IDENTIFICATION OF *ECHINOCOCCUS GRANULOSUS SENSU LATO* COMPLEX AT SPECIES/GENOTYPES LEVEL BY PCR/RFLP AND MULTIPLEX PCR

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1. AIM AND FIELD OF APPLICATION

This method aims at the identification of genotypes/species belonging to the *Echinococcus granulosus sensu lato* (*s.l.*) species complex using PCR-RFLP (Polymerase Chain Reaction - Restriction Fragment Length Polymorphism) followed by multiplex PCR.
This method, applies to DNA extracted from worm or parasitic cyst material of human or animal origin, preserved in ethanol or frozen,
Parasites target of this method are tapeworms of the class Cestoda, whose adult stages live in the small intestine of domestic dogs and wild canids (definitive hosts); definitive hosts become infected by eating organs of the intermediate hosts (usually domestic ungulates) containing the cystic larval of the parasite. Humans are aberrant hosts who acquire infection by ingesting parasite eggs shed through faeces of infected definitive hosts.

2. PRINCIPLE OF THE METHOD

PCR (Polymerase Chain Reaction) is a molecular biology technique that allows the amplification of specific nucleic acid fragments, the initial and terminal nucleotide sequences of which are known (oligonucleotide pair). If a species (or genotype) has a characteristic DNA portion, different in base composition and/or size from those of phylogenetically-related species/genotypes, it is possible to choose an oligonucleotide pair allowing its amplification and, consequently, its unambiguous identification in unknown biological samples. PCR amplification is characterized by a high sensitivity and specificity.

It is possible to combine standard PCR with RLFP (Restriction Fragment Length Polymorphism), i.e. the analysis of DNA restriction fragments. The technique allows the distinction of DNA fragments of different lengths obtained by enzymatic digestion with one or more endonucleases, enzymes able to cut DNA by recognition of short and specific oligonucleotide sequences. Using a single primer pair, it is possible to amplify (PCR) the same portion of DNA from different species and then distinguish them on the basis of digested DNA fragment size (RFLP).

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

As reported in the literature, nuclear and mitochondrial DNA analysis has allowed to split the *E. granulosus s.l*. complex into 8 species/genotypes identified as *E. granulosus sensu stricto* (genotypes G1/G3), *E. equinus* (genotype G4), *E. ortleppi* (genotype G5) and *E. canadensis* (genotypes G6/G7, G8 and G10).

By amplifying, with specific primers, a 444 base pair (bp) DNA fragment belonging to the COX1 (Cytochrome c oxidase I) gene, it is possible to distinguish genotypes G1/G3 (predominant) from other genotypes (G4, G5, G6/G7, G8, G10) thanks to the presence, in the amplified fragment, of a restriction site, cut by the enzyme AluI. After COX1 amplification, the amplified fragment from genotypes G1/G3 iscut into a 209 and a 235 bp fragments, while the amplified fragment from other genotypes is not cut, as it has no restriction site. If the amplified COX1 DNA fragment is not cut, to further distinguish genotypes G4, G5, G6/G7, G8 and G10 a multiplex PCR assay is performed, using 5 oligonucleotide pairs (table A), allowing the allocation of the genotypes on the basis of the PCR product size (table B).

