IDENTIFICATION OF *Opisthorchis* sp. DNA BY PCR

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1 AIM AND FIELD OF APPLICATION

This document describes a method to determine the presence of *Opisthorchis* sp. eggs by PCR. The method can be applied to faecal samples of human or animal origin, preserved in ethanol.

2 PRINCIPLE OF THE METHOD

The Polymerase Chain Reaction (PCR) is a molecular biology technique allowing for the amplification of specific nucleic acid fragments, which initial and terminal nucleotide sequences are known (oligonucleotide pair). If a species (or a genotype) has its own characteristic DNA portion, due to its composition and/or dimension, it is possible to choose an oligonucleotide pair allowing for its amplification. The PCR amplification is characterized by a high sensitivity and specificity.

Tremadotes of the genus *Opisthorchis* and *Clonorchis* are transmitted to humans through the consumption of raw freshwater fishe of the family Cyprinidae. The *Opisthorchis* genus includes the species *O. felineus* and *O. viverrini*, the first species is widespread in Europe and in the Russian Federation; the second one is endemic in Southeast Asia (Laos, Cambodia and Thailand), the related genus *Clonorchis* i salso present in the asian continent. The adult parasites colonize the biliary ducts and cause tiredness, loss of weight, and diarrhoea e. In advanced cases portal hypertension, chronic inflammation and hyperplasia of the biliary tract develop, including the possible invasion of the pancreatic duct. Cirrhosis of the liver, or cholangiocarcinoma, may arise to complicate the late clinical picture of the disease.

The diagnosis of opistorchiasis is made by microscopic examination of faecal samples for the detection of the parasite eggs. The diagnosis can be difficult due to the reduced number of eggs, the intermittent egg production, their small size and their morphology. The *Opisthorchis* sp. eggs may be confused with those of phylogenetically related trematodes or may not be recognized at all, if the examination is carried out by not experienced analyst.

Molecular methods based on PCR allow to identify the presence of parasite eggs in faecal samples of human and animal origin, even when their number is too low to be detected by microscopy.

The Internal Transcribed Spacer 2 (ITS2), placed among the ribosomal genes 5.8S and 28S is a molecular marker frequently used in molecular diagnosis. The ITS1 does not codificate any proteic or functional product therefore is more subjected to genetic variability, this characteristic making it a very useful diagnostic tool.

By applying the PCR analysis to a specific fragment of the ITS2 gene, it is possible to discriminate between *Opishtorchis* and *Clonorchis* genera.

In table A are reported the size of the ITS2 gene fragments obtained from three species of the Opisthorchidae family by amplification with specific oligonucleotide pair.

Table A – Size of the amplification product of the ITS2 gene fragment (in base pairs) from each species

O. felineus	***O. viverrini***	***C. sinensis***
248	**248**	**255**

3 REFERENCES

Muller B., Schmidt J., Mehlhorn H. PCR diagnosis of infections with different species of Opisthorchidae using a rapid clean-up procedure for stool samples and specific primers. Parasitol Res (2007) 100:905-909.

ISO/FDI 20837:2006(E). Microbiology of food and animal feeding stuffs – Polymerase chain reaction (PCR) for the detection of food-borne pathogens - Requirements for sample preparation for qualitative detection

ISO/FDI 20838:2006(E). Microbiology of food and animal feeding stuffs – Polymerase chain reaction (PCR) for the detection of food-borne pathogens - Requirements for amplification and detection for qualitative methods

ISO 22174: 2005. Microbiology of food and animal feeding stuffs – Polymerase chain reaction (PCR) for the detection of food-borne pathogens.

Qiagen: QIAamp Fast DNA Stool Handbook.

QIAxcel User manual.

4 DEFINITIONS

ITS2 (Internal Transcribed Spacer 2), intergenic spacer placed among 5.8S and 28S genes.

Oligonucleotide, a short sequence (15/30 nucleotides) used for the amplification of a specific DNA fragment.

Set O, mix of 2 oligonucleotides amplifying a fragment of the ITS2 gene from species belonging to the Opisthorchidae family.

Reference DNA, genomic DNA extracted from *O. felineus* adults.

DNA extraction positive control, aliquots of human faeces spiked with *O. felineus* eggs, processed in the same working session of test samples, to verify the efficacy of the DNA extraction.

Positive amplification control, a reference DNA; this control is used in the amplification session to verify the efficacy of the PCR.

Negative amplification control, reagent grade water; this control is used in the amplification session to verify the absence of contamination in the PCR reaction.

The definitions and terminology used in the ISO 22174 standard are applied in the present protocol.

