.

6.7 Anti-*Opisthorchis* positive control sera

Sera positive for antibodies anti-*Opisthorchis* coming from individuals affected by opisthorchiasis confirmed by the presence of *Opisthorchis* eggs in the feces.

6.8 Anti-*Opisthorchis* negative control sera

Sera from Opisthorchis free individuals that resulted suitable for blood donation according to the Italian low (negative controls).

6.9 TMB (3, 3', 5, 5' tetramethylbenzidine) peroxidase substrate

This substrate is recommended; if not available, any other peroxidase substrate can be used.

6.10 96-well flat-bottomed microtiter plate

6.11 Conjugate: peroxidase labelled anti–human IgG goat antibodies

Upon opening, rehydrate the freeze-dried material with analitycal grade water, shaking on vortex until its complete dissolution.

The solution can be stored at a temperature 1÷8°C for a week.

To determine the optimal working dilution of the conjugate, perform a titration curve testing the working dilutions greater, minor and equal than the recommended by the supplier for use in ELISA, until identify the optimal one (i.e. the dilution of the conjugate in which the differences in optical density, O.D., between positive and negative controls are maximum, maintaining the minimum background, as can be seen from the O.D. value of the blank).

The conjugate, once rehydrated, if distributed in aliquots and stored frozen at a temperature ≤-50°C remains stable for at least 20 years. After the expiration date, its suitability will be verified, through the O.D. values detected in the positive and negative controls, in the analytical sessions in which it is used.

Before execution of the test, a conjugate aliquot is diluted to the optimum concentration with the dilution solution referred to in point 6.1.

Once diluted, store the conjugate refrigerated (1÷8°C) and use it within 24 hours

7. Procedure

7.1 Test and control samples preparation

Thaw the positive, negative control sera and test sample sera, if thawed. Store them at a temperature 1÷8°C for at least 5 h.

Once thawed, keep them in an ice bath and stir them by vortexing before use.

Dilute 1:200 the test and control sera as follows: in a 1-2 mL conical bottom tube, add 2 µL of serum and 398 µL of diluting solution. Diluted sera can be stored refrigerated (1÷8°C) for up to 24 h.

7.2 Procedure

Withdraw the microtiter plate from the refrigerator

Dispense, in each well, 100µL diluted Ag E/S (6.6) through a dispenser with tips or alternatively a micropipettes with tips; incubate 1h at 37°C.

Wash 3 times in the automatic plate washer with the washing solution (6.2).

Add, in each well, 200 µL blocking solution (6.3) through a dispenser with tips or alternatively a micropipettes with tips; incubate 1 h at 37° C.

Wash 3 times in the automatic plate washer with the washing solution (6.2).

Dispense, in duplicate, 100µL of each diluted positive controls in PS1, PS2, PS3 and PS4 wells, diluted negative controls in NS1, NS2, NS3 and NS4 wells, diluted sample sera in SSxx wells and 100µL of diluter solution in blank wells. Incubate 30 minutes at 37°C.

Microtiter plate scheme

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
A	PS1	PS1	SS1	SS5	SS9	SS13	SS17	SS21	SS25	SS29	SS33	SS37
B	PS2	PS2	SS1	SS5	SS9	SS13	SS17	SS21	SS25	SS 29	SS33	SS37
C	PS3	PS3	SS2	SS6	SS10	SS14	SS18	SS22	SS26	SS30	SS34	SS38
D	PS4	PS4	SS2	SS6	SS10	SS14	SS18	SS22	SS26	SS30	SS34	SS38
E	NS1	NS1	SS3	SS7	SS11	SS15	SS19	SS23	SS27	SS31	SS35	SS39
F	NS2	NS2	SS3	SS7	SS11	SS15	SS19	SS23	SS27	SS31	SS35	SS39
G	NS3	NS3	SS4	SS8	SS12	SS16	SS20	SS24	SS28	SS32	SS36	blank
H	NS4	NS4	SS4	SS8	SS12	SS16	SS20	SS24	SS28	SS32	SS36	blank

Legenda: PS1÷PS4: positive control sera, NS1÷NS4: negative control sera, SS1÷SS39: sample sera to be tested

Wash 3 times in the automatic plate washer with the washing solution (6.2).

Add, to each well, 100µL of the diluted conjugate (6.11) and incubate 1h at 37°C.

