IDENTIFICATION AT THE SPECIES LEVEL OF OOCYSTS OF *Cryptosporidium* spp. BY PCR/RFLP

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1 AIM AND FIELD OF APPLICATION

This document describes a method to determine the species of protozoa belonging to the genus *Cryptosporidium* by PCR/RLFP. The method can be applied to fecal samples of human or animal origin, previously diagnosed as positive for the presence of *Cryptosporidium* spp. oocysts.

2 PRINCIPLE OF THE METHOD

The Polymerase Chain Reaction (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 a 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 reaction is characterized by high sensitivity and specificity. Nested-PCR is a modification of the PCR technique that allow to obtain a high sensibility using two consecutive PCR reactions. In the first reaction an external oligonucleotide pair, while in the second an internal ones is used, targeting the same DNA fragment.

It is possible to combine the PCR with the technique known as “Restriction Fragment Length Polymorphism” (RFLP), that means the analysis of DNA restriction fragments. The technique allows to differentiate PCR fragments by enzymatic digestion with one or more endonucleases, enzymes that cut the DNA by recognition of short and specific oligonucleotide sequences. In our case, it is possible to amplify the same DNA fragment from different species, and to identify the species based on the number and size of restriction fragments (the restriction pattern).

Parasitic protozoa of the genus *Cryptosporidium* infect all vertebrates, including humans, and localize in the gut or the stomach of the host. The infective and environmentally resistant stage, known as the oocyst, causes, upon ingestion, a disease known as cryptosporidiosis. The parasites within the genus *Cryptosporidium* cannot be identified on the basis of their morphology. However, genetic analysis has demonstrated the existence of many species within the genus and, currently, those that are considered pathogenic to humans include *Cryptosporidium hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, *C. canis*, *C. muris*, *C. suis*, *C. andersoni, C. ubiquitum, C. cuniculus* and the ‘monkey’ and ‘skunk’ genotypes. It should be noted that the vast majority of human cases of cryptosporidiosis is due to two species, namely *C. hominis* and *C. parvum.*

Molecular methods based on PCR-RFLP allowed for the identification at the species level of *Cryptosporidium* sp. oocysts present in fecal samples of human and animal origin.

A commonly employed method for molecular diagnostics of *Cryptosporidium* parasites is based on the amplification of a fragment of the gene encoding for a structural protein of the oocyst wall (*Cryptosporidium* Oocyst Wall Prorein, COWP). The sensitivity of the method has been established, and a nested PCR assay can detect the parasite in fecal samples containing <500 oocysts per gram (Pedraza-Diaz et al., 2001). The oligonucleotide pairs used for the two consecutive PCR reactions and the following enzymatic digestion of the amplification product permit to identify *Cryptosporidium* at specie level (Pedraza-Diaz et al., 2001 and Spano et al., 1997).

The fragments of the COWP gene obtained from different *Cryptosporidium* species, by amplification with the two oligonucleotide pairs, are 769 and 553 base pairs, respectively. Table A lists the size of the fragments obtained after enzymatic digestion of the COWP amplification products with the indicated restriction enzymes.

Table A. Size of the restriction fragments (in base pairs) from the COWP gene from each species as obtained with different restriction enzymes.

	Restriction fragments of PCR products from the COWP gene	
Species	***Restriction enzyme Rsa I***	***Restriction enzyme Alu I***
C. parvum	***413, 106, 34***	
C. hominis	***284, 129, 106, 34***	
C. canis	***195, 106, 86, 71, 43, 34, 18***	
C. felis	***406, 86, 61***	
C. meleagridis	***372, 147, 34***	
C. andersoni	***327, 140, 86***	
C. suis	***266, 129, 106, 34, 18***	***424, 129***
C. ubiquitum	***266, 129, 106, 34, 18***	***324, 129, 100***
Horse genotype	***519, 34***	

By using PCR/RFLP, it is possible, based on the restriction patterns generated by the enzyme *Rsa I*, to distinguish the species *C. parvum, C. hominis, C. canis*, *C. felis, C. meleagridis, C. andersoni,* and the horse genotype. Since *C. suis* and *C. ubiquitum* have an identical *Rsa* I restriction pattern, their identification requires another restriction analysis using the enzyme *Alu* I.

