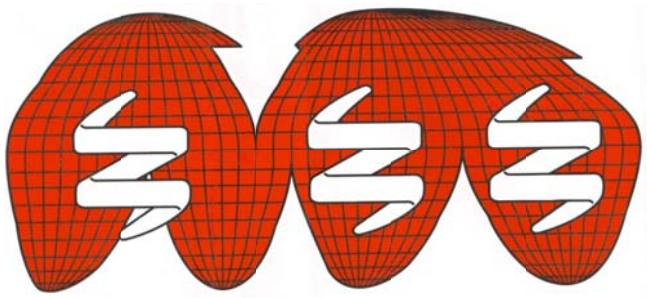

International Commission on Trichinellosis

Guidelines for the Identification of *Trichinella* Muscle Stage Larvae at the Species or Genotype Level

INDEX

1. Aim and field of application

To identify the species or genotype of *Trichinella* larvae by a multiplex PCR analysis or other PCR derived methods. These techniques can be applied to larvae collected from human biopsies or from muscle tissues of animal origin.

2. *Trichinella* morphology

The morphology of adult worms (male, total length 0.62 mm to 1.58 mm, width 25 µm to 33 µm; female, total length 0.952 mm to 3.35 mm, width 26 µm to 43 µm) and of new born larvae (average 110 µm in length, 7 µm in width) does not have any diagnostic importance, since the only stage which can be easily isolated and identified is the muscle larva. Muscle larvae are L1 larvae because developmental molts occur only after their penetration in the gut mucosa of a new host. The following morphological characters can sexually distinguish male and female muscle larvae. Male larva: total length 0.641 mm to 1.07 mm; width 26 µm to 38 µm; intestinal bulb generally close to the convex surface; in some larvae close to the concave surface; intestine crossing the gonad from the convex to the concave surface; in some larvae, crossing the gonad from the concave to the convex surface and then re-crossing to the concave surface; length of rectum of about 40 µm to 50 µm. Female larva: total length 0.71 mm to 1.09 mm, width 25 µm to 40 µm; intestinal bulb generally close to the concave surface; intestine on the concave surface; in some larvae, intestine crossing the gonad from the concave to the convex surface and then re-crossing to the concave surface; length of rectum 17 µm to 35 µm; presence of a thickened subcuticular layer in the region of vulva primordium, i.e. on the convex surface at about 2/3 of the way along the stichosome. However, the recovery of nematodes belonging to genera other than *Trichinella* during routine meat inspection suggests that the persons performing the analyses need to be informed of the possibility of false positives and, consequently, the larva morphology should be kept in mind before the molecular identification.

3. Principle of the methods

The PCR is a molecular biology technique that allows for the amplification of specific nucleic acid fragments, of which the 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.

A modification of the “standard PCR” is the multiplex-PCR, where two or more oligonucleotide pairs are used. In this case, it is possible to amplify with a single PCR analysis more than one sequence at the same time.

Today, 9 sibling species, namely *T. spiralis, T. nativa, T. britovi, T. pseudospiralis, T. murrelli, T. nelsoni, T. papuae, T. zimbabwensis*, *T. patagoniensis*, and 3 genotypes, *Trichinella*-T6, *Trichinella*-T8 and *Trichinella*-T9, have been identified in the genus *Trichinella*. The comparative analysis of three nucleotide sequences belonging to the ITS1, ITS2 and ESV, allows the univocal identification of most epidemiologically relevant taxa: *T. spiralis, T. nativa, T. patagoniensis*, *T. britovi, T. pseudospiralis, T. murrelli, T. nelsoni, T. papuae, T. zimbabwensis* and *Trichinella*-T6. Since the difference between *T. nativa* and *T. patagoniensis* is of only two base pairs, they can be distinguished by PCR amplification and sequencing of the ESV; *T. britovi* and *Trichinella*-T9 can be distinguished by PCR-RFLP of the CO I mitochondrial gene, and; *T. britovi* and *Trichinella*-T8 can be distinguished by PCR amplification and sequencing of the ITS2.

The sizes of the ITS1, ITS2 and ESV fragments produced by the PCR amplification are shown in Table A.