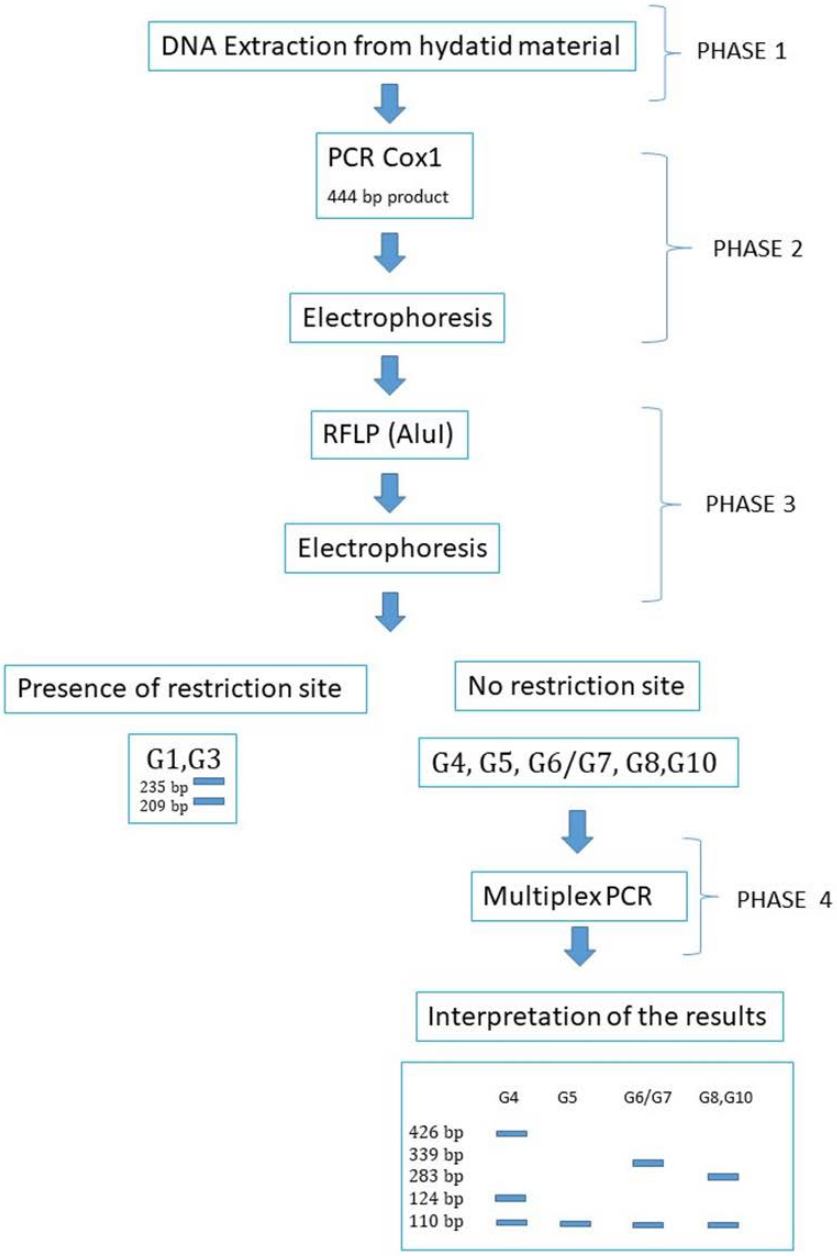
Table A*: Oligonucleotide codes used in the method and their nuclear or mitochondrial targets*

Code	**Nuclear Target**
Elp	Ezrin-radixin-moesin-like protein
Cal	Calreticuline
Code	**Mitochondrial Target**
COX1	Cytochrome c oxidase subunit I
COX2	Cytochrome c oxidase subunit II
ND1	NADH dehydrogenase subunit I

Table B*: Base pair size of the multiplex PCR products expected for each species (genotype) belonging to Echinococcus granulosus sensu lato complex*

Primer pairs	***E. equinus* (G4)**	***E. ortleppi* (G5)**	***E. canadensis* (G6/G7)**	***E. canadensis* (G8, G10)**
Eeq Cal	426 bp	-	-	-
Ecnd G6/7 ND1	-	-	339 bp	-
Ecnd G8/10 Elp	-	-		283 bp
Eeq Cox1	124 bp	-	-	-
Eg complex Cox2	110 bp	110 bp	110 bp	110 bp

The method consists in the following phases:



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4. DEFINITIONS

DNA/parasite – DNA extracted from a single test sample.

Hydatid material – Part of the *E. granulosus s. l.* hydatid cyst; this can be the germinal layer, the hydatid fluid or isolated protoscoleces.

Negative control for the DNA extraction– Reagent grade water (6.16). This control is used in the same working session of test samples to verify the absence of contamination during DNA extraction.