3 REFERENCES

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

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

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Qiagen: QIAamp Fast DNA Stool Handbook.

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4 DEFINITIONS

COWP (*Cryptosporidium* Oocyst Wall Protein), sequence encoding for a structural protein, a component of the oocyst wall.

Oligonucleotide, a short sequence (15/30 nucleotides) used for the amplification of a specific DNA fragment.

Set A, mix of two oligonucleotides that amplify an external fragment of the COWP gene from given species or genotypes.

Set B, mix of two oligonucleotides that amplify an internal fragment of the COWP gene from given species or genotypes.

Reference DNA, genomic DNA extracted from the feces of a calf experimentally infected with oocysts of *C. parvum.*

DNA extraction positive control, aliquots of feces from a calf experimentally infected with oocysts, processed in the same working session of test samples, to verify the efficacy of the DNA extraction.

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

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

Restriction Enzymes, enzymes of bacterial origin able to cut DNA at specific sites, thus allowing DNA fragmentation in a reproducible and specific manner. Restriction enzymes recognize specific short sequences of 4-8 bases in the DNA. Enzyme concentration is measured as “enzymatic units” (U). In this case, 1U corresponds to the amount of enzyme needed to completely digest 1 µg of DNA in 1 hour at the optimal temperature.

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

5 DEVICES/INSTRUMENTS

5.1 Bench centrifuge for 1.5-2.0 mL tubes, 10.000 x g

5.2 Freezer, ≤ -15°C

5.3 Thermoblock with vibration, temperature range +25-100°C

5.4 PCR thermocycler

5.5 Refrigerator, temperature range +1-8°C

5.6 Qiaxcel, capillary electrophoresis system

5.7 Horizontal electrophoretic apparatus

5.8 Digital imaging system

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

5.10 Reagen grade water system production

5.11 Vortex

5.12 Analytical balance, readability 0.1g

5.13 UV Transilluminator

5.14 Orbital shaker

6 REAGENTS AND CHEMICALS

6.1 **InhibitEX Tablet.** Commercially available reagent: QIAamp Fast DNA Stool, QIAGEN. Store according to the manufacturer’s recommendations.

6.2 **Proteinase K.** Commercially available reagent: QIAamp Fast DNA Stool, QIAGEN. Store according to the manufacturer’s recommendations.

6.3 **Lysis buffer.** Commercially available solution: QIAamp Fast DNA Stool, labelled as ‘AL

solution'. Store at room temperature.

6.4 **Absolute ethanol**. Commercially available reagent.

6.5 **Recovery column.** Commercially available material: QIAamp Fast DNA Stool, QIAGEN, labelled as QIAmp Mini Spin Columns.

6.6 **Collection tubes.** Commercially available material: QIAamp Fast DNA Stool, QIAGEN, labelled as Collection tubes (2 ml).

6.7 **Washing buffers.** Commercially available solutions: QIAamp Fast DNA Stool, QIAGEN. Prepare according to the manufacturer's recommendations, and label the solutions as 'AW1'and 'AW2'. Store at room temperature.

6.8 **Elution buffer.** Commercially available solution: QIAamp Fast DNA Stool, QIAGEN, labelled as 'Buffer AE'. Store at room temperature.

6.9 **PCR master mix.** Commercially available solution to perform PCR amplification experiment. Store according to the manufacturer's recommendations. If large volumes of PCR master mix are purchased, the product can be divided into 1-2 mL aliquots and stored according to the manufacturer's recommendations.

6.10 **Set A.** The oligonucleotide mixture (6.12) used for the PCR; the mixture is obtained combining an equal volume of the oligonucleotides BCOWPF and BCOWPR (6.12) diluted at 20 pmol/µL with reagent grade water. The final concentration corresponds to 10 pmol/µL of each oligonucleotide; 100 µL aliquots are prepared and stored frozen (5.2) for up to 10 years.