5 DEVICES/INSTRUMENTS

5.1 Bench centrifuge for 1.5-2.0 mL tubes, min 20,000 g

5.2 Freezer ≤ -15°C

5.3 Thermoblock with vibration, temperature range 25-100°C

5.4 PCR thermocycler

5.5 Refrigerator, 1-8°C

5.6 Horizontal electrophoretic apparatus

5.7 Digital imaging system

5.8 Adjustable volume pipettes, range: 1-1000µL

5.9 Reagent grade water system production

5.10 Vortex

5.11 Balance, readability 0.1g

5.12 UV Transilluminator

5.13 Qiaxcel, capillary vertical electrophoretic apparatus

6 REAGENTS AND CHEMICALS

6.1 **InhibitEX Buffer.** Commercially available reagent: QIAamp Fast DNA Stool, QIAGEN. Store according to the manufacturer's recommendations.

6.2 **Proteinase K.** Commercially available reagent: QIAamp Fast DNA Stool, QIAGEN. Store according to the manufacturer's recommendations.

6.3 **Lysis buffer.** Commercially available solution: QIAamp Fast DNA Stool, labelled as 'AL solution'. Store according to the manufacturer's recommendations.

6.4 **Absolute ethanol**. Commercially available reagent.

6.5 **Binding column.** Commercially available material: QIAamp Fast DNA Stool, QIAGEN, labelled as "QIAmp Mini Spin Columns".

6.6 **Collection tubes.** Commercially available material: QIAamp Fast DNA Stool, QIAGEN, labelled as "Collection tubes" (2 mL).

6.7 **Washing buffers.** Commercially available solutions: QIAamp Fast DNA Stool, QIAGEN, prepared according to the manufacturer's recommendations and labelled as "AW1" and "AW2". Store according to the manufacturer's recommendations.

6.8 **Elution buffer.** Commercially available solution: QIAamp Fast DNA Stool, QIAGEN, labelled as "Buffer AE". Store according to the manufacturer's recommendations.

6.9 **PCR master mix.** Commercially available solution to perform PCR amplification experiment (e.g., QIAgen HotStarTaq Master Mix Kit). Store according to the manufacturer's recommendations. If large volumes of PCR master mix are purchased, the product can be divided into 1-2 mL aliquots.

6.10 **SetO.** The oligonucleotide mixture (6.11) used for the PCR; the mixture is obtained combining an equal volume of the oligonucleotides OpITS2f and OpITS2r (6.11) diluted at 20 pmoli/µL with reagent grade water or Milli-Q (6.18). The final concentration corresponds to 10 pmol/µL of each oligonucleotide; 100 µL aliquots are prepared and stored frozen for up to 10 years.

6.11 **Oligonucleotides.** Commercially available reagents (Table B). The lyophilized products is reconstituted with reagent grade water according to the manufacturer's recommendations, at a concentration of 100 pmol/µL. This operation is recorded, dated and signed, on the oligonucleotide technical sheet provided by the manufacturer. The lyophilized product can be stored frozen for up to 20 years; the reconstituted product can be stored frozen for up to 10 years.

Table B. Oligonucleotide sequences of set-O (6.11), their codes and amplified nucleotide sequence.

Oligonucleotide sequences	Codes	Amplified sequence
5'-CGAGGGTCGGCTTATAAAC-3' 5'- AGCCTCAACCAAAGACAAAG-3'	OpITS2f OpITS2r	ITS2

6.12 **Loading buffer**, Commercially available product allowing electrophoresis of DNA molecules. It can be included in the PCR master mix (see 6.9). Store according to the manufacturer's recommendations.

6.13 **Agarose**, Commercially available product suitable for performing DNA molecule electrophoresis. Store according to the manufacturer's recommendations.

6.14 **50x TAE solution**, Commercially available product (2M Tris-acetate, 50mM EDTA, pH 8.2–8.4 at 25°C). Store at room temperature for up to 24 months.

6.15 **1x TAE solution,** 1000 mL preparation: take 20 mL of the 50x solution and bring to 1000 mL with water. The solution must prepared prior to the use.

6.16 **DNA intercalating agent**. Commercial product able to fit in the DNA double helix, used to view the amplification product on agarose gel. Store according to the manufacturer's recommendations.

6.17 **L50,** Commercially available product containing markers for DNA molecular weight multiple

of 50 bp, within the 50-500 bp range. Store refrigerated according to manufacturer's recommendations.

6.18 **Milli-Q grade water.**

6.19 **QIAxcel high resolution kit:** Commercially available product from Qiagen. Include a cartridge for samples separation by electrophoresis and solutions for samples preparation and run. Store according to manufacturer's recommendations.