Locus	*T. spiralis*	*T. nativa*	*T. patagoniensis*	*T. Britovi*[1]	*T. Pseudospiralis*	*T. murrelli*	*Trichinella T6*	*T. nelsoni*	*T. papuae*	*T. zimbabwensis*
ESV	173	129	127	129	292-360[2]	129	129	155	240	264
ITS1				253			210			
ITS2						316		404		

[1] and *Trichinella* T8 and *Trichinella* T9;

[2] a multiple band pattern (with 1, 2 or 3 bands) of variable size can be detected in this range.

Table A - Dimension of the expected amplification products (in base pairs) for each taxon.

Using the multiplex-PCR technique with 5 oligonucleotide pairs, it is possible to identify larvae with only one amplification assay at the species or genotype level.

4. References

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Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, NY, Vol. 1, 2, 3.

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Zarlenga DS, Chute MB, Martin A, Kapel CM. 1999. A multiplex PCR for unequivocal differentiation of all encapsulated and non-encapsulated genotypes of *Trichinella*. Int. J. Parasitol. 29:1859-67.

5. Definitions

CDC, Centers for Disease Control and Prevention, Office of Health and Safety (www.cdc.gov/od/ohs/biosfty/bmbl4/b4af.htm).

DNA/larva, DNA extracted from one or more larvae

ESV (Expansion Segment 5), sequence belonging to domain 4 of the nuclear ribosomal gene

ITS1 (Internal Transcribed Spacer 1), interspaced sequence 1 of the nuclear ribosomal gene

ITS2 (Internal Transcribed Spacer 2), interspaced sequence 2 of the nuclear ribosomal gene

MSL, muscle stage larvae

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

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

PCR, Polymerase Chain Reaction

Positive control for the amplification, a reference DNA supplied by RLT; this control is used in the amplification session to verify the efficacy of the multiplex-PCR system

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

Reference larvae, larvae belonging to a known *Trichinella* species or genotype and with the ISS code (http://www.iss.it/site/Trichinella/index.asp), supplied by the RLT or another reference laboratory

Reference DNA, purified DNA belonging to a known *Trichinella* species or genotype with the ISS code (http://www.iss.it/site/Trichinella/index.asp), supplied by RLT or another reference laboratory

RLT, Reference Laboratory for Trichinellosis of the World Organization for Animal Health (OIE) and of the International Commission of Trichinellosis (ICT), Rome, Italy (http://www.iss.it/site/Trichinella/index.asp)

SetB, mix of 5 oligonucleotide base pairs amplifying specific sequences of single species

Test sample, one or more MSL collected from a single infected host and preserved in ethanol, to be identified

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

6. Devices/instruments

6.1 Stereo microscope, magnification 60÷100x

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

6.3 Freezer ≤-15°C

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

6.5 Magnetic separation stand

6.6 PCR thermocycler

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

6.8 Horizontal electrophoretic apparatus

6.9 Analytical balance, readability 0.1g

6.10 UV transilluminator

6.11 Digital imaging system

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

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

6.14 Vortex

7. Reagents and chemicals

The source manufactures of reagents reported below at points 7.1 – 7.6, are for reference purposes only and other suppliers can be used at the discretion of the user but changes will likely require optimization of the protocol.

7.1 **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.

7.2 **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.

7.3 **Paramagnetic resin.** Commercial suspension: DNA IQ™ System kit, Promega, code DC6701 or DC6700. Store according to the manufacturer’s recommendations.

7.4 **Washing buffer.** Commercial solution: Tissue and Hair Extraction Kit, Promega, code DC6740. Once prepared, label the solution with “WB+”. Store according to the manufacturer’s recommendations.

7.5 **Eluting buffer.** Commercial solution: Tissue and Hair Extraction Kit, Promega, code DC6740. Store according to the manufacturer’s recommendations.

7.6 **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.

7.7 **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 5 years; the reconstituted product can be stored frozen up to 18 months.