6.11 **Set B.** The oligonucleotide mixture (6.12) used for the PCR; the mixture is obtained combining an equal volume of the oligonucleotides Cry9 and Cry15 (6.12) diluted at 20 pmol/µL with reagent grade water. The final concentration corresponds to 10 pmol/µL of each oligonucleotide; 100 µL aliquots are prepared and stored frozen (5.2) for up to 10 years.

6.12 **Oligonucleotides.** Commercially available reagents (Table B); the lyophilized products is reconstituted with analytical grade water (5.9) at a concentration of 100 pmol/µL. This operation is recorded, dated and signed, on the oligonucleotide technical sheet provided by the manufacturer. The lyophilized product can be stored frozen (5.2) for up to 20 years; the reconstituted product can be stored frozen (5.2) for up to 10 years.

Table B. Oligonucleotide sequences of set-A (6.11), their codes and amplified nucleotide sequence.

Oligonucleotide sequences	Codes	Amplified sequence
5'-CCGCTTCTCAACAACCATCTTGTCCT 3' 5'-CGCACCTGTTCCCACTCAATGTAAACCC-3'	BCOWPF BCOWPR	COWP (external primers)
5'-GGACTGAAATACAGGCATTATCTTG-3' 5'-GTAGATAATGGAAGAGATTGTG-3'	Cry9 Cry15	COWP (internal primers)

6.13 **Loading buffer.** Commercially available product allowing electrophoresis of DNA molecules. Store according to the manufacturer's recommendations.

6.14 **QIAxcel DNA High Resolution kit.** Commercially available product to use in Qiaxcel apparatus (5.7). It includes ready to use gel cartridges and buffer for preparing and running the samples. Store according to the manufacturer's recommendations.

6.15 **Alignment markers.** Commercially available product to use in Qiaxcel apparatus (5.7). Store according to the manufacturer's recommendations.

6.16 **DNA size markers.** Commercially available product to use in Qiaxcel apparatus (5.7). Store according to the manufacturer's recommendations.

6.17 **Agarose.** Commercially available product suitable for performing electrophoresis of DNA molecules. Store at room temperature for up to 24 months.

6.18 **50x TAE solution.** Commercially available product (2M Tris-acetate, 50mM EDTA, pH 8.2–8.4 at 25°C) to be used as buffer for electrophoresis. Store at room temperature for up to 24

months.

6.19 **1x TAE solution.** 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.

6.20 **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.

6.21 **L50.** Commercially available 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 (5.5) according to manufacturer's recommendations.

6.22 **L100.** Commercially available product containing markers for DNA molecular weight up to 1000 bp. All commercial products containing molecules of within the 100-1000 bp range can be used. Store refrigerated (5.5) according to manufacturer's recommendations.

6.23 **Milli-Q grade water.**

6.24 **Reference fecal sample.** feces containing *C. parvum* oocysts. Store refrigerated (5.5) for up to **10 years**.

6.25 **Reference DNA:** genomic DNA extracted from the fecal sample containing oocysts of *C. parvum*. Store frozen (5.2) for up to **10 years**.

6.26 **Restriction enzymes *Rsa* I and *Alu* I:** Commercially available products suitable for DNA enzymatic digestion (for example, New England Biolabs, Rsa I, cod. R0167S; Alu I, cod. R0137S). Store according to the manufacturer's recommendations. The oligonucleotide sequences recognized by each enzyme is reported in Table C.

Table C - Oligonucleotide sequences recognized by the reported enzymes

Restriction enzyme	Sequence recognized
***Rsa* I**	5'…GT▼AC…3' 3'…CA▲TG…5'
***Alu* I**	5'…AG▼CT…3' 3'…TC▲GA…5'

6.27 **Restriction buffers.** Commercially available products suitable for DNA enzymatic digestion with defined pH and saline concentration. The buffers are usually sold with the corresponding restriction enzyme. Store according to the manufacturer's recommendations.

