Identification of *Trichinella* Muscle Stage Larvae at the species level by Multiplex PCR

INDEX

1. AIM AND FIELD OF APPLICATION 2
2. PRINCIPLE OF THE METHOD 2
3. REFERENCES 2
4. DEFINITIONS 3
5. DEVICES/INSTRUMENTS 3
6. REAGENTS AND CHEMICALS 4
7. PROCEDURE
   - 7.1 Sample preparation 5
   - 7.2 Method 6
8. RESULTS 9
9. CHARACTERISTICS OF THE METHOD 10
10. SAFETY MEASURES 10

1. AIM AND FIELD OF APPLICATION

To identify the species or genotype of single *Trichinella* larvae preserved in ethanol by a multiplex PCR analysis. This method can be applied to larvae collected from human biopsies or from muscle 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, 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.

Till now, eight sibling species, namely *T. spiralis, T. nativa, T. britovi, T. pseudospiralis, T. murrelli, T. nelsoni, T. papuae,* and *T. zimbabwensis* and 3 genotypes, *Trichinella*-T6, *Trichinella*-T8 and *Trichinella*-T9, have been identified in the genus *Trichinella*. All of the *Trichinella* species and genotypes differ among them by the composition and/or dimension of the nucleotide sequences of different loci; consequently, the comparative analysis of three nucleotide sequences belonging to the ITS1, ITS2 and ESV, allows the univocal identification of most of the epidemiologically relevant taxa: *T. spiralis, T. nativa, T. britovi, T. pseudospiralis, T. murrelli, T. nelsoni, T. papuae, T. zimbabwensis* and *Trichinella*-T6.

The size of the ITS1, ITS2 and ESV fragments produced by the amplification with nucleotide pairs are shown in Table A.

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

	T. spiralis	***T. nativa***	***T. britovi***	***T. pseudospiralis***	***T. murrelli***	***Trichinella T6***	***T. nelsoni***	***T. papuae***	***T. zimbabwensis***
ESV	173	127	127*	310-350**	127	127	155	240	264
ITS1			253			210			
ITS2					316		404***		

*The presence of satellite bands can be frequently observed in capillary electrophoresis.
** Multiple banding within the specified molecular weight range can be observed with both capillary electrophoresis and agarose gel.
***The 404 bp band helps to recognize *T. nelsoni* in agarose electrophoresis but it is not essential.

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

3. REFERENCES

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

La Rosa G, Marucci G, Pozio E. 2003. Biochemical analysis of encapsulated and non-encapsulated species of *Trichinella* (Nematoda, Trichinellidae) from cold- and warm-blooded animals reveals a high genetic divergence in the genus. Parasitol. Res. 91:462-6.

OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2004, V Edition, pp. 380-386.

Pozio E, Foggin CM, Marucci G, La Rosa G, Sacchi L, Corona S, Rossi P, Mukaratirwa S. 2002. *Trichinella zimbabwensis* n.sp. (Nematoda), a new non-encapsulated species from crocodiles (*Crocodylus niloticus*) in Zimbabwe also infecting mammals. Int. J. Parasitol. 32:1787-99.

Pozio E, La Rosa G. 2003. PCR-derived methods for the identification of *Trichinella* parasites from animal and human samples. Methods Mol. Biol. 216:299-309.

Pozio E, Zarlenga DS. 2005. Recent advances on the taxonomy, systematics and epidemiology of *Trichinella*. Int. J. Parasitol. 35:1191-204.

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

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

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.

4. 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 a single larva

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

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

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; 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

ITRC, International *Trichinella* Reference Center

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

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

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

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 96-PCR Plate Centrifuge

5.4 Freezer ≤-20°C

5.5 Fridge 1-8°C

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

5.7 Magnetic separation stand for 1.5-2 ml tubes

5.8 PCR thermocycler

5.9 Qiaxcel, Capillary electrophoresis system

5.10 Horizontal electrophoretic apparatus

5.11 Analytical balance, readability 0.1g

5.12 UV transilluminator

5.13 Digital imaging system

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

5.15 Analytical grade water system production

5.16 Vortex

5.17 BioSprint 96, automated DNA purification system

6. REAGENTS AND CHEMICALS

6.1 **Phosphate-buffered saline (PBS),** pH 7.3±0.2

KH_2PO_4	0.34 g
Na_2HPO_4	1.21 g
NaCl	8.0 g
distilled and deionized water	up to 1000 mL

Add the components specified above in about 750 mL of distilled and deionized water. Stir with magnetic stirrer until completely dissolved. Check the pH (7.3±0.2) and make up to volume. Store at room temperature. Stability: 6 months.

6.2 **DigiQ** – lysis solution made by mixing 2 µL PK (6.25), 2 µL DTT 1M (6.24) e 16 µL PBS (6.1).

6.3 **LisiQ** - lysis solution made by mixing 78 µL RLT (6.5) e 2 µL of paramagnetic resin (6.4).

6.4 **Qiagen MagAttract Suspension G (code 1026901).** Commercial suspension containing paramagnetic resin. Store according to the manufacturer's recommendations.