Negative control for the amplification– Reagent grade water (6.16). This control is used in the amplification session to verify the absence of contamination.

Positive control for the DNA extraction – Reference hydatid material (6.17). This control is used in the same working session of test samples to verify the proper functioning of the DNA extraction system.

Positive control for the amplification – Reference DNA (6.18 and 6.19). This control is used in the metacestode DNA amplification session to verify the proper functioning of the PCR system.

Positive control for the enzymatic digestion– PCR product amplified from reference *E. granulosus s.s.* (genotypes G1/G3) DNA (6.18). This control is used in the enzymatic digestion sessions to verify the proper functioning of the RFLP system.

Cal – Calreticuline.

COX1 – Cytochrome c oxidase subunit I.

COX2 – Cytochrome c oxidase subunit II.

Elp – Ezrin-radixin-moesin-like protein.

ND1 – NADH dehydrogenase subunit I.

Endonuclease or restriction enzyme – Enzyme of bacterial origin able to cut the DNA at a specific site, allowing the DNA fragmentation in a reproducible and specific manner. Restriction enzymes cut the DNA chain into specific site that are sequences of 4-8 nucleotides, different for each enzyme. Enzyme concentration is expressed as “enzymatic units” (U), where 1U corresponds to the amount of enzyme needed to completely digest 1µg of DNA at the optimum temperature.

Oligonucleotide – Short DNA sequence (15/30 nucleotide bases) used to amplify a DNA specific fragment.

SetA – Mix of two oligonucleotides amplifying a 444 bp DNA fragment present in the COX1 gene of all *E. granulosus s.l.* genotypes.

SetB – Mix of five oligonucleotide pairs amplifying specific sequences of the *E. granulosus s.l.* genotypes G4, G6/G7, G8, and G10.

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

5. DEVICES/INSTRUMENTS

5.1 Bench top centrifuge for 1.5 mL tubes, minimum 10,000 rpm.

5.2 Vortex.

5.3 Freezer ≤-15°C.

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

5.5 Thermoblock with vibration, temperature range 25÷100°C.

5.6 PCR thermocycler.

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

5.8 Stereomicroscope, magnification 60-500x.

5.9 Qiaxcel, capillary electrophoresis system.

6 REAGENTS AND DISPOSABLE MATERIAL

6.1 **Scissors** or disposable surgical blade.

6.2 **Tweezers**. Commercial product.

6.3 **Tubes**: 0.2 mL, 1.5 mL, 5 mL. Commercial product suitable for molecular biology experiments.

6.4 **Pasteur pipettes**: 1 mL. Commercial product suitable for molecular biology experiments.

6.5 **Petri dishes**. Commercial product

6.6 **Ethanol 70%.** For 30 mL solution: 21.87 mL of Ethanol 96% are added to 8.13 mL of distillated water. Store up to 12 months at room temperature.

6.7 **Lysis buffer.** Commercial solution contained in the Qiagen DNeasy Blood & Tissue Kit, identified as "ATL". Store according to the manufacturer's recommendations.

6.8 **Lysis buffer**. Commercial solution contained in the Qiagen DNeasy Blood & Tissue Kit identified as "AL". Store according to the manufacturer's recommendations.

6.9 **Proteinase K.** Broad-spectrum protease, used to digest proteins in nucleic acids extraction protocols. Commercial solution. Store according to the manufacturer's recommendations.

6.10**Spin column**. Commercial product contained in the DNeasy Blood & Tissue Kit Qiagen and identified as "DNeasy Mini spin column".

6.11 **Collection tube**. Commercial product contained in the DNeasy Blood & Tissue Kit Qiagen and identified as "collection tube" (2 mL).

6.12 **Ethanol 96%**. Commercial solution. Store according to the manufacturer's recommendations.

6.13 **Washing buffer.** Commercial solution contained in the Qiagen DNeasy Blood & Tissue Kit and identified as "AW1". Store according to manufacturer's recommendations.