7 PROCEDURE

7.1 Sample preparation

The tubes containing the feces with oocysts of *Cryptosporidium* must be intact and show no sign of spillage. If the conditions are not suitable, the test is not performed.

7.2 Method

7.2.1 *DNA extraction from the fecal sample to be tested*

If not otherwise stated, the procedure is performed at room temperature.

Each working session requires that a reference sample (6.21) is used for DNA extraction, and labelled as "positive extraction control".

a) Transfer 1.0 mL of each fecal sample containing 50% ethanol in single 1.5 mL tubes
b) Centrifuge (5.1) the tubes at 8,000 g for 5 minutes
c) Discard the supernatant and add enough water to reconstitute the starting volume
d) Centrifuge again as in point 'b'

e) Repeat the washing step as in point 'c'
f) Transfer 200 µL of each fecal sample in 2 mL tubes
g) Add 1 mL of InhibitEX buffer (6.1) and vortex for 1 minute to homogenize the sample
h) Incubate for 10 minutes at 95°C in the thermomixer (5.3). Set the shaking at 1400 rpm during the lysis step
i) Centrifuge (5.1) for 1 minute at 12,000 g
j) Put 25 µL of proteinase K (6.2) in a clean 1.5 mL tube
k) Transfer 600 µL of the supernatant (step i) in the tube containing proteinase K (step j)
l) Add 600 µL of lysis buffer AL (6.3) and vortex for 15 seconds
m) Incubate in a thermomixer (5.3) for 10 minutes at 70°C
n) Add 600 µL of absolute ethanol and vortex briefly
o) For each sample, put one recovery column (6.5) into a collection tube (6.6)
p) Transfer 600 µL of lysate (from point r) into the recovery column (6.6) and centrifuge (5.1) for 1 minute at 13.400 g
q) Discard the collection tube (6.6) and put recovery column (6.5) into a clean collection tube (6.6)
r) Repeat step k-l twice.
s) Add 500 µL of washing buffer AW1 (6.7) to the recovery column and centrifuge (5.1) for 1 minute at 12,000 g
t) Discard the collection tube (6.6) and put recovery column (6.5) into a clean collection tube (6.6)
u) Add 500 µL of washing buffer AW2 (6.7) to the recovery column and centrifuge (5.1) for 3 minutes at 12,000 g
v) Transfer the recovery column (6.5) in a clean 1.5 mL tube
w) Add 100 µL of elution buffer ATE (6.8) to the recovery column (6.5) and incubate for 1-2 minutes
x) Centrifuge (5.1) for 1 minute at 12,000 g, discard the recovery column (6.5), and save the tube containing the DNA extract
y) The DNA prepared is labeled 'DNA/fecal sample' and are stored frozen (5.2) for up to 10 years.

7.2.2 PCR amplification

If not specified otherwise, keep tubes on ice, use aerosol-free tips and wear disposable gloves.

At each working session, use a positive and a negative amplification control. Use reference DNA (6.22) as positive control, and water (6.20) as negative control.

The following procedure uses a PCR master mix at a 2x concentration. If the concentration is different, modify the procedure following the manufacturer's recommendations.

a) Thaw: DNA/fecal samples, 2x PCR MasterMix (6.10), SetA (6.11), and amplification positive control (reference DNA, 6.22)
b) Mark with a progressive number an adequate number of 0.2 mL PCR tubes
c) Prepare an adequate cumulative volume of amplification mix. Calculate the volume based on a single sample amplification mix (Table D) and of the total number of samples plus two reactions (one for the positive amplification control and one for the negative control)

Table D. Amplification mix for a single sample: components and volumes

2x PCR MasterMix (6.9)	25 µL
H_2O (6.23)	19 µL
SetA (6.10)	1 µL
Total	45 µL

d) Mix the amplification mix by vortexing and centrifuge (5.1) at maximum speed for a few seconds
e) Transfer 45 µL of the cumulative amplification mix to each PCR tube (point "b")

f) Add 5 µL of the DNA/fecal samples to be tested to each tube

g) Close the tubes, mix by vortexing and centrifuge (5.1) at maximum speed for a few seconds

h) Start the amplification cycle (Table E) on the thermocycler device (5.4); wait until the temperature reaches 94°C and insert the tubes in the thermoblock by pausing the instrument. Close the lid and restart the program