6.5 **RLT - lysis buffer (code 79216)**. Commercial solution, store according to the manufacturer's recommendations.

6.6 **AW1 - washing buffer (code 19081).** Commercial solution, store according to the manufacturer's recommendations.

6.7 **RPE** - **washing buffer (code 1018013).** Commercial solution, store according to the manufacturer's recommendations.

6.8 **AE – elution buffer (code 19077).** Commercial solution, store according to the manufacturer's recommendations.

6.9 **Rod cover –** disposable plastic tool used to prevent direct contact between the magnet head and the solution.

6.10 **PCR master mix hotstart.** commercial solution suitable for PCR amplification. Store according to the manufacturer's recommendations.

6.11 **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 B. Oligonucleotide sequences of setB (6.12), their codes and amplified nucleotide sequences.

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

6.12 **SetB.** Oligonucleotide mixture used for the multiplex-PCR; the mixture is obtained combining an equal volume of the oligonucleotides (6.13); the final concentration corresponds to 10 pmol/µL; aliquots are prepared and stored frozen up to 10 years.

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

6.14 **Agarose**. Commercial product suitable for performing DNA molecule electrophoresis. Store according to the manufacturer's recommendations.

6.15 **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.

6.16 **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.

6.17 **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.

6.18 **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 50-500 bp range can be used. Store refrigerated according to manufacturer's recommendations.

6.19 **TE 1x solution**. Commercial product 10mM Tris-HCl (pH 8,0), 1mM EDTA-Na_2, pH 7.9–8.1 at 25°C. Store according to the manufacturer's recommendations.

6.20 **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.

6.21 **Milli-Q grade water.**

6.22 **Reference Larvae.** *Trichinella* single muscle larvae stored in ethanol (95-99%) supplied by ITRC. Reference larvae are supplied by ITRC after isolation from mouse muscle tissues by HCl-pepsin digestion (see the procedure "Experimental infection of mice by Trichinella spp. muscle stage larvae", consultabile all'indirizzo https://www.iss.it/en/-/standard-operating-procedures-sops-); store frozen for up to 10 years.

6.23 **Reference DNAs.** Genomic DNA purified from reference larvae. Store frozen for up to 10 years.

6.24 **1,4-Dithiothreitol (DTT)**, commercial product 1M. Store refrigerated according to manufacturer's recommendations.

6.25 **Proteinase K (PK),** commercial product 18mg/ml. Store refrigerated according to manufacturer's recommendations.

6.26 **QIAxcel DNA High Resolution kit -** commercial product from Qiagen to be used with Qiaxcel (5.9). It includes separation cartridge, running and washing buffers. Store according to manufacturer's recommendations.

6.27 **Alignment markers -** commercial product to be used with Qiaxcel (5.9). Store according to manufacturer's recommendations.

6.28 **DNA size markers -** commercial product to be used with Qiaxcel (5.9). Store according to manufacturer's recommendations.

6.29 **Loading buffer -** commercial product. Store according to manufacturer's recommendations.

7. PROCEDURE

7.1 Sample preparation

- Test samples are inspected to verify the integrity of muscle stage larvae.
- The ethanol containing larvae is transferred into a petri dish and observed under the stereo microscope. A maximum of 5 larvae are collected and individually placed in 1.5 mL conical tubes or in 5 wells of a 96 wells PCR plate. Excess ethanol is removed and the minimum volume is left.

- Spin tubes/plate containing larvae at maximum speed for a few seconds.
- Store the tubes/plate frozen. Under these conditions, larvae can be stored for the DNA extraction for up to 5 years.
- If the larvae are absent or are not suitable, the test isn't performed.

7.2 Method

7.2.1 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 a sufficient volume of the DigiQ (6.2) and LisiQ (6.3) solutions according to the manufacturer's recommendations.
- DNA purification can be done in 1.5 mL tubes as well as in 96 wells plate, according to operator's needs.

7.2.1.1 DNA purification in 1.5 ml tubes

a) Centrifuge the tubes containing the larvae to be identified at maximum speed for a few seconds.
b) Add 20 µL of DigiQ solution (6.2).
c) Incubate at 42°C for 15-30 min in the thermoblock. During incubation, shake at 1,400 vibrations/min.
d) Centrifuge, as in point "a".
e) Add 40 µL of LisiQ solution (6.3).
f) Add 4 µL of paramagnetic resin (6.4) after resuspending it by vortexing.
g) Incubate for 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 AW1 (6.6) 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 RPE 1x (6.7) 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 RPE (6.7) 3 times.
q) After the last washing, leave the tubes open to let the resin particles dry for 15-20 min.
r) Add 60 µL of the AE buffer (6.8) 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 and transfer it to a new 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 10 years.