6.14 **Washing buffer.** Commercial solution contained in the Qiagen DNeasy Blood & Tissue Kit and identified as "AW2". Store according to manufacturer's recommendations.

6.15 **Elution buffer**. Commercial solution contained in the Qiagen DNeasy Blood & Tissue Kit and identified as "AE". Store according to manufacturer's recommendations.

6.16 **Milli-Qgrade-water**. Resistivity ≥ 18 MΩ/cm. Commercially available.

6.17 **Reference hydatid material**. Hydatid material belonging to *E. granulosus s. l.* complex genotypes G3, G4, G5, G6/G7, G8 and G10, stored in ethanol 70%, to be used as positive extraction control. Store up to 10 years.

6.18 **Reference DNA G1/G3.** Genomic DNA extracted from hydatid material belonging to *E. granulosus s. s*. complex genotypes G1/G3. Store frozen (5.3) up to 10 years.

6.19 **Reference DNA G4, G5, G6/G7, G8, and G10.** Genomic DNA extracted from hydatid material belonging to *E. granulosus s.l*. complex genotypes G4, G5, G6/G7-G8, and G10. Store frozen (5.3) up to 10 years.

6.20 **Restriction enzyme AluI:** Commercially available product suitable for DNA enzymatic digestion protocols (e.g. New England Biolabs, AluI, cod. R0137S). Store according to manufacturer's recommendations. The oligonucleotide sequence cleaved by the enzyme is reported in Table C.

Table C - *Oligonucleotide sequence cleaved by the restriction enzyme AluI*

Restriction enzyme	Cleaved sequence
AluI	5′...AG▼CT...3′ 3′...TC▲GA...5′

6.21 **Restriction buffer.** CutSmart®Buffer: commercial solution with defined pH and saline concentration. The buffer is provided together with the restriction enzyme, suitable for DNA enzymatic digestion protocols. Store according to manufacturer's recommendations.

6.22 **QIAxcel DNA High Resolution kit.** Commercial product to be used with QIAxcel (5.9). It contains high-resolution gel cartridge and buffers for sample preparation and analysis. Store according to manufacturer's recommendations.

6.23 **Alignment marker.** Commercial product to be used with QIAxcel (5.9). Store according to manufacturer's recommendations.

6.24 **DNA size marker.** Commercial product to be used with QIAxcel (5.9). Store according to manufacturer's recommendations.

6.25 **PCR master mix HotStart.** Commercial solution suitable for PCR amplification. Store according to manufacturer's recommendations.

6.26 **PCR master mix HotStart Multiplex.** Commercial solution suitable for multiplex PCR amplification. Store according to manufacturer's recommendations

6.27 **Oligonucleotides.** Commercial preparation. The lyophilized product is reconstituted according to manufacturer's recommendations, at a concentration of 100 pmol/µL with MilliQ grade water (6.15). The lyophilized product can be stored frozen (5.3) up to 20 years. The reconstituted product can be stored frozen up to 10 years.

6.28 **SetA.** Oligonucleotide mixture (6.27) used for PCR. The mixture is obtained combining an equal volume of the oligonucleotides EgCO1.1 and EgCO1.2 (Table D) diluted at 10 pmol/µL. The final concentration is 5 pmol/µL. Aliquots of 100µL are prepared and stored frozen (5.3) up to 10 years.

Table D: *Oligonucleotide mitochondrial sequences of SetA, codes and amplified genes.*

Sequence	Code	Gene
5' – TTTTTTGGCCATCCTGAGGTTTAT – 3'	EgCO1.1	COX1
5' – TAACGACATAACATAATGAAAATG – 3'	EgCO1.2	COX1

6.29 **SetB**. Oligonucleotide mixture (6.27) used for Multiplex PCR. The mixture is obtained combining an appropriate volume of the oligonucleotides listed in Table E. Aliquots of 150µL are prepared and stored frozen (5.3) up to 10 years.