Table E. Amplification cycle

Pre-denaturation #	5 min/94°C
Amplification	30 s/94°C 30 s/58°C 60 s/72°C
Number of cycles	40
Final extension	7 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 (5.1) the tubes at maximum speed for a few seconds

l) Leave the tubes on ice or in a refrigerator (5.5) until the Nested PCR is performed.

7.2.3 Nested PCR

a) Mark with a progressive number an adequate number of 0.2 mL PCR tubes

b) Prepare an adequate cumulative volume of amplification mix. Calculate the volume based on a single sample amplification mix (Table F) and of the total number of samples plus one (negative control)

Table F. Amplification mix for a single sample: components and volumes

2x PCR MasterMix (6.9)	25 µL
H_2O (6.23)	19 µL
Set B (6.11)	1 µL
Totale	45 µL

c) Mix the amplification mix by vortexing and centrifuge (5.1) at maximum speed for a few seconds

d) Transfer 45 µL of the cumulative amplification mix to each PCR tube (point "b")

e) Add 5 µL of amplification product obtained from the first PCR reaction (7.2.2, point l) in each corresponding PCR tube

f) Close the tubes, mix by vortexing and centrifuge (5.1) at maximum speed for a few seconds

g) Start the amplification cycle (Table G) on the thermocycler device (5.4); wait until the temperature reaches 94°C and insert the tubes in the thermoblock by pausing the instrument. Close the lid and restart the program

Table G. Amplification cycle

Pre-denaturation #	5 min/94°C
Amplification	30 s/94°C 30 s/48°C 60 s/72°C
Number of cycles	40
Final extension	7 min/72°C

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

h) At the end of the amplification phase (nested PCR), centrifuge (5.1) the tubes at maximum speed for a few seconds

i) Leave the tubes on ice or in a refrigerator (5.5) before the electrophoresis

7.2.4 Visualisation of results

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

7.2.4.1 *Capillary gel electrophoresis*

a) Switch on the Qiaxcel instrument (5.6) 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" (6.10); 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 (6.14);

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 (6.16);

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 - 500bp (6.10). In "Sample Information" enter the names of the samples in the corresponding boxes;

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 (6.9) and that the alignment Marker has been loaded, then select the appropriate boxes; finally select "Run";

j) visualize the results by selecting the "Absolute migration time" mode from the "Image options" menù and process the data with the "Start analysis" command;

k) scroll through the electropherogram of each sample to check peaks above the highest band of the alignment marker (6.15);

l) print the results to archive;

m) at the end of the run, close the program and turn off the instrument.

If the instrument 5.6 is out of service for an extended period proceed with agarose gel following the protocol below:

a) Assemble the electrophoresis apparatus (5.7) according to the manufacturer's recommendations. For the gel preparation, use a comb suited for the number of test samples

b) Weight (5.12) 2 g of agarose (6.17) and add it to 100 mL of TAE 1x (6.19) in a glass beaker

c) Gently resuspend the agarose 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 during boiling

f) Allow the agarose solution to cool

g) Before it solidifies (at about 47°C), add DNA intercalating agent (6.20) 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 (6.19) and gently pull out the comb

l) Load in each well 10 µL of the amplification product (7.2.3 point “i”), adding in each tube the loading buffer (6.13), if not present in the PCR master mix

m) Load the first and the last well with 10 µL of the L100 solution (6.22)

n) Connect the electrophoresis apparatus with the power supply (5.7), set 10 v/cm of gel

o) Run the gel for about 30 min or until the fastest dye, contained in the loading buffer 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 distinguish all bands of the molecular weight marker ranging from 250 to 2000 bp. If the separation is incomplete, continue the run.

q) At the end of the run, transfer the gel to the imaging system (5.8) and print the result.