Oligonucleotide sequences	code	Amplified sequence
5'-GTT.CCA.TGT.GAA.CAG.CAG.T-3' 5'-CGA.AAA.CAT.ACG.ACA.ACT.GC-3'	cp-I.F cp-I.R	ESV
5'-GCT.ACA.TCC.TTT.TGA.TCT.GTT-3' 5'-AGA.CAC.AAT.ATC.AAC.CAC.AGT.ACA-3'	cp-II.F cp-II.R	ITS1
5'-GCG.GAA.GGA.TCA.TTA.TCG.TGT.A-3' 5'-TGG.ATT.ACA.AAG.AAA.ACC.ATC.ACT-3'	cp-III.F cp-III.R	ITS1
5'-GTG.AGC.GTA.ATA.AAG.GTG.CAG-3' 5'-TTC.ATC.ACA.CAT.CTT.CCA.CTA-3'	cp-IV.F cp-IV.R	ITS2
5'-CAA.TTG.AAA.ACC.GCT.TAG.CGT.GTT.T-3' 5'-TGA.TCT.GAG.GTC.GAC.ATT.TCC-3'	cp-V.F cp-V.R	ITS2

Table B. Oligonucleotide sequences of setB (7.7), their codes and amplified nucleotide sequences.

7.8 **SetB.** The oligonucleotide mixture (7.7) used for the multiplex-PCR; the mixture is obtained by combining an equal volume of the 10 oligonucleotides (7.7); the final concentration corresponds to 10 pmol/µL; 100µL aliquots are prepared and stored frozen up to 24 months.

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

7.10 **Agarose**. Commercial product suitable for performing electrophoresis of amplified DNA. Store at room temperature for up to 24 months.

7.11 **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.

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

7.13 **Ethidium bromide solution**. Commercial product: stock 10 mg/L. For the working solution, 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 mutagenic, carcinogenic and teratogenic; wear disposable gloves and handle the solution containing this substance very carefully.

7.14 **L50.** Commercial product: DNA molecular weight markers that migrate in increments of 50 bp. Any commercial product of similar size distribution i.e., 50 bp within the 50-500 bp range, can be used. Store refrigerated according to manufacturer's recommendations.

7.15 **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 for up to 12 months.

7.16 **TE 0.1x solution.** See 7.15. For 100 mL preparation of 0.1x TE, dilute 10 mL of the 1x solution to 100 ml with water. Filter with 0.22 µm filter and store aliquots of 10 mL frozen for up to 24 months.

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

7.18 **Reference Larvae.** *Trichinella* MSL stored in ethanol (95-99%) and with the ISS code supplied by RLT, Rome, Italy (http://www.iss.it/site/Trichinella/index.asp) or another reference laboratory. Reference larvae are supplied after isolation from mouse muscle tissues by HCl-pepsin digestion (see the procedure in OIE 2004). Store frozen for up to 5 years.

7.19 **Reference DNAs.** Genomic DNA purified from reference larvae. Reference DNAs are supplied (1ng/µL) by RLT. They are produced by RLT from a pool of reference larvae according to the protocols described in Sambrook et al. (1989). Store frozen for up to 5 years.

8. Procedure

8.1 Sample preparation

8.1.1 Mixed larvae

- Test samples are inspected to verify the integrity of MSL.
- If the worms are fresh or frozen, one can proceed directly to isolating DNA from MSL. If MSL have been preserved in ethanol, larvae should be washed in water or PBS 3 times prior to DNA isolation.

8.1.2 Individual larvae

- Test samples are inspected to verify the integrity of MSL.
- The ethanol containing MSL is transferred into a petri dish and observed under the stereo microscope. A maximum of 5 MSLs are collected and placed in 1.5 mL conical tubes, one MSL 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 5 years.

8.2 Method

8.2.1 DNA extraction from a mixed population of larvae

- Total nucleic acids can be isolated from a preparation of mixed MSL by any one of a multitude of methods for isolating genomic DNA from tissues including conventional proteinase K:SDS digestion followed by organic extraction. Numerous kits are commercially available for this purpose; however, isolation should involve the use of proteinase K to digest through the parasite cuticle. These procedures need not require removal of RNA; the presence of total RNA during PCR will not affect PCR amplification.

