Molecular identification of nematode larvae different from those of *Trichinella* genus detected by artificial digestion method of muscle tissue

STANDARD OPERATING PROCEDURE

SUMMARY

1. SCOPE

This document describes a method, based on PCR amplification and sequencing of molecular markers that can be used to identify at the species, genus or family level nematode larvae isolated by artificial digestion of muscle tissues from different host species.

2. INTRODUCTION

The gold standard method for detecting *Trichinella* sp. larvae in muscle tissues of animals intended for human consumption is the artificial digestion (Commission Regulation, 2015). However, the detection of nematode larvae outside their natural niche (i.e., the muscle cell) can lead to an incorrect diagnosis of *Trichinella* infection during routine inspections. In fact, larvae of nematodes living in or migrating to different niches (e.g., gut lumen, liver, lungs, lymphatic or blood vessels) can contaminate muscle tissues and be mistakenly identified as belonging to the genus *Trichinella*. The size and shape of these larvae are often sufficiently different from those of a *Trichinella* larva; however in other cases, a thorough knowledge of *Trichinella* larva morphology is needed. It is well known that the morphological identification of nematode larvae at the species, genus or family level is difficult or impossible to be achieved, given the lack of specific characters, which are quite often present only at the adult stage.
The use of short DNA sequences as taxon “barcodes” to differentiate or discover new species is increasingly taking hold inside the scientific community. Molecular target regions as Internal Transcribed Spacer 1 (ITS1), cytochrome C oxidase subunit 1 (COI) and ribosomal DNA genes (18S, 12S) could allow a fast, objective and efficient identification of these nematodes when the morphological identification is hard or not possible.

3. REFERENCES

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4. DEFINITIONS

ITS1 (Internal Transcribed Spacer 1), interspaced sequence 1 of the nuclear ribosomal DNA gene cluster.
COI (cytochrome C oxidase subunit 1), mitochondrial gene encoding subunit 1 of cytochrome C oxidase.
18S, part of the nuclear ribosomal DNA gene cluster encoding a component of the small eukaryotic ribosomal subunit (40S).
12S, mitochondrial gene encoding the 12S ribosomal RNA.

5. EQUIPMENT

5.1 Stereo microscope, magnification 60÷100x
5.2 Bench top centrifuge for 1.5 mL tubes, minimum 10,000xg
5.3 Freezer ≤-20°C
5.4 Thermo-block 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 0.1g
5.10 UV transilluminator
5.11 Digital imaging system
5.12 Adjustable volume pipettes, volume range: 1-10µL, 2-20µL, 20-100µL, 50-200µL, 200-1000µL
5.13 Analytical grade water system production, resistivity ≥ 18 Mohm/cm
5.14 Vortex
5.15 Power supply for gel electrophoresis

6. REAGENTS

6.1 Incubation buffer. Commercial solution: DNA IQ™ System kit, Promega, code DC6701 or DC6700. Once prepared, label the solution with “IB+”. Store according to the manufacturer’s recommendations.
6.2 Lysis buffer. Commercial solution: Tissue and Hair Extraction Kit, Promega, code DC6740. Once prepared, label the solution with “LB+”. Store according to the manufacturer’s recommendations.
6.3 Paramagnetic resin. Commercial suspension: DNA IQ™ System kit, Promega, code DC6701 or DC6700. Store according to the manufacturer’s recommendations.
6.4 Washing buffer. Commercial solution: Tissue and Hair Extraction Kit, Promega, code DC6740. Store according to the manufacturer’s recommendations.
6.5 Elution buffer. Commercial solution: Tissue and Hair Extraction Kit, Promega, code DC6740. Store according to the manufacturer’s recommendations.

6.6 HotStarTaq DNA Polymerase. 2x commercial solution, Qiagen, codes: 203203, 203205, 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 10 years.