Table E: *Oligonucleotide sequences of SetB, codes, amplified genes and final concentration.*

Sequence	Code	Gene	Final concentration
5' – TGGTCGTCTTAATCATTTG – 3'	Eg complex F	COX2	1 pmol/µL
5' – CCACAACAATAGGCATAA – 3'	Eg complex R	COX2	1 pmol/µL
5' – GCTTATTTAGGATCCCA – 3'	Eeq Cal F	Cal	10 pmol/µL
5' – TCGTTTTTGCCAGTG – 3'	Eeq Cal R	Cal	10 pmol/µL
5' – GTTGGGTTGGATGTT – 3'	Eeq Cox1 F	COX1	4 pmol/µL
5' – CAAAACAGGATCACTCTT – 3'	Eeq Cox1 R	COX1	4 pmol/µL
5' – CTGCAGAGGTTTGCC – 3'	Ecnd G6/7 ND1 F	ND1	2.5 pmol/µL
5' – CACAACAGCATAAAGCG – 3'	Ecnd G6/7 ND1 R	ND1	2.5 pmol/µL
5' – CCTAGTCTTCCCATGATA – 3'	Ecnd G8/10 Elp F	Elp	7.5 pmol/µL
5' – ACAGAAGGCATATCCA – 3'	Ecnd G8/10 Elp R	Elp	7.5 pmol/µL

7 PROCEDURE

7.1 Test samples

The status of test samples has to be verified. Test samples consist of whole hydatid cysts (7.1.1), cyst's germinal layer (7.1.2), or protoscoleces (7.1.3), worm or part of it.

7.1.1 Whole hydatid cyst

a. Transfer the hydatid cyst into a Petri dish (6.5);

b. In case of an intact and fluid-filled cyst, incise the wall with disposable scissors or surgical blade (6.1) using tweezers (6.2) to facilitate the flow-out of the cyst's fluid. Transfer the drained cyst into a new Petri dish (6.5). Observe under the stereomicroscope if protoscoleces are present in the cyst's fluid.

c. If protoscoleces are present in the cyst's fluid, aspirate them from the Petri dish with a 1 mL Pasteur pipette (6.4) and aliquot it into 1.5 mL tubes (6.3); continue with the extraction (7.1.2).

d. If liquid is absent, take a flap of about 0.5-1 cm^2 of just the inner layer of the cyst (germinal layer), if possible, and transfer it into a 1.5 mL tube (6.3). If isolation of the germinal layer is not possible, take a full-thickness flap of the cyst wall of about 0.5-1 cm^2 and transfer it into a 1.5 mL tube (6.4). Continue with the extraction (7.1.3). Place the remaining of the cyst back in the original tube, adding 70% ethanol (6.6), if needed.

Note: it is preferable to carry out the DNA extraction starting from protoscoleces; in the absence of

protoscoleces, start the DNA extraction from the germinal layer or from the cyst wall.

7.1.2 Protoscoleces

a) Centrifuge a 1.5 mL tube containing the hydatid fluid with the protoscoleces (5.1) for 3 minutes at 3,000 rpm and discard the supernatant by aspiration, being careful to not touch the pellet;

b) leave the tube with the cap open for 5 minutes to facilitate the evaporation of the residual ethanol.

7.1.3 Germinal layer or cyst wall

a) Centrifuge (5.1) for 3 minutes at 3,000 rpm and discard the supernatant by aspiration, being careful to not touch the pellet;

b) leave the tube with the cap open for 5 minutes to facilitate the evaporation of the residual ethanol.

7.1.4 Worms

a) withdraw worm from the tube containing ethanol and put it in a new tube
b) leave the tube with the cap open for 5 minutes to facilitate the evaporation of the residual ethanol

7.2 Method

7.2.1 PHASE 1: DNA extraction

If not otherwise specified, the procedure is carried out at room temperature, using barrier tips and wearing disposable gloves.

Each working session requires a positive extraction control (hydatid reference material) (6.17) and a negative extraction control (water) (6.16).