8.2.2 DNA extraction from one single larva

- If not otherwise specified, the procedure is carried out at room temperature.
- Each working session requires that a reference larva be submitted to the DNA extraction procedure and identified as “positive control for the extraction”.
- Before starting the procedure, prepare sufficient volumes of the IB+ (7.1) and LB+ (7.2) solutions according to the manufacturer’s recommendations.
  a) Centrifuge the tubes containing the MSL to be identified at maximum speed for a few seconds.
  b) Add 20 µL of IB+ (7.1).
  c) Incubate at 55°C for 30-60 min in the thermoblock. During incubation, shake at 1,400 vibrations/min.
  d) Centrifuge, as in 8.2.2a
  e) Add 40 µL of LB+ (7.2).
  f) Add 4 µL of paramagnetic resin (7.3). Be sure to completely suspend resin by vortexing before dispensing.
  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; avoid dislodging the resin particles from

the sides of the tube.

j) Add 100 µL of LB+ (6.2) and suspend the resin particles by vortexing.
k) Place the tubes in the magnetic separation stand, as in point 8.2.2h.
l) Discard the liquid phase by aspirating.
m) Add 100 µL of WB+ 1x (6.4) and suspend the resin particles by vortexing.
n) Place the tubes in the magnetic separation stand, as in point 8.2.2h.
o) Discard the liquid phase by aspirating.
p) Repeat the washing step, from 8.2.2m to 8.2.2o, with WB+ (7.4) 3 times.
q) After the last wash, leave the tubes open to let the resin particles dry for 15-20 min.
r) Add 40 µL of the elution buffer (7.5) and gently suspend 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 8.2.2h
u) Collect 30 µL of the liquid phase and transfer it to a 1.5 mL tube.
v) The resulting extract is defined as "larval DNA" and stored frozen. Under these conditions, it can be stored for up to 5 years.

8.2.3 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 (7.19) as positive control and water (7.17) as negative control.
- For DNA isolated from a mixed population of larvae, PCR amplification can be performed as described below; however, the amount of DNA to be tested (Step 8.2.3f) must be determined empirically beginning with dilutions of 1:200 of stock isolated MSL DNA and adjusting the volume of water accordingly in the final reaction mix.

a) Thaw DNA/larva, 2x PCR MasterMix, SetB, 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).

2x PCR MasterMix (6.6)	15 µL
H_2O	4 µL
SetB (7.7)	1 µL
Total	20 µL

Table C – single sample amplification mix: components and volumes

d) Mix the amplification by vortexing and centrifuge at maximum speed for a few sec.
e) Transfer 20 µL of the cumulative amplification mix (8.2.3c) to each PCR tube (8.2.3b).
f) Add 10 µL of the larval DNA (8.2.2.v) 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 after pausing the instrument.

Pre-denaturation	4 min/95°C
Amplification	10 s/95°C 30 s/55°C 30 s/72°C
Number of cycles	35
Final extension	3 min/72°C

Table D – amplification cycles

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 (7.9).

m) Vortex and centrifuge the tubes at maximum speed for a few sec.

n) Keep tubes on ice or refrigerated until ready for electrophoresis.

8.2.4 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 2 gr agarose (7.10) in 100 mL TAE 1x (7.12) in a glass beaker.

c) Gently suspend the powder by rotation.

d) Boil the agarose suspension for 30 sec. If the agarose is not completely dissolved, continue to boil for another 30 sec. Repeat as necessary until the solution is homogeneous.

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 (7.13).

h) Shake gently to uniformly dissolve the ethidium bromide and pour the agarose in the gel tray previously prepared (8.2.4.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 (7.12) and gently pull out the comb.

l) The first and last wells are loaded with 15 µL of the L50 solution (7.14).

m) Load in each well, 20 µL of the amplification product (point 8.2.1.n), giving attention to the progressive numbering of the tubes (point 8.2.3.b).

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

o) Run the gel for about 30 min or until the fastest dye, contained in the loading buffer (7.9), 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 one can easily distinguish all bands of the molecular weight marker ranging from 50 - 500

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.