Table A. Oligonucleotides sequences

Code	Sequence	Target
025 026	5'-TAACAAGGTTTCCGTAGGTG-3' 5'-AGCTRGCTGCGTTCTTCATCGA-3'	ITS1
JB3 JB4.5	5'-TTTTTTGGGCATCCTGAGGTTTAT-3' 5'-TAAAGAAAGAACATAATGAAAATG-3'	COI
SSU18A SSU26R	5'-AAAGATTAAGCCATGCATG-3' 5'-CATTCTTGGCAAATGCTTTCG-3'	18S
12Sf 12Sr	5'-GTTCCAGAATAATCGGCTA-3' 5'-ATTGACGGATG(AG)TTTGTACC-3'	12S

6.8 Loading buffer 6x. Commercial product allowing DNA gel electrophoresis. Store according to the manufacturer's recommendations.

6.9 Agarose. Commercial product suitable for performing DNA gel electrophoresis. Store according to the manufacturer's recommendations.

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

6.11 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.

6.12 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 according to the manufacturer's recommendations.
NOTE: Ethidium bromide is potentially mutagenous, carcinogenic and teratogenic; wear disposable gloves and handle the solution containing this substance very carefully.

6.13 L100. Commercial product containing DNA fragments multiples of 100 bp for use as molecular weight standards for agarose gel electrophoresis. All commercial products containing DNA fragments within the 50-1000 bp range can be used. Store refrigerated according to manufacturer's recommendations.

6.14 TE 1x solution. Commercial product 10mM Tris-HCl (pH 8,0), 1mM EDTA-Na2, pH 7.9–8.1 at 25°C. Store refrigerated according to manufacturer's recommendations.

6.15 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.

6.16 Milli-Q grade water. Resistivity ≥ 18 Mohm/cm or commercially available DNA Nuclease-Free Water for molecula biology application.

6.17 Buffer PBI Commercial solution: QIAquick PCR Purification Kit (Qiagen). Store according to the manufacturer's recommendations.

6.18 Buffer PE Commercial solution: QIAquick PCR Purification Kit (Qiagen). Store according to the manufacturer's recommendations.

6.19 Buffer EB Commercial solution: QIAquick PCR Purification Kit (Qiagen). Store according to the manufacturer's recommendations.

7. PROCEDURE

7.1 DNA extraction

If not otherwise specified, the procedure is carried out at room temperature. 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) Transfer the parasite (or a part of it) on the bottom of a 1.5 mL Eppendorf tube by using a stereo microscope. If the sample contains ethanol, it is necessary to wait its complete evaporation before adding the lysis buffer.
b) Add 20 µL of IB+ (6.1) and incubate at 55°C for 30-60 min in the thermoblock. During incubation, shake at 1,400 vibrations/min.
c) Add 40 µL of LB+ (6.2).
d) Add 4 µL of paramagnetic resin (6.3) after re-suspending it by vortexing.
e) Incubate in the thermoblock at 25°C for 5-10 min. During incubation, shake at 1,400 vibrations/min.
f) Place the tubes in the magnetic separation stand and wait for 30 s, so that the paramagnetic resin particles are blocked by the magnetic field on the tube wall.
g) Discard the liquid phase by aspiration, avoiding the dislodging of the resin particles.
h) Add 100 µL of LB+ (6.2) and re-suspend the resin particles by vortexing.
i) Place the tubes in the magnetic separation stand, as in point "f".
j) Discard the liquid phase by aspiration.
k) Add 100 µL of WB 1x (6.4) and re-suspend the resin particles by vortexing.
l) Place the tubes in the magnetic separation stand, as in point "f".
m) Discard the liquid phase by aspirating.
n) Repeat the washing step, from "k" to "m", with WB (6.4) for a total of 3 times.
o) After the last washing, leave the tubes open to let the resin particles dry for 15-20 min. at room temperature.
p) Add 50 µL of the elution buffer (6.5) and gently re-suspend the resin particles, do not vortex.
q) Incubate at 65°C for 5 min. During incubation, shake at 1,400 vibrations/min.
r) Place the tubes in the magnetic separation stand, as in point "f".
s) Collect the liquid phase and transfer it to a new 1.5 mL tube.
t) The resulting extract is defined as "DNA" and stored frozen. Under these conditions, it can be stored for up to 5 years.

