Molecular identification of *Sarcocystis* species

STANDARD OPERATING PROCEDURE

SUMMARY

1. SCOPE

This document describes molecular methods to detect and identify *Sarcocystis* spp. in meat for human consumption, in particular zoonotic species representing a risk to the human health. The procedure starts with sampling of infected muscles, proceeding with DNA extraction, PCR amplification of cytochrome C oxidase subunit 1 (COI) gene, as informative marker, and sequencing of the PCR products. Therefore, the identification at the species level of a given *Sarcocystis* specimen is obtained through the comparison of its COI sequence with COI known sequences.

2. INTRODUCTION

Sarcocystis is a large taxonomic group of ubiquitous apicomplexan parasites that includes more than 150 related species with a wide range of potential hosts that includes reptiles, birds and mammals. *Sarcocystis* spp. have a complex life cycle that requires two different hosts: an intermediated host as prey and a final host as predator. These parasites are present in the form of cysts (sarcocysts) in the muscles of herbivorous or omnivorous animals (intermediate hosts) who acquire the infection by ingestion of food or water contaminated with the sporocysts of the parasite. The infected animals are potential preys to carnivores or omnivores. Finally, predators who ingested infected meat constitute the definitive hosts for *Sarcocystis*. In fact, the life cycle is completed in the gut of definitive hosts with the emission of ready-to-infect sporocysts in the stools.

It has been shown that, in particular regions of South East Asia, humans can be an aberrant intermediate hosts after the ingestion of sporocysts of endemic species. However, most commonly, human represents a definitive host for two species, *Sarcocystis hominis* and *Sarcocystis suihominis;* the infection could be acquired through the consumption of raw or undercooked meat. As definitive host, the human sarcocystosis is localized in the small intestine associated with gastric and intestinal symptoms.

Zoonotic sarcocysts can be present in all kind of muscles of herbivores or omnivores (i. e. pigs and wild boars) and therefore meats from domesticated or wild animals represent potential sources for human infections. Despite the potential hazards for the human health, a routine procedure to identify zoonotic species in meat for human consumption is still lacking. Since zoonotic species are not morphologically distinguishable from other species, a molecular approach based on PCR and sequencing would be an appropriate choice for the genetic identification of *Sarcocysts*. Indeed, genetic typing is increasingly used to identify and classify *Sarcocystis* species even if a taxonomy, based on the sequences of genetic loci, is still incomplete. Actually, the mitochondrial gene cytochrome C oxidase subunit 1 (COI) is considered one of the most suitable target to assign a genetic barcode to a given *Sarcocystis* species. Moreover, many COI sequences of *Sarcocystis* from mammalians, including *S. hominis* and *S. suihominis,* are now available in genomic databases.

3. REFERENCES

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4. DEFINITIONS

COI (cytochrome C oxidase subunit 1), mitochondrial gene encoding subunit 1 of cytochrome C oxidase.

5. EQUIPMENT

5.1 Scalpel
5.2 Compressorium (two glass plates to compress thin slices of tissue for microscopic examination)
5.3 Stereo microscope, magnification 60÷100x
5.4 Siringe needles
5.5 Tubes 1.5 mL, 2 mL and 5 mL
5.6 Analytical balance, readability 0.1g
5.7 Adjustable volume pipettes, volume range: 1-1000µL
5.8 Vortex
5.9 Thermo-block with mixer, temperature range 25÷100°C
5.10 Bench top centrifuge for 1.5 mL tubes, minimum 13,000xg
5.11 Magnetic separation stands suitable for 5 mL and 1.5 mL tubes
5.12 Freezer ≤-20°C
5.13 PCR thermocycler
5.14 Refrigerator, temperature range +1 ÷ +8°C
5.15 Analytical grade water system production, resistivity ≥ 18 Mohm/cm
5.16 Horizontal electrophoretic apparatus
5.17 Power supply for gel electrophoresis
5.18 UV transilluminator
5.19 Digital imaging system

6. REAGENTS

6.1 Lysis buffer A. Commercial solution e.g.: Wizard magnetic DNA purification system for food-Promega

6.2 Proteinase K solution 20 mg/ml
6.3 DTT solution
6.4 Lysis buffer B. Commercial solution e.g.: Wizard magnetic DNA purification system for food- Promega
6.5 Precipitation solution. Commercial solution e.g.: Wizard magnetic DNA purification system for food- Promega
6.6 MagneSil PMPs. Commercial solution e.g.: Wizard magnetic DNA purification system for food- Promega.
6.7 Isopropanol.
6.8 Ethanol 70%. Absolute ethanol, in water.
6.9 Nuclease free water.
6.10 PCR master mix 2x commercial solution, e.g. GoTaq Green Master Mix-Promega.
6.11 Milli-Q grade water. Resistivity ≥ 18 Mohm/cm or commercially available DNA Nuclease-Free Water for molecular biology application
6.12 Oligonucleotides. Commercial preparation (Table A); the lyophilized products is reconstituted with TE 0.1x, according to the manufacturer's recommendations, at a concentration of 100 pmol/µL; the lyophilized product can be stored frozen for up to 20 years; the reconstituted product can be stored frozen up to 10 years.