8.2.5 Interpretation of Results

- The size of the amplification bands revealed by the electrophoresis is evaluated by their comparison with the reference molecular weight standards L50. The visual evaluation is considered sufficient and adequate, since the differences among species are macroscopical (see Table A)
- The amplification test is considered valid if:
  a) the amplification of the positive control shows an amplification product 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 in Table A;

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

9 Results

The results are expressed as follows:

- If the amplification band is of 173 bp, the larva is identified as belonging to *T. spiralis.*
- If the amplification band is of 129 bp, the larva is identified as belonging to *T. nativa*.
- If the amplification band is of 127 bp, the larva is identified as belonging to *T. patagoniensis.*
- If the amplification bands are of 129 bp and 253 bp, the larva is identified as belonging to *T. britovi* or *Trichinella* T8 or *Trichinella* T9*.*
- If the amplification band pattern (1, 2 or 3 bands) is in the range between 292 and 360 bp, the larva is identified as belonging to *T. pseudospiralis.*
- If the amplification bands are of 129 bp and 316 bp, the larva is identified as belonging to *T. murrelli.*
- If the amplification bands are of 129 bp and 210 bp, the larva is identified as belonging to *Trichinella* T6.
- If the amplification bands are of 155 bp and 404 bp, the larva is identified as belonging to *T. nelsoni.*
- If the amplification band is of 240 bp, the larva is identified as belonging to *T. papuae.*
- If the amplification band is of 264 bp, the larva is identified as belonging to *T. zimbabwensis.*
- If the test is classified as valid and the sample shows an unexpected band of a size not reported in Table A, the identification is defined “impossible”.

Whenever possible, five larvae should be tested for each test sample. The isolate identification is considered valid if at least one larva can be identified. In the case of an invalid result, an additional 5 larvae should be tested.

10 PCR and sequencing of the ESV to distinguish between *T. nativa* and *T. patagoniensis*.

When using multiplex-PCR, larvae belonging to the *T. nativa* display a band pattern very similar (only two base pairs of difference, 129 bp versus 127 bp) to the band pattern of *T. patagoniensis* larvae. These two species can be distinguished by the amplification with the primer set cp-I.F and cp-I.R, and sequencing of the ESV. *T. nativa* yields a fragment of 129 bp, whereas *T. patagoniensis* yields a fragment of 127 bp. The allineament is shown in Table E.

T. nativa GTTCCATGTGAACAGCAGTTGGACATGGGTCAGTCGATCCTAAGAAAACGG CGAAAGCTTGTTCGAATTTGCGA
T. patagoniensis GTTCCATGTGAACAGCAGTTGGACATGGGTCAGTCGATCCTAAGAAAACGG CGAAAGCTTGTTCGAATTTGCGA
T. nativa CATGAATTGTAAGACTGTGTG--AAT-- TGTGTGTGTGTGCAGTTGTCGTATGTTTTCG-129bp
T. patagoniensis CATGAATTGTAAGACTGTGTGTGAAT--TGGGTGTG---- CAGTTGTCGTATGTTTTCG-127bp

Table E - Allineament of the ESV of *T. nativa* and *T. patagoniensis*. Primers are marked in grey.

11 PCR-RLFP to distinguish between *T. britovi* and *Trichinella* T9

When using multiplex-PCR, larvae belonging to the T9 genotype display the same band pattern as *T. britovi* larvae. These two taxa can be distinguished by PCR-RFLP based on the CO I mitochondrial gene sequence. According to the available information, the T9 genotype is circulating only in Japan; for this reason, it is suggested that this method be used only if the MSL with a *T. britovi* band pattern by multiplex-PCR originated from this country.

Reagents: (i) 2x PCR Master Mix; (ii) oligonucleotides for CO I, as target locus: L6625, F 5'-TTYTGGTTYTTCGGKCACCC-3'; H7005, R 5'-ACGACGTAGTAGGTRTCRTG-3'; (iii) reference DNA from *T. britovi* and *Trichinella* T9 (ITRC, Rome, Italy); and (iv) purified DNA of the larvae to be tested.

Equipment**:** a thermocycler

Procedures: the procedure consists of two steps: (i) PCR amplification of samples; and (ii) restriction analysis of the PCR products.