Use an operational identification code to mark an adequate number of 1.5 mL tubes (6.3).

a) Add 180 µL of ATL Lysis Buffer (6.7) and 20 µL of Proteinase K (6.9) to the sample (7.1.2 or 7.1.3); resuspend the solution by vortexing (5.2);

b) incubate the solution in the thermoblock (5.5) at 56 °C, shaking at 850 rpm for 60 minutes or until the sample is completely lysed;

c) resuspend the solution by vortexing (5.2) and centrifuge at the maximum speed for a few seconds;

d) add 200 µL of Lysis Buffer AL (6.8) to the sample and mix thoroughly by vortexing. (5.2);

e) add 200 µL of 96% ethanol (6.12) to the sample and resuspend the solution by vortexing (5.2);

f) transfer the solution to the spin column (6.10);

g) centrifuge (5.1) at 8,000 x rpm for 1 minute;

h) transfer the column to a new collection tube (6.11);

i) add 500 µL of washing buffer AW1 (6.13) to the column and centrifuge (5.1) at 8,000 rpm for 1 minute;

j) transfer the column to a new collection tube and add 500 µL of washing buffer AW2 (6.13), centrifuge (5.1) at 14,000 rpm for 3 minutes;

k) transfer the column to a 1.5 mL tube (6.3), add 100 µL of AE elution buffer (6.15), leave for 1 minute at room temperature, then centrifuge at 8,000 rpm for 1 minute.

The resulting extract is placed in a box identified as "DNA/parasite", and stored in the freezer (5.3)

7.2.2 PHASE 2: PCR amplification

Amplification controls

At each session, two positive amplification controls are used, i.e. a reference DNA genotype G1/G3 (6.18) and a reference DNA from one of the genotypes G4, G5, G6 / G7, G8, or G10 (6.19). Two positive controls are necessary to verify the correct amplification reaction (PHASE 2) and the digestion reactions with endonucleases (PHASE 3). Furthermore, the use of a negative amplification control i.e. water (6.16), is required.

If not otherwise specified, the procedure is carried out at room temperature, using barrier tips and wearing disposable gloves.

Amplification reaction

The procedure involves the use of a 2x concentration PCR master mix; in case of different master

mix concentration, change the protocol according to the manufacturer's specifications.

a) Thaw: DNA/ metacestode, 2x PCR master mix (6.25), SetA (6.28), and the 2 positive amplification controls (6.18 and 6.19);

b) mark with a progressive code an adequate number of 0.2 mL PCR tubes (6.3);

c) prepare an adequate cumulative volume of the amplification mix. Evaluate the volume on the basis of a single sample amplification mix (Table F) considering the total number of samples to analyze, the extraction and the amplification controls, plus one additional reaction;

d) mix the amplification mixture by vortexing (5.2) and, if necessary, centrifuge (5.1) at the maximum speed for a few seconds;

e) transfer 48 µL of the cumulative amplification mixture into each of the PCR tubes (point 'b');

f) add 2 µL of DNA/parasite preparation (7.2.1) into each PCR tube;

g) close the tube, mix by vortexing (5.2) and centrifuge (5.1) at the maximum speed for a few seconds;

h) start the amplification cycle (Table G) on the thermocycler device (5.6); wait for the temperature to reach 95°C and put the tubes in the thermoblock by pausing the instrument;

i) at the end of the amplification phase, centrifuge (5.1) the tubes at the maximum speed for a few seconds;

j) leave the tubes on ice or in the refrigerator (5.4) until starting the electrophoresis.

Table F – *Amplification mix for each sample: reagents and related volumes*

2x PCR MasterMix	25 µL
SetA	2.5 µL
H_2O	20.5 µL
DNA	2 µL
Total	50 µL

Table G – *Amplification cycles*

Initial Denaturation	95 °C x 15 min	
Denaturation	94°C x 30 sec	38 cycles
Amplification	55°C x 30 sec	
Extension	72°C x 30 sec	
Final Extension	72°C x 5 min	

Display of amplification results

The amplification results are displayed through capillary 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 "Cartryge 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 analyzed 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 (6.21);

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 (6.24);

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": PCR + data

"Alignment Marker": 15bp - 1kbp

i) in "Run Check" check that all the selected rows are occupied by tubes containing PCR products to be analyzed or QX DNA dilution buffer (6.22) and that the alignment Marker has been loaded, then select the appropriate boxes; finally select "Run";

j) 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 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.