Table A. Oligonucleotides sequences

Code	Sequence	Target
SF2	5'-GGTATCTTYAGCGTTGTTGGTACTC-3'	COI gene
SR9	5'-ATATCCATACCRCCATTGCCCAT-3'	COI gene

6.13 Agarose. Commercial product suitable for performing DNA gel electrophoresis.
6.14 TAE solution 50x. Commercial product (2M Tris-acetate, 50mM EDTA, pH 8.2–8.4 at 25°C). Store at room temperature for up to 24 months.
6.15 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.
6.16 Ethidium bromide solution. Commercial product 10 mg/L. For the working condition, dilute 1:100,000; for 100 mL solution, add 1.0 µL. Store according to the manufacturer's recommendations. NOTE: Ethidium bromide is potentially mutagenous, carcinogenic and teratogenic; wear disposable gloves and handle the solution containing this substance very carefully.
6.17 L100. Commercial product containing DNA fragments multiples of 100 bp for use as molecular weight standards for agarose gel electrophoresis. All commercial products containing DNA fragments within the 50-1000 bp range can be used.
6.18 Loading buffer 6x. Commercial product allowing DNA gel electrophoresis.
6.19 Buffer PBI Commercial solution, e.g. QIAquick PCR Purification Kit (Qiagen).
6.20 Buffer PE Commercial solution, e.g. QIAquick PCR Purification Kit (Qiagen).
6.21 Buffer EB Commercial solution, e.g. QIAquick PCR Purification Kit (Qiagen).
6.22 TE 1x solution. Commercial product 10mM Tris-HCl (pH 8,0), 1mM EDTA-Na_2, pH 7.9–8.1 at 25°C.
6.23 TE 0.1x solution. TE 1x diluted solution. For 100 mL preparation: 10 mL of 1x solution plus 90 mL water. Filter with 0.22 µm filters and prepare aliquots of 10 mL. Store frozen for up to 24 months.

7. PROCEDURE

7.1 Picking up single cysts from slice of muscle tissue.
a) Cut small slices (<5 cm in length) of muscles (esophagus or diaphragm are recommended).

b) Place the muscle slices between the two glass plates of a compressorium.
c) Press the tissue tightening the screws.
d) Examine the tissue under a stereo microscope (sarcocysts appear like whitish spindle-shaped cysts of few millimeters)
e) Once identified one or more sarcocyst/s remove the upper glass plate.
f) Pick up the cysts with a needle and put them in a 2 mL tube.
g) Add 200 µL of water and start with DNA extraction (7.3).

7.2 Harvesting pieces of muscle with sarcocysts (alternative approach to 7.1).

a) Collect the pieces of tissue with the suspected cysts.
b) Cut the tissue with a scalpel to have small pieces.
c) Weight 200 mg of meat (in case adjust to 200 mg with water), place in a 2 mL tube and start with DNA extraction (7.3).

7.3 DNA extraction (the recommended kit is Wizard magnetic DNA purification system for food-Promega)

NOTE: If not otherwise specified, the procedure is carried out at room temperature.

a) Take aliquots of isolate sarcocysts (200 µL in water) or aliquots of meat (200 mg) and place in a 2 mL tube.
b) Add 400 µL of Lysis Buffer A, 50 µL proteinase K and 50 µL DTT. Cap the tube and vortex vigorously for 5-10 seconds.
c) Incubate at 55°C for 60-90 min with shaking at 1,000 rpm in thermoblock, until cyst or meat are dissolved.
d) Add 250 µL of Lysis Buffer B and vortex for 10-15 seconds. Incubate the tube 10 minutes at room temperature.
e) Add 750 µL of Precipitation Solution. Vortex vigorously.
f) Spin 10 minutes in a microcentrifuge at maximum speed (14,000 rpm).
g) Transfer the supernatant (liquid phase) to a new 5 mL tube.
h) Mix the bottle of MagneSil PMPs for 15-30 seconds. The MagneSil PMPs must be thoroughly resuspended before being dispensed.
i) Add 50 µL of MagneSil PMPs to the supernatant and vortex.
j) Add 0.8 volume of isopropanol. Invert the tube 10-15 times. Incubate for 5 minutes at room temperature with occasional mixing.
k) Place the tube onto a magnetic separation stand and leave for 1 minute, (resin will form a dark-grey visible sediment on the tube wall toward the magnetic stand). Discard the liquid phase by aspiration, avoiding the dislodging of the resin.
l) Remove the tube from the stand and add 250 µL of Lysis Buffer B. Invert the tube 2-3 times and place the tube back in the stand. Allow the MagneSil PMPs to separate for 1 minute and discard the liquid phase by aspiration as above.
m) Resuspend the resin in 1 ml of ethanol 70% as washing solution and transfer in a new 1.5 mL tube.
n) Place the tube on the magnetic separation stand for 1 minute. Discard the liquid phase by aspiration.
o) Repeat the washing step with ethanol 70% solution (from “m” to “n”) twice more, three washes needed.
p) After the last wash aspire the liquid as much of as possible and discard it.
q) Air-dry the resin with the open tubes (15-30 minutes at room temperature or 10 minutes at 65°C).
r) Add 100 µL of the Nuclease Free Water, vortex briefly and incubate at 65°C for 5 minutes in thermoblock at 800 rpm
s) Place the tube in the magnetic stand again.
t) Collect the liquid phase in a new 1.5 mL tube.

u) This DNA solution can be stored at 4°C for 1-2 weeks or up to 5 years frozen at -20° C.

