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

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ANNEX A - Production of excretory/secretory antigens from adult worms of *Opisthorchis felineus*
1. **Aim and field of application**

To determine the presence of anti-\textit{Opisthorchis} sp. antibodies by an enzyme linked immunosorbent assay 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 \textit{Opisthorchis} (\textit{Opisthorchis viverrini} and \textit{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: \textit{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, \textit{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 opisthorchiasis 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). \textit{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 \textit{O. felineus} excretory/secretory (E/S) antigens partially purified in conditions capable of maintaining the antigens' native form.

Control and test sera, properly diluted, are distributed in the wells, allowing any anti- \textit{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**


4. Definitions

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibodies</td>
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<tr>
<td>Ag E/S</td>
<td>Excretory/Secretory antigens</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>h</td>
<td>Hours</td>
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<td>min</td>
<td>Minutes</td>
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<tr>
<td>RT</td>
<td>Room temperature</td>
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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 at 450 nm
- Freezer ≤ -15°C
- Freezer ≤ -30°C
- Ice maker
- Incubator 36 ÷ 38°C
- Magnetic stirrer
- Adjustable volume dispenser (e.g., Multipette Eppendorf®)
- pH meter ± 0.3
- 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

KH₂PO₄ 0.34 g
Na₂HPO₄ 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; store the solution refrigerate (1 °C). Stability: 3 months

6.3 Carbonate buffered saline, pH 9.6 ± 0.2

Na₂CO₃ 1.12 g
NaHCO₃ 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 RT. 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 °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 50.00 mL

The solution should be prepared immediately before use, as follows: place 0.25 g BSA (bovine serum albumin) 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 °C), the solution must be used within 24 h.

6.6 Sera and conjugate diluter

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

The solution should 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 it to volume. If refrigerated (1 °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 RT.

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 should be reconstituted using analytical grade water. The reconstituted antigens should then be brought to a final concentration of 2µg/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 should be used at the optimal dilution calculated by checking board titration. The dilution should be prepared on ice immediately before use.

6.12 Anti-Opisthorchis sp. seropositive control sera

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

6.14 Sera to be tested

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

7. Procedure

7.1 Preparing test and control samples

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. Analytical 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 should 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

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

8.1.1 The OD value of the negative control sera should be lower than the cut off value determined by the validation method (i.e., 17%);

8.1.2 The OD value of the positive control sera has to be ≥ to the lowest OD value among reference sera used for the calculation of the diagnostic sensitivity;

8.1.3 The difference in OD between the 2 measures made on the same positive control sample in strict conditions of repeatability has to be ≤ 0.2 unit absorbance, and on the same negative control sample it has to be ≤ 0.1 unit absorbance.

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.

8.2 Calculate the mean of the 2 duplicates for each positive serum (PS) and for each test serum (TS).

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

8.4 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:

\[ E_I (%) = \frac{\text{OD mean duplicates TS} - \text{OD}_b}{\text{OD mean duplicates highest PS} - \text{OD}_b} \times 100\% \]

where:

- \( E_I \geq 17\% \), Opisthorchis positive serum
- \( E_I < 17\% \), Opisthorchis negative serum

9. Characteristics of the method

This method was studied in 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


3. Definitions

OD, optical density

4. Devices/Instruments

- Incubator 36-38°C with 4-5% CO2
- Spectrophotometer, MIPI 11.50
- Freezer ≤ -15°C
- Refrigerator, 1°C ÷ 8°C
- Freezer < -50°C
- Amicon® pressure concentrating chamber
- Adjustable micropipettes (up to 2 μL, 20 μL, 200 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

\[
\begin{align*}
\text{KH}_2\text{PO}_4 & \quad 0.34 \text{ g} \\
\text{Na}_2\text{HPO}_4 & \quad 1.21 \text{ g}
\end{align*}
\]
5.1 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 (it must be 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: 1 month.

5.2 PBS, pH 7.3 ± 0.2 with antibiotics (Penicillin and Streptomycin) 5 X

<table>
<thead>
<tr>
<th>PBS</th>
<th>Penicillin/Streptomycin solution</th>
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<tr>
<td>900 mL</td>
<td>100 mL</td>
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</table>

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

5.3 Complete medium

| RPMI 1640 | 285 mL |
| 1M HEPES | 3 mL |
| 200mM Glutamine | 3 mL |
| 100mM Na-pyruvate | 3 mL |
| Penicillin/Streptomycin | 6 mL |

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

5.4 Penicillin (5000 mg/mL)-Streptomycin (5000 units/mLsolution), Euroclone®, ECB 3055D

5.5 RPMI 1640 culture medium, Euroclone®, ECB 9006L

5.6 HEPES-1M Buffer solution, Euroclone®, EUUMO 180D

5.7 L-Glutamine 0.2M solution,

5.8 Sterile 0.22 μm syringe filters

5.9 YM-3 filters Amicon ®

5.10 Coomassie Protein Assay Reagent®, Pierce ®, 1856209

5.11 Slide-A-Lyzer Dialysis Cassettes, 3.5K MWCO, 3 - 12 ml Capacity Pierce ®, 66110

5.12 Cocktail of protease inhibitors (Sigma P8465, Saint Louis, Mo USA)

5.13 Opisthorchis felineus adults worms

5.14 Sterile conical tubes 15, 50 mL

5.15 6 wells plates for cell cultures

6 Procedure

a) Place the solutions 5.1, 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/Penicillin/Streptomycin solution). 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% CO₂ at 37°C for 24 h.

g) Check worms viability and bacterial and fungal contamination by microscopy. Collect the media discarding dead parasites. Add fresh complete media to the plates with living parasites. Steeps ‘e’, ‘f’ and ‘g’ should be repeated every 24 hours whenever the parasites are still alive.

h) Filter the media through a 0.2 μm filter. Discard the worms.
i) Concentrate the filtered media 100 X in the Amicon® pressure concentrating chamber using a YM-3 filter at +4°C. The obtained solution may be clarified by washing with PBS in the Amicon® chamber or by dialysing for at least 4 h in PBS. Keep the excretory/secretory antigen solution (E/S Ag) on ice until freezing.

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

k) Determine the protein concentration by the Bradford method.

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

m) Aliquot the antigen in 1 mL vials at a total protein concentration of 0.1÷2.5 mg and store at ≤-15°C.

n) 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 5 ‘g’;
- Worms viability, controlled as described in point 5 ‘g’;
- Optical density ratio of 280nm/260nm of the final antigen solution \( \geq 1 \), as specified in point 5 ‘k’.

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).