Identification of *Anisakidae* Larvae at the species level by multiplex PCR

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1. Aim and field of application

To identify the species or genotype of single *Anisakidae* larvae preserved in ethanol by a multiplex PCR analysis. This method can be applied to larvae, or their portions, collected from human biopsies or from tissues of animal origin.

2. Principle of the method

The PCR is a molecular biology technique that allows for the amplification of specific nucleic acid fragments, which initial and terminal nucleotide sequences are known (oligonucleotide pair). If a species (or 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. Multiplex-PCR is an application of standard PCR where two o more pairs of primers are used at the same time. In such a way, it is possible to amplify multiple sequences at the same time during the same reaction.

The larval stages of nematodes of the Anisakidae family parasitize fishes, cephalopods and shrimps and the *Anisakidae* adult worms parasitize fishes, marine birds and mammals. The larvae of the genus *Anisakis* and *Pseudoterranova* are responsible, if ingested by humans, of the disease known as anisakiasis. Larvae, obtained from human patiens or infected hosts, can be morphologically identified, however larvae of the genus *Anisakis* and *Pseudoterranova* cannot be distinguished.

Molecular methods based on the multiplex PCR allowed, based on the different size of the PCR fragments amplified from a portion of the ITS (Internal Transcribed Spacer) region, to distinguish at the species level single larvae of *Anisakis* spp. from that of *Pseudoterranova, Contracaecum* and *Hysterotilacium spp.,* and, within *Anisakis* spp., between *A. pegreffii*, A*. simplex s.l.* (including the hybrid genotype *A. simplex/A. pegreffii*), *A. physeteris* (including *A. brevispiculata and A. paggiae*) and *A. typica*.

The sizes of the fragments produced by the amplification with nucleotide pairs specific for each member of the Anisakidae family, are shown in *Table A*.

Table A – Expected size of the ITS fragments (base pairs) for each species obtained with the reported primers.

Primer 5'	*Primer 3'*	*A. pegreffii*	*A. simplex* s.l. e *A. simplex*/pegreffii ibrido	*A. physeteris*[a]	*A. typica*	*Contracaecum osculatum*	C. rudolphii (A, B, C)	*Pseudoterranova* spp.	*Hysterotilacium aduncum*
ASPf	RevB	588bp	588bp						
APE1	"	672bp							
APyf	"			143bp					
ATf	"				427bp				
COf	"					799bp			
CRf	"						307bp		
PDf	"							370bp	
HAf	"								991bp

[a] can amplify also *Anisakis brevispiculata* and *A. paggiae*

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4. Definitions

ITS (Internal Transcribed Spacer 1), interspaced sequence of the nuclear ribosomal gene including the ITS-1, the 5.8S gene, ITS-2 gene with the addition of 70 bp of the 28S gene

Oligonucleotide, short sequence (15/30 nucleotide bases) used to amplify a DNA specific fragment

Multiplex Primers Mix, mix of 9 oligonucleotide base pairs amplifying ITS from each species

Reference larvae, larvae of *Anisakis pegreffi* in ethanol, supplied by Dipartimento di Scienze di Sanità Pubblica "G. Sanarelli", Università Sapienza, Roma

Reference DNA, purified DNA from larvae of *Anisakis pegreffi*, supplied by Dipartimento di Scienze di Sanità Pubblica "G. Sanarelli", Università Sapienza, Roma

Positive control for the DNA extraction, a reference larva analysed in the same working session of test samples, to verify the efficacy of the DNA extraction session

DNA/larva, DNA extracted from a single larva

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

Negative control for the amplification, reagent grade water; this control is used in the amplification session to verify the efficacy of the PCR

PCR, Polymerase Chain Reaction.

The definitions and terminology used in the UNI EN ISO 22174 standard are applied in the present method.