7.2.5 Interpretation of the nested PCR amplification results

The amplification test is considered valid if:

I. the extraction positive control shows an amplification product of 553 bp;

II. the negative control does not show any amplification product or, eventually, only bands related to unincorporated oligonucleotides and/or primer dimers;

III. The amplification positive control shows a band of 553 bp.

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

- amplification band greater than 50 bp;
- amplification band included between the two bands of the Alignment marker (6.15);
- 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

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.

The size of the amplification bands revealed by the electrophoresis is evaluated by their comparison with the DNA molecular weight L100 (6.22) and with extraction and amplification positive controls. The visual evaluation is considered sufficient and adequate.

If an unexpected band is present, the enzymatic digestion is not performed, the result of the test is expressed as “undeterminable species”.

If a test sample shows no amplification, the presence of inhibitors is tested by adding reference DNA (6.25) to the DNA of the test sample and then performing a new PCR reaction as described in the paragraph 7.2.5.

If the expected 553 bp fragment is not amplified, a new DNA extraction from the test sample is performed. Whenever even the amplification of the newly extracted DNA, does not generate an amplification band of 553 bp, the result of the test is expressed as “undeterminable species”

The species identification is obtained by comparing the restriction pattern (number and size of the fragments generated by enzymatic digestion) of test samples with those reported in Table B.

7.2.6 Test for the presence of inhibitors by PCR with reference DNA

If nor otherwise stated, keep tubes on ice, use tips with barrier and wear disposable gloves.

At each working session use a positive amplification control, reference DNA (6.25).

The following procedure foresee a 2x PCR master mix, if the concentration is different, modify the procedure following the manufacturer's recommendations.

a) Thaw: DNA/fecal samples (7.2.1 point "t"), 2x PCR MasterMix (6.9), SetA (6.10), amplification positive control (reference DNA, 6.25)

b) Mark with a progressive number an adequate number of 0.2 mL PCR tubes

c) Prepare an adequate cumulative volume of amplification mix. Calculate the volume on the basis of a single sample amplification mix (Table H) and of the total number of samples plus two (1 for the positive amplification control and 1 for the negative control).

Table H – Amplification mix for a single sample: components and volumes

2x PCR MasterMix (6.9)	25 µL
H_2O (6.23)	14 µL
Set A (6.10)	1 µL
Reference DNA (6.25)	5
Totale	45 µL

d) Mix the amplification mix by vortexing and centrifuge (5.1) at maximum speed for a few seconds

e) Transfer 45 µL of the cumulative amplification mix (7.2.6 point "c") to each PCR tube (point "b").

f) Add to each tube 5 µL of the DNA/fecal samples (7.2.1 point "t") to be tested.

g) Close the tubes, mix by vortexing and centrifuge (5.1) at maximum speed for a few seconds

h) Start the amplification cycle on the thermocycler device (5.4) according to table E, paragraph 7.2.2. Wait until the temperature reaches 95°C and insert the tubes in the thermoblock by pausing the instrument. Close the lid and restart the cycle.

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

l) Leave the tubes on ice or in a refrigerator (5.5) until the Nested PCR, see paragraph 7.2.3..

7.2.6.1 Visualization of the results

For result visualization, follow the procedure described at point 7.2.4.

7.2.6.2 Result interpretation of the amplification to verify the inhibitor presence

To interpret the results, see the paragraph 7.2.5.

The test is valid if:

- The positive control amplification shows a band of 553 bp;
- The negative control amplification does not show any amplification product or, eventually, only bands related to unincorporated oligonucleotides and/or primer dimers

7.2.7 Enzymatic DNA digestion with endonucleases

If nor otherwise stated, keep tubes on ice.

At each working session, independent digestions with the enzyme *Rsa* I (6.26) are performed. To verify the correct execution of the digestion, the PCR amplification product of the reference DNA (6.25) is also digested.