6.20 **Alignment marker:** Commercially available product from Qiagen. Store according to manufacturer's recommendations.

6.21 **DNA size marker:** Commercially available product from Qiagen. Store according to manufacturer's recommendations.

6.22 **Faecal reference sample:** human faeces spiked with *O. felineus* eggs. Store frozen for up to 10 years.

6.23 **Reference DNA of *O. felineus*:** genomic DNA extracted from adults of *O. felineus*. Store frozen for up to 10 years.

7 PROCEDURE

7.1 Sample preparation

The faecal sample be preserved in ethanol, the tubes must be intact and show no sign of spillage. If the conditions are not suitable, the test will not be performed.

7.2 Method

7.2.1 *DNA extraction from the faecal sediment sample*

If not otherwise stated, the procedure is performed at room temperature.

Each working session requires that a reference sample (6.21) is processed for DNA extraction and labelled as "positive extraction control".

NB: before starting, wash fecal material with reagent grade water, centrifuge 5 minutes at 5,000 x g to eliminate the ethanol and resuspend the pellet in an adequate volume of reagent grade water.

a) Transfer 200 µL of each faecal sample in 2 mL tubes, previously marked with the identification code
b) Add 1 mL of lysis buffer (6.1) and vortex to homogenize the sample
c) Incubate for 10 minutes at 95°C in the thermomixer. Set the shaking at 1,400 rpm during the lysis step
d) Centrifuge 1 minute at 20,000 g
e) Put 25 µL of proteinase K (6.2) in a 2 mL tube.
f) Transfer 600 µL of supernatant (point "d") in the tube containing the proteinase K (point "e")
g) Add 600 µL of lysis buffer AL (6.4) to the same tube and vortex briefly
h) Incubate 10 minutes in a thermomixer at 70°C
i) Add 600 µL of absolute ethanol and vortex briefly
j) For each sample, set a binding column (6.5) into a collection tube (6.6)
k) Transfer 600 µL of lysate (from point "i") into the binding column (6.5) and centrifuge 1 minute at 12,000 g
l) Discard the collection tube (6.6) and transfer the binding column (6.5) into a fresh collection tube (6.6)
m) Repeat twice from point "k" to "l"
n) Add 500 µL of washing buffer AW1 (6.7) to the binding column (6.5) and centrifuge 1 minute at 20,000 x g

o) Discard the collection tube (6.6) and transfer the binding column (6.5) into a fresh collection tube (6.6)

p) Add 500 µL of washing buffer AW2 (6.7) to the binding column (6.5) and centrifuge 3 minutes at 20,000 g

q) Transfer the binding column (6.5) in a 1.5 mL tube

r) Add 100 µL of elution buffer ATE (6.8) to the binding column (6.5) and incubate for 1-2 minutes

s) Centrifuge 1 minute at 20,000 g, discard the binding column (6.5), and store the 1.5 mL tube containing the DNA extract

t) The obtained DNA extract, labelled "DNA/fecal sample", is stored frozen for up to 10 years.

7.2.2 PCR amplification

If not specified otherwise, keep tubes on ice or in a cooler rack, use aerosol-free tips and wear disposable gloves.

At each working session, use a positive and a negative amplification control. Use reference DNA (6.23) as positive control and water (6.18) as negative control.

The following procedure uses a PCR master mix at a 2X concentration. If the concentration is different, modify the procedure following the manufacturer's recommendations.

a) Thaw: DNA/faecal samples, 2x PCR MasterMix (6.10), SetO (6.11) and amplification positive control (reference DNA, 6.23).

b) Mark with a progressive number an adequate number of 0.2 mL PCR tubes.

c) Prepare an adequate cumulative volume of amplification mix. Calculate the volume on the basis of a single sample amplification mix (Table C) and of the total number of samples plus three reactions (extraction positive control, amplification positive control and amplification negative control).

*Table C. A*mplification mix for a single sample: components and volumes

2x PCR MasterMix (6.9)	15 µL
H_2O	12 µL
SetO (6.10)	1 µL
Total	28 µL

d) Mix the amplification mix by vortexing and centrifuge at maximum speed for few seconds

e) Transfer 28 µL of the cumulative amplification mix to each PCR tube (point "b").

f) Add to each tube 2 µL of the DNA/fecal samples to be tested.

g) Close the tubes, mix by vortexing and centrifuge at maximum speed for few seconds

h) Start the amplification cycle (Table D) on the thermocycler device; wait until the temperature reaches 94°C and insert the tubes in the thermoblock by pausing the instrument. Close the lid and restart the program.