5. Devices/instruments

5.1 Stereo microscope, magnification 60÷100x

5.2 Bench top refrigerated centrifuge for 1.5 mL tubes, minimum 10,000xg

5.3 Freezer ≤-15°C

5.4 Thermoblock with vibration, temperature range 25÷100°C

5.5 Magnetic separation stand

5.6 PCR thermocycler

5.7 Refrigerator, temperature range +1÷+8°C

5.8 Horizontal electrophoretic apparatus

5.9 Analytical balance, readability 0.1 g

5.10 UV transilluminator

5.11 Digital imaging system

5.12 Adjustable volume pipettes, volume range: 1-10 µL, 2-20 µL, 20-100 µL, 50-200 µL, 200-1000 µL

5.13 Analytical grade water system production, resistivity ≥ 18 Mohm/cm

5.14 Vortex

5.15 Orbital shaker

6. Reagents and chemicals

6.1 **Incubation buffer.** Commercial solution: DNA IQ™ System kit, Promega, code DC6701 or DC6700. Once prepared, label the solution with “IB+”. Store PK and DTT and their aliquots at -20°C , whereas store the incubation buffer at +4°C. **6.2** **Lysis buffer.** Commercial solution: Tissue and Hair Extraction Kit, Promega, code DC6740. Once prepared, label the solution with “LB+”.Store DTT and its aliquots at -20°C , whereas store the lysis buffer at +4°C. .

6.3 **Paramagnetic resin.** Commercial suspension: DNA IQ™ System kit, Promega, code DC6701 or DC6700. Store at room temperature.

6.4 **Washing buffer.** Commercial solution: Tissue and Hair Extraction Kit, Promega, code DC6740. Once prepared, label the solution with "WB+". Store at room temperature **6.5 Eluting buffer.** Commercial solution: Tissue and Hair Extraction Kit, Promega, code DC6740. Store at room temperature **6.6 2x PCR master mix.** 2x commercial solution, Promega, codes: M7501, M7502, M7505 (composition: dATP 400 µM, dCTP 400 µM, dGTP 400 µM, dTTP 400 µM, MgCl2 3mM, Taq DNA polymerase 50 U/mL), other commercial PCR master mixes should be considered suitable for PCR amplification. Store according to the manufacturer's recommendations.

6.7 **Oligonucleotides.** Commercial preparation (*Table B*); the lyophilized products is reconstituted with TE 0.1x, according to the manufacturer's recommendations, at a concentration of 100 pmol/µL; the lyophilized product can be stored frozen for up to 20 years; the reconstituted product can be stored frozen up to 10years.

6.8 **10X multiplex Primers Mix.** The oligonucleotides mixture (6.7) used for the multiplex PCR; the mixture is obtained combining a defined volume of the oligonucleotides reported in *Table B* in TE 0.1x (6.16); the final concentration corresponds to 3 pmol/µL, except for APEf, at 4 pmoli/µL, and RevB, at 6 pmoli/µL. 100µL aliquots are prepared and stored frozen up to 10years.

Table B – Oligonucleotides present in the 10X Multiplex Primers' Mix (6.7 e 6.8), their codes and amplified nucleotide sequences.

Oligonucleotide sequence	Code	Target sequence
5'- TTGCAATCACTTCTCTCAGATTG -3'	ATf	ITS
5'- GGCTGGTTGATGAACTGTTG -3'	APyf	ITS
5'- GACATTGTTATTTCATTGTATGTGTTGAAAATG -3'	ASPf	ITS
5'- GAGCAGCAGCTTAAGGCAGAGGC -3'	APE1	ITS
5'- CGAGTACTTTTTATGGTCGTGAAGT -3'	PDf	ITS
5'- TGATATGCTTGAAAGGCAGG -3'	COf	ITS
5'- CGACAAGCAGTGTCCCTTTG -3'	CRf	ITS
5'- GCCTTCCATATGCGCGTATA -3'	HAf	ITS
5'- GCCGGATCCGAATCCTGGTTAGTTTCTTTTCC-3'	RevB T	ITS

6.9 **Loading buffer 6x**. Commercial product allowing DNA molecule electrophoresis to be performed. Store according to the manufacturer's recommendations.

6.10 **Agarose**. Commercial product suitable for performing DNA molecule electrophoresis. Store at room temperature according to the manufacturer's recommendations.

6.11 **TAE solution 50x**. Commercial product (2M Tris-acetate, 50mM EDTA, pH 8.2–8.4 at 25°C). Store according to the manufacturer's recommendations.

6.12 **TAE solution 1x.** 1000 mL preparation: take 20 mL of the 50x solution and bring to 1000 mL with water. Store at room temperature for up to 1 month.

6.13 **Ethidium bromide solution**. Commercial product 10 mg/L. For the working condition, dilute 1:100,000; for 100 mL solution, add 1.0 µL. Store in the dark at room temperature for up to 24 months.

NOTE: Ethidium bromide is potentially mutagenous, carcinogenic and teratogenic; wear disposable gloves and handle the solution containing this substance very carefully.