Interpretation of the results

The size of the amplification products is identified by visual comparison of the position of the test bands with that corresponding to the molecular weights of the "DNA size marker" (6.24) and with those of the positive extraction and amplification controls on the virtual gel.

Since all the genotypes/species belonging to the *E. granulosus s.l.* complex produce an amplification band of 444 bp, the amplification test is considered valid if:

i) the positive amplification controls show an amplification product of 444 bp. If no band of 444 bp is observed or bands of different sizes are present, the PCR reaction must be repeated using new reagents;

ii) the negative amplification control does not show amplification products or possibly only bands referable to unused oligonucleotides and/or their derivatives (primer dimers). If bands different from oligonucleotides or primer dimers are observed, the PCR reaction must be repeated using new reagents and eliminating the amplification reagents used previously.

Evaluation of the test sample

If an amplification product of the expected size (444 bp) is present, the enzyme digestion reaction with endonucleases is carried out (7.2.5) (PHASE 3).

If there are no amplification products, a new extraction (PHASE 1) and DNA amplification (PHASE 2) are carried out.

If the result is still negative (no amplification), even after having repeated the extraction and subsequent PCR steps, the result is "not determinable"

7.2.3 PHASE 3: enzymatic DNA digestion with endonucleases

Where not expressly indicated, keep tubes on ice or in a cool support, use tips with barrier and wear disposable gloves.

The digestion reaction step consists in digesting the amplification product (PHASE 2) with *AluI* (6.20) restriction enzyme, including in the session the amplification product of reference DNA G1/G3 (6.18) and one of the reference DNA genotypes that does not present the restriction site (6.19).
The following procedure involves the use of restriction enzymes at a concentration of 10 U/µL and 10x concentrated restriction buffers. In case different concentrations area available, modify the protocol according to the manufacturer's specification.

a) Thaw the PCR products (7.2.2 point "j") and the 10x restriction buffer (6.20). Keep the restriction enzyme on ice (6.20);

b) mark with a progressive code an adequate number of 1.5 mL tubes (6.3);

c) prepare an adequate volume of digestion mixture. Calculate the volume on the basis of the digestion mixture (Table H), considering the total number of samples to be analyzed and including in

the count the positive control (product of amplification of the genotype G1/G3), the negative control (product of amplification of one of the genotypes G4, G5, G6 / G7, G8, or G10), plus one additional reaction;

Table H- Digestion mix for each sample: reagents and related volumes

10x Restriction Mix	2 µL
AluI Enzyme (10U)	1 µL
PCR product	10 µL
H_2O	7 µL
Total	20 µL

d) mix the cumulative digestion mix by vortexing (5.2) and, if necessary, centrifuge at the maximum speed for a few seconds;

e) transfer 10 µL of the digestion mix (point "d") into each of the 1.5 mL tubes (6.3);

f) add 10 µL of PCR product to be analyzed to each tube (7.2.2 point "j");

g) close the tube, mix by vortexing (5.2), and centrifuge (5.1) at the maximum speed for a few seconds;

h) incubate the samples at 37 °C in the thermoblock (5.5) for 3 h;

i) after the incubation, centrifuge (5.1) the tubes at the maximum speed for a few seconds;

j) mix the samples by vortexing (5.2) and centrifuge (5.1) the tubes at the maximum speed for a few seconds;

k) leave the tubes on ice or in the refrigerator (5.4) until analyzed by electrophoresis.

Results display of enzymatic digestion

Results display of enzymatic digestion is carried out as reported in paragraph 7.2.3.