Table D. Amplification cycle

Pre-denaturation [#]	5 min/95°C
Amplification	30 s/95°C 30 s/62°C 30 s/72°C
Number of cycles	35
Final extension	3 min/72°C

[#] pre-denaturation length may vary according to the Master Mix manufacturer's recommendations

i) At the end of the amplification step, centrifuge the tubes at maximum speed for few seconds.

l) Leave the tubes on ice or in a refrigerator before the electrophoresis.

7.2.3 Visualisation of results

a) Assemble the electrophoresis apparatus (5.6) according to the manufacturer's recommendations. For the gel preparation, use a comb suited for the number of samples to be tested.

b) Weight 2 g of agarose (6.13) and add it to 100 mL of TAE 1x (6.15) in a glass beaker.

c) Gently resuspend the agarose powder by rotation.

d) Boil the agarose suspension for 30 sec. If the solution is not homogeneous, continue to boil for another 30 sec.

e) Restore with water the volume lost during boiling.

f) Allow the agarose solution to cool.

g) Before it solidifies (at about 47°C), add DNA intercalating agent (6.16) according to the manufacturer's instructions.

h) Shake gently to dissolve uniformly the DNA intercalating agent and pour the agarose in the gel tray previously prepared (point "a").

i) Wait for the gel to solidify, which requires at least 30 min.

j) Place the tray with the gel in the electrophoresis apparatus.

k) Cover the gel with TAE 1x bufer (6.15) and gently pull out the comb.

l) Load in each well 10 µL of the amplification product (7.2.2 point "n"), adding loading buffer (6.12) according to the manufacturer's instructions.

m) Load the first and the last well with 10 µL of the L50 solution (6.17).

n) Connect the electrophoresis apparatus with the power supply and set 10 v/cm of gel.

o) Run the gel for about 30 min or until the fastest dye contained in the loading buffer reaches a distance of 1 cm from the gel border.

p) After 30 min, switch off the power supply, place the gel under UV illumination and check the band separation. The electrophoresis run is adequate if it is possible to distinguish easily all bands of the molecular weight marker ranging from 250 to 2000 bp. If the separation is incomplete, continue the run.

q) At the end of the run, transfer the gel to the imaging system (5.7) and print the result.

7.2.4 Interpretation of the PCR amplification results on agarose gel

The size of the amplification bands revealed by the electrophoresis is evaluated by comparison with the reference molecular weight L50 (6.17) and with the positive extraction and amplification controls (6.22, 6.23). The visual evaluation is considered sufficient and adequate (see Table A).

The amplification test is considered valid if:

1) the amplification of the positive control (6.23) shows an amplification product as in Table A;
2) the amplification of the negative control does not show any amplification product or, eventually, only bands related to unincorporated oligonucleotides and/or primer dimers

In case the sample shows one or more unexpected bands (differing from those in Table A) or if it shows no amplification, the presence of inhibitors will be tested by mixing equal volumes of *O. felineus* reference DNA (6.23) and test sample DNA (7.2.1 point "t"), and amplifying the mix by PCR reaction as described in the paragraph 7.2.7.

7.2.5 Visualisation of results on capillary electrophoresis (alternative to 7.2.3)

a) Switch on the Qiaxcel instrument (5.13) and the relative Qiaxcel ScreenGel management software on the PC;

b) Access the "Process Profile" panel; indicate under "Cartridge Type" the option "DNA HighRes";

indicate the desired profile and Experiment Directory;

c) Move the tube tray to the "Access Position" by selecting the "Load Position" item from the "Status Information" panel;

d) Insert in MARKER1 position the 12 tubes containing at least 10 µL of the chosen "Alignment Marker" (6.27); then return the tray to the initial position by selecting the "Park Position" from the "Status Information" panel;

e) Starting from the "A" row, position the samples to analyze (minimum volume 10 µL) in rows of 12 tubes. If the samples to be analysed are not enough to complete the row of 12, add the required number of tubes containing the QX DNA dilution buffer (minimum volume 10 µL) supplied with the QIAxcel DNA High Resolution kit (6.19);

f) For each analysis session (which may include up to a maximum of 8 runs of 12 samples), include a tube containing the DNA size marker (6.21);

g) In "Run Parameters" set the option 0M500 under "Method"; select the runs on the virtual plate in the "Sample Row Selection" side panel;

h) In "Sample Selection" set the run parameters as follows:

"Plate ID": insert first and last sample code of the plate

"Alignment Marker": select the chosen alignment marker (6.20)

i) In "Sample Information" enter the names of the samples in the corresponding boxes.

j) in "Run Check" check that all the selected rows are occupied by tubes containing PCR products to be analyzed or QX DNA dilution buffer and that the alignment Marker has been loaded, then select the appropriate boxes; finally select "Run";

k) At the end of the run, close the program and turn off the instrument.