6.14 **L50.** Commercial product containing markers for DNA molecular weight multiple of 50 bp. All commercial products containing molecules of multiples of 50 bp within the 50-1000 bp range can be used. Store refrigerated according to manufacturer's recommendations.

6.15 **TE 1x solution**. Commercial product 10mM Tris-HCl (pH 8,0), 1mM EDTA-Na_2, pH 7.9–8.1 at 25°C.

Store refrigerated according to the manufacturer's recommendations.

6.16 **TE 0.1x solution.** TE 1x diluted solution. For 100 mL preparation: take 10 mL from 1x solution and add 90 mL water. Filter with 0.22 µm filters and prepare aliquots of 10 mL. Store frozen for up to 24 months.

6.17 **Milli-Q grade water.** Resistivity ≥ 18 Mohm/cm

6.18 **Reference Larvae.** Larvae of *A. pegreffi* stored in ethanol (95-99%) supplied by Dipartimento di Scienze di Sanità Pubblica "G. Sanarelli", Università Sapienza, Roma. Store frozen for up to 10 years.

6.19 **Reference DNA.** Genomic DNA purified from a reference larva. Reference DNA (1ng/µL), is supplied by Dipartimento di Scienze di Sanità Pubblica "G. Sanarelli", Università Sapienza, Roma, and prepared according to the protocols described in Sambrook et al. (1989). Store frozen for up to 10 years.

7. Procedure

7.1 Sample preparation

- Test samples are inspected to verify the presence of larvae and the preservation conditions. If the condition are not suitable, the test will not be performed.
- The ethanol containing the larvae is transferred into a Petri dish and observed under the stereo microscope. A maximum of 3 larvae are collected and placed in 1.5 mL conical tubes, one larva in each tube. Excess ethanol is removed and the minimum volume is left.
- Spin tubes containing larvae at maximum speed for a few seconds.
- Store the tubes frozen. Under these conditions, larvae can be stored for the DNA extraction for up to 10 years.

7.2 Method

7.2.1 *DNA extraction from single larva*

- If not otherwise specified, the procedure is carried out at room temperature.
- Each working session requires the DNA extraction of a reference larva identified as "positive control for the extraction".
- Before starting the procedure, prepare a sufficient volume of the IB+ (6.1) and LB+ (6.2) solutions according to the manufacturer's recommendations.

a) Centrifuge the tubes containing the larvae to be identified at maximum speed for a few seconds.
b) Add 100 µL of IB+ (6.1).
c) Incubate at 55°C for 30-60 min in the thermoblock. During incubation, shake at 1,400 vibrations/min.
d) Centrifuge, as in point "a".
e) Add 200 µL of LB+ (6.2).
f) Add 10 µL of paramagnetic resin (6.3) after resuspending it by vortexing.
g) Incubate for 5-10 min at 25°C in the thermoblock. During incubation, shake at 1,400 vibrations/min.
h) Place the tubes in the magnetic separation stand and wait for 30-60 sec, so that the paramagnetic resin particles are blocked by the magnetic field on the tube wall.
i) Discard the liquid phase by aspirating, avoiding the dislodging of the resin particles.
j) Add 100 µL of LB+ (6.2) and resuspend the resin particles by vortexing.
k) Place the tubes in the magnetic separation stand, as in point "h".
l) Discard the liquid phase by aspirating.

m) Add 100 µL of WB+ 1x (6.4) and resuspend the resin particles by vortexing.
n) Place the tubes in the magnetic separation stand, as in point "h".
o) Discard the liquid phase by aspirating.
p) Repeat the washing step, from "m" to "o", with WB+ (6.4) 3 times.
q) After the last washing, leave the tubes open to let the resin particles dry for 15-20 min.
r) Add 100 µL of the eluting buffer (6.5) and gently resuspend the resin particles, do not vortex.
s) Incubate at 65°C for 5 min. During incubation, shake at 1,400 vibrations/min.
t) Place the tubes in the magnetic separation stand, as in point "h".
u) Collect the liquid phase (about 90-100 µL) and transfer it to a 1.5 mL tube.
v) The resulting extract is defined as "DNA/larva" and stored frozen. Under these conditions, it can be stored for up to 10 years.

7.2.2 Multiplex PCR amplification

- Unless otherwise clearly stated, store tubes on ice; use tips with barrier and wear disposable gloves.
- At each working session, use a positive and a negative amplification control. Use reference DNA (6.19) as positive control and water (6.17) as negative control.

a) Thaw DNA/larva, 2x PCR MasterMix, 10X multiplex Primers Mix., and positive amplification controls.
b) Mark with a progressive number an adequate number of 0.2 µL PCR tubes.
c) Prepare an adequate cumulative volume of the amplification mix. Evaluate the volume on the basis of a single sample amplification mix (*Table C*) and of the total number of samples plus two (1 for the positive amplification control and 1 for the negative one).