Wash 3 times in the automatic plate washer with the washing solution.

Add 100µL TMB substrate (6.9) per well; incubate 10 min at room temperature.

Stop the reaction by adding 50µL of the stop solution (6.4) per well and read the reaction in the spectrophotometer at 450nm.

8. Interpretation of the results

To evaluate the obtained results:

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

Subtract from each mean value the mean O.D. value of the blanks (OD_b).

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

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

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

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

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

- ✓ The O.D. value of the negative control sera is lower than the *cut off* value determined by the validation method (i.e., 17%);
- ✓ The O.D. value of the positive control sera is $\geq$ to the lowest O.D. value among reference sera used for the calculation of the diagnostic sensitivity;
- ✓ The difference in O.D. between the two measures, made on the same positive control sample in strict conditions of repeatability, is $\leq$ 0.15 absorbance unit; the difference, in O.D., between the two measures made on the same negative control serum is < 0.05 absorbance unit, in at least 6 out of 8 controls included in the analytical session.

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.

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 Acronyms

O.D., optical density

Ag E/S, escretory/secretory *Opisthorchis felineus* antigens

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

Refrigerated centrifuge

5. Reagents and chemicals

5.1 *Opisthorchis felineus* adults worms.

5.2 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 the compounds in 750 mL of analytical grade water under magnetic stirring. Control the pH (7.3±0.2) and then bring the solution to the final volume. Sterilize by filtration with a 0.22 µm filter. Store the solution refrigerate at 1÷8°C. Stability: 6 months.

5.3 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.

5.4 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.

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

5.6 Sterile 0.22 μm syringe filters.

5.7 Device for concentrating by ultrafiltration, cut-off 5kDa.

5.8 Device for dialysis, membrane of 3.5K MWCO.

5.9 Cocktail of protease inhibitors.

Sterile conical tubes 15, 50 mL.

6 wells plates for cell cultures.

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) In a sterile 50 ml conical tube, wash the worms, adults trematodes collected from experimentally infected hamsters, five times by sedimentation 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) Under the laminar flow hood, suspend the worms in warm complete medium.

e) Place 2-5 worms in each well of a six wells culture plate containing 5mL of complete medium,

f) Incubate the plates in 5% CO_2 at 37±1°C for 24 h.

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

h) Discard dead parasites.

i) Under the laminar flow hood, collect the medium in a sterile 50 ml conical tube. Add fresh complete medium to the plates with living parasites.

j) Incubate the plates in 5% CO_2 at 37±1°C

k) Repeat steps “g” and “h” every 24 hours whenever the parasites are still alive. Finally, discard the worms.

l) Filter the medium, containing the escretory/secretory antigens, through a 0.22 μm filter, after each daily collection.

m) Keep the E/S Ag at a temperature 1÷8°C until concentration (step “n”), if stored more than 24 hours, the E/S Ag should be frozen.

n) Concentrate the E/S Ag with device for ultrafiltration (cut-off 5kDa) by centrifugation at 3,000 g for 30 minutes in a refrigerated centrifuge (temperature 1÷8°C).

o) Repeat the concentration step until the final volume is almost 100 times more concentrated of the initial volume.

p) Retrieve the concentrated E/S Ag in a 50 mL tube and store at temperature 1÷8°C until dialysis.

q) Dialyze the concentrated E/S Ag versus PBS at temperature 1÷8°C for at least 4 hours.

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

s) If the OD ratio is < 1, the solution is discharged because contaminated with DNA.

t) Determine the protein concentration of the E/S Ag by the Bradford method.

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

v) Assign to each aliquot an identification number as follow progressive number/years. The aliquots may be stored frozen or lyophilized.

w) Frozen (T< -50°C) E/S Ag aliquots are stable for 10 years.

x) Lyophilized E/S Ag aliquots if preserved refrigerated (1÷8°C) are stable for 20 years, in absence of humidity inside the vial.

y) When necessary, reconstitute lyophilized aliquot with distilled analytical grade water, aliquot and froze (T< -50°C). The product is stable for 10 years in this condition. Each sub aliquots is identified with number/year/x (number and year are the same of the aliquot of resuspended E/S Ag, x is a alphabetic letter).

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’;

OD ratio of 280nm/260nm of the final antigen solution $\geq$ 1, as specified in point 6 ‘r’.

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.)