Alignment of the molecular weight references

Visualize the results by selecting the "Absolute migration time" mode from the "Image options" menù and process the data with the "Start analysis" command. Scroll through the electropherogram of each sample and identify the peaks relating to the alignment markers by comparison with the electropherogram of the negative control. Eliminate the peaks lower and higher than those of the alignment marker. Then, process again the data with the "Start analysis" command in "Relative migration time" mode by selecting the corresponding option.

Print the obtained result to archive.

The standard methods for daily use are described above, for all other results interpretation refer to the user manual of the instrument.

7.2.6 Interpretation of the PCR amplification results on capillary electrophoresis

The data analysis shall consider only those bands satisfying the following requirements:

1) Band size bigger than 50 bp;
2) Comprised between the two Alignment marker bands (6.20);
3) Intensity of the emission peak greater than a threshold value of 5%.

In case of overlapped peaks, only the peak showing the higher intensity will be considered; if peak values are comparable, the result is rejected.

The size of the amplification bands revealed by the electrophoresis is evaluated by:

i) visual comparison with the “DNA size marker” (6.21) and with the positive extraction and amplification controls on the virtual gel;

ii) comparison between the band size calculated by the software and the expected band size;

If the sample shows one or more unexpected bands (differing from those in Table A) or no amplification, the presence of inhibitors will be tested by mixing equal volumes of *O. felineus*

reference DNA (6.23) and test sample DNA (7.2.1 point "t"), and amplifying the mix by PCR reaction as described in the paragraph 7.2.7.

7.2.7 Test for the presence of inhibitors by PCR with O. felineus reference DNA

If nor otherwise stated, keep tubes on ice, use tips with barrier and wear disposable gloves.

At each working session, use reference DNA (6.23) as positive.

The following procedure uses a PCR master mix at a 2X concentration. If the concentration is different, modify the procedure following the manufacturer's recommendations.

a) Thaw: DNA/fecal samples, 2x PCR MasterMix (6.10), SetO (6.11), amplification positive control (reference DNA, 6.23).
b) Mark with a progressive number an adequate number of 0.2 µL PCR tubes.
c) Prepare an adequate cumulative volume of amplification mix. Calculate the volume on the basis of a single sample amplification mix (Table F) and of the total number of samples added of two reactions (one for the positive amplification control and one for the negative control).

Table E– Amplification mix for a single sample: components and volumes

2x PCR MasterMix (6.10)	15 µL
H_2O	8 µL
SetO (6.11)	1 µL
Reference DNA (6.23)	2 µL
Totale	28 µL

d) Mix the amplification mix by vortexing and centrifuge at maximum speed for few seconds.
e) Transfer 28 µL of the cumulative amplification mix to each PCR tube (point "b").
f) Add to each tube 2 µL of the DNA/fecal samples to be tested.
g) Close the tubes, mix by vortexing and centrifuge (5.1) at maximum speed for a few seconds.
h) Start the amplification cycle on the thermocycler device (5.4) according to table D, paragraph 7.2.2
i) At the end of the amplification step, centrifuge (5.1) the tubes at maximum speed for a few seconds.
i) Leave the tubes on ice or in a refrigerator (5.5) until the electrophoresis.

7.2.8 Results

To visualize the results, follow the procedure described at point 7.2.3. and 7.2.5

7.2.9 Interpretation of the PCR amplification results on agarose gel and capillary electrophoresis

If the expected 248 bp fragment from *O. felineus* is amplified, the presence of inhibitors is excluded and the sample will be judged as "negative", the results of the test is expressed as "negative".

If the expected 248 bp fragment is not amplified, a new DNA extraction from the test sample is performed.

8 RESULTS

The results are expressed as follows:

If the amplification product show a size of 248 bp, the sample is classified "positive" for *Opisthorchis* spp".

If the test is valid and the sample shows amplification products different from those reported in table A, the sample is classified "negative".

9 CHARACTERISTICS OF THE METHOD

The present method has been characterized in terms of sensitivity, specificity and repeatability, and the validation process confirmed that the method is suitable for the specific aim.

10. SAFETY MEASURES

This method has to be carried out only by authorized personnel. The analyst shall wear individual protection devices (gloves and lab coat) while performing the test.