Table C – single sample amplification mix: components and volumes

2x PCR MasterMix (6.6)	25 µL
10x Multiplex PCR Primers Mix	5 µL
H_2O	22 µL
Total	48 µL

d) Mix the amplification mix by vortexing and centrifuge at maximum speed for a few sec.
e) Transfer 48 µL of the cumulative amplification mix to each PCR tube (point "b").
f) Add 2 µL of the larval DNA to be tested to each tube.
g) Close the tubes, mix by vortexing and centrifuge at maximum speed for a few sec.
h) Start the amplifying cycle (*Table D*) on the thermocycler device; wait for the temperature to reach 95°C and insert the tubes in the thermoblock by pausing the instrument.

Table D – amplification cycles

Pre-denaturation	2 min/95°C
Amplification	30 s/95°C 30 s/55°C 45 s/72°C
Number of cycles	30
Final extension	7 min/72°C

i) At the end of the amplification phase, centrifuge the tubes at maximum speed for a few sec.
l) Add 5.0 µL of loading buffer 6x (6.9).
m) Vortex and centrifuge the tubes at maximum speed for a few sec.

n) Keep tubes on ice or refrigerated until starting electrophoresis.

7.2.3 Result display by gel electrophoresis

a) Assemble the electrophoresis apparatus according to the manufacturer's recommendations. For the gel preparation, use a comb suited for the number of samples.
b) Add 1.6 gr agarose (6.10) in 100 mL TAE 1x (6.12) in a glass beaker.
c) Gently resuspend the 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 by boiling.
f) Allow the agarose solution to cool.
g) Before it solidifies (at about 47°C), add 1.0 µL of ethidium bromide solution (6.13).
h) Shake gently to dissolve uniformly the ethidium bromide 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 buffer (6.12) and gently pull out the comb.
l) The first or last well are loaded with 15 µL of the L50 solution (6.14).
m) Load in each well 10 µL of the amplification product (point 7.2.1 "n"), respecting the progressive numbering of the tubes (point 7.2.1 "b").
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 (6.9), 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 easily distinguish all bands of molecular weight marker ranging from 250 to 1000 bp. If the separation is incomplete, continue the run.
q) At the end of the run, transfer the gel to the imaging system and print the result.

7.2.4 Result Interpretation by gel electrophoresis

The size of the amplification bands revealed by the electrophoresis is evaluated by comparison with the reference molecular weight L50 (6.14) and with the positive controls of extraction and amplification. The visual evaluation is considered sufficient and adequate (see *Table A*).

The amplification test is considered valid if:

a) the amplification of the positive control shows an amplification product as in *Table A*;
b) the amplification of the negative control does not show any amplification product or, eventually, only bands related to unused oligonucleotides and/or primer dimer
c) the positive control of the extraction product shows an amplification product as in *Table A*;

The species identification is made comparing the size of the amplification fragment(s) produced by the sample(s) with those shown in *Table A*.

In case the sample shows an unexpected band, the identification of the sample at the species level is not possible.

8. Results

The results are expressed as follows:

If the amplification band is comparable with 588bp, the sample is identified as *A. simplex sl.*

If the amplification bands are comparable with the doublet 588bp-672bp, the sample is identified as *A. pegreffi.*

If the amplification band is comparable with 143bp, the sample is identified as *A. physeteris.*

If the amplification band is comparable with 427bp, the sample is identified as *A. typica.*

If the amplification band is comparable with 370bp, the sample is identified as *Pseudoterranova spp.*

If the amplification band is comparable with 799bp, the sample is identified as *Contracaecum osculatum*

If the amplification band is comparable with 307bp, the sample is identified as *Contracaecum rudolphii (A, B, C).*

If the amplification band is comparable with 991bp, the sample is identified as *Hysterotilacium aduncum.*

In case the digestion test was valid but the sample displays a profile of bands not comparable with those reported in *Table A*, the identification at the species level is considered “impossible”.

9. Characteristics of the method

This method has been characterised in terms of repeatability and specificity. The results of the validation protocol are used to confirm that the method is suitable for the expected aim and are reported in the validation report, which can be sent upon request.

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 general safety measures, refer to the CDC guidelines.