7.2 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.

a) Thaw DNA, 2x PCR MasterMix, SetB, and a positive amplification control.
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 (6.12)	15 µL
H_2O	4 µL
SetB (6.13)	1 µL
Totale	20 µL

d) Mix the amplification mix by vortexing and centrifuge at maximum speed for a few seconds.
e) Transfer 20 µL of the cumulative amplification mix to each PCR tube (point "b").
f) Add 10 µL of the DNA to be tested to each tube.
g) Close the tubes, mix by vortexing and centrifuge at maximum speed for a few seconds.
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

	ITS1	COI	18S	12S
Pre-denaturation*	15 min/95°C	15 min/95°C	15 min/95°C	15 min/95°C
Amplification	30 s 95°C 30 s 45°C 1 min 72°C	30 s 95°C 30 s 50°C 1 min 72°C	30 s 95°C 1 min 52°C 1 min 72°C	30 s 95°C 30 s 50°c 1 min 72°C
Number of cycles	35	35	35	35
Final extension	7 min/72°C	7 min/72°C	7 min/72°C	7 min/72°C

*Pre-denaturation time may vary depending on PCR Master Mix manufacturer.

i) At the end of the amplification phase, centrifuge the tubes at maximum speed for a few seconds.
j) Keep tubes on ice or refrigerated until starting electrophoresis.

7.3 Result display

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 1.5 gr agarose (6.9) in 100 mL TAE 1x (6.11) in a glass beaker.
c) Gently resuspend the powder by rotation.
d) Heat the agarose suspension up to boiling point or until the solution became clear and homogeneous.
e) Restore with water the volume lost by boiling.
f) Allow the agarose solution to cool.
g) Before it solidifies, add 1.0 µL of the ethidium bromide solution (6.12).
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 solidification.
j) Place the tray with the gel in the electrophoresis apparatus.
k) Cover the gel with TAE 1x buffer (6.11) and gently pull out the comb.
l) Load the first well with 15 µL of the L100 solution (6.13).
m) Mix 15 µL of the amplification product with the proper amount of loading buffer and load the mix in each well, respecting the progressive numbering of the tubes.
n) Connect the electrophoresis apparatus with the power supply and set 10 v/cm of gel.
o) Run the gel until the fastest dye, contained in the loading buffer (6.9), reaches a distance of 1 cm from the gel border.
p) Switch off the power supply, place the gel under UV illumination and check the band separation. The electrophoresis run is adequate if all the bands of the molecular weight marker are well separated. If the separation is incomplete, continue the run.

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

7.4 PCR products purification

In case of positive amplification, the PCR products will be purified for sequencing.

a) Add 5 volumes of Buffer PBI (6.17) to 1 volume of the PCR sample and mix.
b) Apply the sample to the QIAquick column and centrifuge for 60 s.
c) Discard flow-through. Place the QIAquick column back into the same tube.
d) Add 0.75 ml of washing Buffer PE (6.18) to the QIAquick column and centrifuge for 60 s.
e) Discard flow-through and place the QIAquick column back in the same tube and centrifuge the column for additional 60 s.
f) Place QIAquick column in a clean 1.5 mL microcentrifuge tube.
g) To elute DNA, add 50 µl Buffer EB (6.19) or water (pH 7.0–8.5) to the centre of the QIAquick membrane and centrifuge the column for 1 min.

7.5 DNA sequencing

The purified PCR products are quantified and sent to a DNA Sequencing Service Company together with the proper oligonucleotides.

7.6 Sequence analysis

The obtained sequences are compared with the GenBank database by Basic Local Alignment Search Tool (BLAST) to find regions of similarity.

8. 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 guidelines of CDC (http://www.who.int/ihr/publications/bioriskmanagement_1/en/).