7.4 PCR amplification

NOTE: Unless otherwise clearly stated, store tubes on ice; use tips with barrier and wear disposable gloves.

At each working session, use a positive and a negative amplification control.

a) Thaw DNA, 2x PCR Master Mix, forward and reverse primers, and a positive amplification control.
b) Mark, with a progressive number, an adequate number of 0.2 µL PCR tubes.
c) Prepare an adequate cumulative volume of the amplification mix. Calculate the cumulative volume on the basis of recipe in table B by multiplying by the total number of samples to analyse plus two controls (1 for the positive amplification and 1 for the negative one).

Table B – single sample amplification mix: components and volumes.

PCR master mix 2x	25 µL
SF2 primer (forward) 10 pmol/µl	1 µL
SR9 primer (revers) 10 pmol/µl	1 µL
Water	18 µL
Final volume	45 µL

d) Vortex the amplification mix and centrifuge at maximum speed for few seconds.
e) Transfer 20 µL of the cumulative amplification mix to each PCR tube (point “b”).
f) Add 10 µL of the DNA to be tested to each tube.
g) Close the tubes, mix by vortexing and centrifuge at maximum speed for few seconds.
h) Insert the tubes in the thermocycler (Table C) and start the amplifying cycle as follows.

Table C – amplification cycle

Pre-denaturation*	Amplification steps	Number of cycles	Final extension
95 °C for 5 min	95°C, 30 sec 51°C, 30 sec 72°C, 90 sec	45	72 °C for 10 min

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

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

7.5 Electrophoresis of the amplification products

a) Prepare the electrophoresis apparatus and assemble a gel tray with a proper comb considering number of samples plus the size marker.
b) In a glass beaker, add 1.0 gr of agarose in 100 mL TAE 1x .
c) Dissolve the agarose powder by gentle rotation and weight the beaker.
d) Boil the agarose suspension until the solution became clear and homogeneous.
e) Restore the original weight with water (to compensate the evaporation of boiling water)
f) Let cool the agarose solution until 50°-60°C.

g) Add 1.0 µL of ethidium bromide solution.
h) Shake gently to disperse homogeneously ethidium bromide and pour the agarose in the gel tray previously prepared (point “a”).
i) Wait until the gel is completely solidified.
j) Place the tray with the gel in the electrophoresis apparatus.
k) Cover the gel with TAE 1x buffer and gently pull out the comb.
l) Load the first well with 15 µL of the L100 solution.
m) Mix 10 µL of the amplification product with the proper amount of loading buffer, if not present in the PCR master mix, and load 10 µL of each PCR reaction in a separate well
n) Connect the electrophoresis apparatus with the power supply and set 10 v/cm of gel.
o) Run the gel until the dye of the sample mix reaches 1 cm from the gel border.
p) Switch off the power supply and place the gel over a UV-transilluminator to check the band separation of the molecular size marker (i. e. L100), continue electrophoretic run if the marker bands are not clearly separated. The electrophoretic run is sufficient when all the bands of the marker are well separated.
q) At the end of the run, transfer the gel to a digital imaging system with UV-transilluminator and take a picture of the gel to record the run.

7.6 Interpretation of PCR results

Samples, positive for *Sarcocystis* COI, produce a unique amplification band of approximately 1 Kb (the exact size depends on the species). Therefore, all the PCR samples showing a ≈ 1 Kb band need to be purified for the sequencing.

7.6 PCR product purification

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

7.7 DNA sequencing with Sanger method

The purified and quantified PCR product is sent to a DNA Sequencing Service according to the company request. In general terms, from each PCR product will be prepared two aliquots to be sequenced on both strands using separately the forward primer (SF2) and the reverse primer (SR9). Follow the indications of your provider for the PCR product amount required for the custom sequencing service.

7.8 Sequence analysis

Sanger DNA sequences are in general supplied by commercial providers as ABI files, therefore a software to read and edit this format may be useful. Two suitable and free applications for this task are bioedit-7 and codoncode. The aim of the editing process is the compilation of a text file, or a FASTA file, with a COI consensus sequence, combining forward and reverse sequences from each PCR product. These consensus sequence can be directly compared with released sequences in GenBank by using Basic Local Alignment Search Tool (BLAST) on-line program.

7.9 Species identification

The identification of the *Sarcocystis* species will be based on the sequence identity with other well characterized *Sarcocystis* isolates.

8. SAFETY MEASURES

This method has to be carried out only by authorized personnel. The operator should wear individual protection devices during the test performance. For general safety measures, refer to the guidelines of CDC (http://www.who.int/ihr/publications/biorisk management_1/en/).