7.2.1.2 DNA purification in 96 wells plate

a) Centrifuge the plate containing the larvae to be identified at maximum speed for a few seconds.
b) Add 20 µL of DigiQ (6.2) in each well and mark the plate as "lysis".
c) Incubate at 42°C for 15-30 min in the thermoblock. During incubation cover the plate with a protective film and shake it at 800 vibrations/min.
d) Add 80 µL of LisiQ solution (6.3) in each well.
e) Prepare a washing plate by dispensing 100 µL of AW1 buffer (6.6) in each well and mark it as "W1".

f) Prepare two washing plates by dispensing 100 µL of RPE buffer (6.7) in each well and mark it as "W2" and "W3".

g) Prepare an empty clean plate to host the rod cover (6.9).

h) Prepare the elution plate by dispensing 60 µL of AE buffer (6.8) in each well and mark it as "DNA".

i) Switch on the Biosprint (5.17) and launch the "MI-02 Qiagen" program.

j) Load the plates inside the Biosprint's slots according to the order showed on the display. All the plates have to be placed in their slots with the same orientation.

k) Start the "MI-02 Qiagen" program.

l) At the end of the program, recover the "DNA" plate and discard the others.

m) Store a 4°C until use.

The resulting extract is defined as "larval DNA" and can be stored frozen. up to 10 years.

7.2.2 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. Use reference DNA (6.23) as positive control and water (6.21) as negative control.

a) Thaw "larval DNA", PCR MasterMix, SetB, and positive amplification controls.

b) Mark with a progressive number an adequate number of 0.2 mL PCR tubes or mark a plate with the date.

c) Prepare an adequate cumulative volume of the amplification mix. Calculate the volume on the basis of table C and of the total number of samples to analyse, including PCR controls and one "virtual" sample every 10 real samples.

Table C – single sample amplification mix: components and volumes

2x PCR MasterMix (6.6)	15 µL
H_2O	4 µL
SetB (6.7)	1 µL
Total	20 µL

d) Mix the amplification mix by vortexing and centrifuge at maximum speed for a few sec.

e) Transfer 20 µL of the cumulative amplification mix to each PCR tube or plate well (point "b").

f) Add 10 µL of the larval DNA to be tested to each tube or plate well.

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/plate in the thermoblock by pausing the instrument.

Table D – amplification cycles

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

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

i) At the end of the amplification phase, centrifuge the tubes/plate at maximum speed for a few sec.

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

7.2.3 Result display

The results display can be done by agarose gel electrophoresis as well as by capillary gel electrophoresis.

7.2.3.1 Electrophoresis

a) Switch on the Qiaxcel instrument (5.9) 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) Place the samples to analyze (minimum volume 10 µL) in rows of 12 tubes starting from the "A" row. 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 (6.26);

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 (6.28);

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

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

j) In "Run Check" verify that all the selected rows are occupied by tubes containing PCR products to be analyzed or QX DNA dilution buffer (6.26) 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.

If the Qiaxcel instrument (5.9) is out of order, proceed with the agarose gel as follow:

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 (6.14) in 100 mL TAE 1x (6.16) 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 DNA intercalating agent 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 buffer (6.16) and gently pull out the comb.

l) The first and last wells are loaded with 15 µL of the L50 solution (6.18).

m) Add 5 µL of loading buffer (6.28) to each sample, if not already present in the PCR master mix

m) Load in each well 20 µL of the amplification product 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 for about 30 min or until the fastest dye, contained in the loading buffer (6.29), 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 50 and 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.

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.

7.2.4 Result Interpretation

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;

7.2.4.1 *Result Interpretation capillary gel electrophoresis*

In the analysis only samples that satisfy the following condition will be considered valid:

- amplification band greater than 50 bp;
- amplification band included between the two bands of the Alignment marker (6.27);
- peak intensity greater than 5% of the threshold value

In case of overlapping peaks only the more intense peak will be considered, if peaks of similar intensity are present the sample will be considered not valid

In case of *T. britovi, T. pseudospiralis, T. papuae* and *T. zimbabwensis* satellite bands in the ESV marker are considered acceptable.

The amplification size are evaluated:

i) by visual comparison with the bands of the “DNA size marker” (6.28) and with the bands of the positive controls on the virtual gel image produced by the software

ii) by comparing the value of the band size estimated by the software with the expected amplification size.

If a test sample shows an unexpected band, the species identification is not possible, the result of the test is expressed as “undeterminable species”.

7.2.4.2 *Result Interpretation agarose gel electrophoresis*

The size of the amplification bands revealed by the electrophoresis is evaluated by their comparison with the reference molecular weight L50 (6.18). The visual evaluation is considered sufficient and adequate, since the differences among species are macroscopical (see 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.

If an unexpected band is present, the species identification is not possible, the result of the test is expressed as “undeterminable species”.

8. 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 127 bp, the larva is identified as belonging to *T. nativa.*
- If the amplification bands are of 127 bp and 253 bp, the larva is identified as belonging to *T. britovi.*
- If the amplification band is in the range between 310 and 350 bp, the larva is identified as belonging to *T.*

pseudospiralis.

- If the amplification bands are of 127 bp and 316 bp, the larva is identified as belonging to *T. murrelli.*
- If the amplification bands are of 127 bp and 210 bp, the larva is identified as belonging to *Trichinella* T6.
- If the amplification bands are of 155 bp with or without the co-presence of the 404 bp band, 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 species identification is not possible, the result of the test is expressed as “undeterminable species”.
- 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.

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 received 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 guidelines of CDC.