(i) PCR amplification: The total volume of the amplification reaction is 50 μl.

1) the amplification solution is prepared by adding sequentially: 25 μl 2x PCR Master Mix, 13 μl water, 1 μl of each primer, and 10 μl of DNA extract from the single larvae to be tested;
2) the amplification cycle, preceded by a pre-denaturation step of 4 min/94°C, is: 60 sec/94°C, 60 sec/48°C, 60 sec/72°C, for 35 cycles, followed by a final extension step of 3 min/72°C. It is recommended that a hot start Taq DNA polymerase be used instead of a standard enzyme;
3) at the end of the amplification, centrifuge the tubes at maximum speed for a few seconds;

4) add the DNA loading buffer and load the samples onto a 2% agarose gel to view the result (both *T. britovi* and *Trichinella* T9 display a 419 bp band). The amount of DNA product present in each sample should be evaluated by eye; and

5) store the amplified products at -20°C until use.

(ii) Restriction analysis; combine all reagents which keeping tubes on ice.

1) for each sample, transfer at least 100 ng of the amplified product into a 1.5 ml reaction tube;

2) set the volume to 10 µl with water;

3) add to the reaction tube: 5 µl of 10x Mse I reaction buffer, 1 µl of Mse I (10 U/µl) restriction enzyme, and water up to 50 µl. When working with multiple samples, a quantity of amplification solution that is sufficient for all of the samples can be prepared all at once; aliquot the solution in 1.5 ml tubes containing the PCR products to be tested;

4) mix and incubate at 37°C for 60 min;

5) stop the reaction by heating at 65°C for 20 min and then spinning for a few sec;

6) add the DNA loading buffer, visualize the results by 2% agarose gel electrophoresis as described for PCR;

7) evaluate the results according to the expected size pattern: for *T. britovi* five bands of 22, 62, 64, 70, and 201 bp; for *Trichinella* T9, two bands of 92 and 327 bp.

12 PCR to distinguish between *T. britovi* and *Trichinella* T8

By multiplex-PCR, the larvae of *Trichinella* T8 display the same band pattern as *T. britovi* larvae. These two taxa can be distinguished by PCR based on a 21 bp deletion in the ITS2 sequence of *Trichinella* T8. According to the available information, the T8 genotype is circulating only in sub-Saharan Africa; it is thus recommended that this method only be used if the larvae with a *T. britovi* band pattern by multiplex-PCR originate from this region.

Reagents: (i) oligonucleotides: ITS2, as target: ITS2G.F 5'-CCGGTGAGCGTAATAAAG-3', ITS2G.R, 5'-TACACACAACGCAACGAT-3'; (ii) reference DNA from *T. britovi* and from *Trichinella* T8 (ITRC, Rome, Italy); and (iii) purified DNA of *Trichinella* larvae to be tested (see section 63.2.1.4).

Equipment: a thermocycler

PCR amplification: the total volume of the amplification reaction should be of 30 µl.

1) the amplification solution is prepared adding sequentially: 15 µl 2x PCR Master Mix, 3 µl water, 1 µl of each primer and 10 µl of DNA extract from a single MSL or control DNA. When working with multiple samples, a quantity of amplification solution that is sufficient for all of the samples can be prepared all at once; aliquot the solution into the PCR tubes containing the DNA samples to be tested;

2) close the tubes, mix by a vortexing, and centrifuge at maximum speed for a few sec;

3) the amplification cycle, preceded by a pre-denaturation step of 4 min/94°C, is: 30 sec/94°C, 30 sec/51°C, 60 sec/72°C, for 35 cycles, followed by a final extension step of 3 min/72°C. It is recommended that a hot start Taq DNA polymerase be used instead of a standard enzyme;

4) at the end of the amplification, centrifuge the tubes at maximum speed for a few sec;

5) add the DNA loading buffer, and visualize the results by 2% agarose gel electrophoresis;

6) evaluate the result according to the expected size pattern: *T. britovi*, band of 125bp; *Trichinella*T8, band of 104 bp (Figure 63.2).

13 Safety measures

This method should 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.