In the procedure, the restriction enzyme (6.26) is used at the initial concentration of 10 U/µl and concentrated restriction enzyme buffers at 10x. In case of different concentrations, adjust the protocol according to the manufacturer's instructions.

a) Thaw nested PCR products (7.2.3 point "i") and the 10x restriction enzyme buffer (6.27). Keep restriction enzyme on ice.

b) Mark with a progressive number an adequate number of 0.2 mL tubes

c) Prepare an adequate cumulative volume of the enzymatic digestion mix for each restriction enzyme. Evaluate the volume based on a single sample enzymatic digestion mix (Table I) and of the total number of samples plus the positive control

Table I. Enzymatic digestion mix for a single sample: components and volumes

10x restriction buffer (6.27)	2,0 µL
Restriction enzyme (6.26)	10U (1 µL)
PCR product (7.2.3 point "i")	10 µL
H_2O	7 µL
Total	20 µL

d) Mix each enzymatic digestion mix by vortexing and centrifuge (5.1) at maximum speed for a few seconds

e) Transfer 10µL of the cumulative amplification mix (7.2.7 point "c") to each tube (point "b")

f) Add 10 µL of the nested PCR product (7.2.3 point "i") to be tested

g) Close the tubes, mix by vortexing and centrifuge (5.1) at maximum speed for a few seconds

h) Incubate the tubes at 37°C for 4 h in the thermoblock (5.3) without shaking

i) At the end of the reaction, centrifuge (5.1) the tubes at maximum speed for a few seconds

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

7.2.7.1 Visualization of results

To visualize the results, follow the procedure described at point 7.2.4.

7.2.7.2 Interpretation of the results of enzymatic digestion

To interpret the results, follow the procedure described at point 7.2.5.

The size of the fragments generated by enzymatic digestion and revealed by the electrophoresis is evaluated by comparison with the DNA size marker (6.16) or DNA molecular weight L50 (6.21) and with the digestion control. As the difference between species are large, visual evaluation is considered sufficient and adequate.

The test will be considered valid if the digestion positive control shows a restriction pattern (number and size of fragments) in agreement with Table A.

The species identification is obtained by comparison of the restriction patterns of each test sample with the restriction patterns listed in Table A.

If a test sample shows one or more unexpected bands, the species identification is not be possible, the result of the test is expressed as "undeterminable species".

8 RESULTS

The results are expressed as follows:

If the restriction pattern with Rsa I is 413, 106, 34bp, the sample is identified as *C. parvum.*

If the restriction pattern with Rsa I is 284, 129, 106, 34bp, the sample is identified as *C. hominis.*

If the restriction pattern with Rsa I is 372, 147, 34bp, the sample is identified as *C. meleagridis.*

If the restriction pattern with Rsa I is 406, 86, 61bp, the sample is identified as *C. felis.*

If the restriction pattern with Rsa I is 195, 106, 86, 71, 43, 34, 18bp, the sample is identified as *C. canis.*

If the restriction pattern with Rsa I is 327, 140, 86bp, the sample is identified as *C. andersoni.*

If the restriction pattern with Rsa I is 519, 34bp, the sample is identified as *horse.* genotype

If the restriction pattern with Rsa I is 266, 129, 106, 34, 18bp and the restriction pattern with Alu I is 424, 129bp, the sample is identified as *C. suis.*

If the restriction pattern with Rsa I is 266, 129, 106, 34, 18bp and the restriction pattern with Alu I is 324, 129, 100pb, the sample is identified as *C. ubiquitum.*

If the test is valid but a test sample show one or more bands not present in Table A, the species identification is “impossible”, the result of the test is expressed as “undeterminable species”.

9 CHARACTERISTICS OF THE METHOD

The present method has been characterized in terms of sensitivity, specificity and and repeatability. The results of the validation process confirmed that the method is suitable for the specified aim, and were evaluated by the Italian accreditation body (ACCREDIA) to accredit it according to the ISO/IEC 17025.

10. SAFETY MEASURES

This method has to be carried out only by authorized personnel. The operator should wear individual protection devices (gloves and lab coat) while performing the test.