Interpretation of the enzymatic digestion results

The size of the amplification products is detected by visual comparison of the position of the tests bands with that corresponding to the molecular weights of the "DNA size marker" (6.24) and with those of the positive extraction and amplification controls on the virtual gel.

Expression of enzymatic digestion results

The digestion test is considered valid if the band profile of the positive digestion control with AluI consists of approximately 209 bp and 235 bp bands.

If the test is valid and the analyzed sample shows digestion bands similar to those of the positive digestion control, the sample is identified as *E. granulosus s.s.* (genotypes G1/G3).

If no bands are observed or bands do not have the appropriate restriction profile, the multiplex PCR step is carried out (PHASE 4)

7.2.4 PHASE 4: amplification by multiplex PCR

Where not expressly indicated, keep tubes on ice or in a cool support, use tips with barrier and wear disposable gloves.
In each session, a positive control, i.e. reference DNA (6.19), and a negative control, i.e. water (6.16), are used to check the performance of the amplification reaction.
The following procedure involves the use of a 2x concentration master mix PCR, in case a different concentration is available, change the protocol according to the manufacturer's specifications.

a) Thaw: DNA/parasite, 2x Multiplex PCR master mix (6.26), Set B (6.29), positive amplification control (6.19);

b) mark with a progressive number an adequate number of 0.2 mL PCR tubes (6.3);

c) prepare an adequate volume of cumulative amplification mixture. Calculate the volume on the basis of the amplification mixture (Table I), considering the total number of samples to be analyzed, one positive control, one negative control and one additional reaction;

d) mix the amplification mixture by vortexing (5.2) and, if necessary, centrifuge (5.1) at the maximum speed for a few seconds;

e) transfer 48 µL of the cumulative amplification mixture into each PCR tubes (point 'b');

f) add 2 µL of DNA/parasite preparation (7.2.1) into each PCR tube;

g) close the tube, mix by vortexing (5.2) and centrifuge (5.1) at the maximum speed for a few seconds;

h) start the amplifying cycle (Table J) on the thermocycler device (5.6), wait for the temperature to reach 95°C and insert tubes in the thermocycler by pausing the instrument;

i) at the end of the amplification phase, centrifuge (5.1) the tubes at the maximum speed for a few seconds;

j) leave the tubes on ice or in the refrigerator (5.4) until analysis by electrophoresis.

Table I - *Amplification mixture of the single sample: components and relative volumes*

2x PCR MasterMix	25 µL
SetB	10 µL
H_2O	13 µL
DNA	2 µL
Total	50 µL

Table J- *Amplification cycles*

Initial Denaturation	95 °C x 15 min	
Denaturation	94°C x 30 sec	35 cycles
Amplification	56°C x 30 sec	
Extension	72°C x 60 sec	
Final Extension	72°C x 5 min	

Multiplex PCR results display

The display of results of the multiplex PCR amplification is carried out as reported in paragraph 7.2.3.

Interpretation of multiplex PCR amplification results

The size of the amplification products is identified by visual comparison of the position of the test bands compared with that of the molecular weights of the "DNA size marker" (6.23) and with those of positive extraction and amplification controls on the virtual gel.

8. INTERPRETATION OF MULTIPLEX PCR RESULTS

Express the assay results in the test report as follows:

i) If the amplification bands are 110 bp, 124 bp and 426 bp, the sample is identified as *E. equinus* (genotype G4). The presence of specific bands of 110 bp and 426 bp or 110 bp and 124 bp is also considered valid for the recognition of this species.

ii) If a single amplification band of 110 bp is present, the sample is identified as *E. ortleppi* (genotype G5).

iii) If the amplification bands are 110 bp and 339 bp, the sample is identified as *E. canadensis* (genotypes G6 / G7).

iv) If the amplification bands are 110 bp and 283 bp, the sample is identified as *E. canadensis* (genotypes G8, G10).

9. CHARACTERISTICS OF THE METHOD

This method has been characterized in terms of repeatability and specificity. The results of the validation protocol confirmed 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.