Molecular detection of Sarcocystis spp. in meat samples

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Sarcocystis

- Phylum Apicomplexa
- More than 150 species
- Ubiquitous
- Two hosts to complete the life cycle:

  An intermediate host (prey)

  A definitive host (predator)

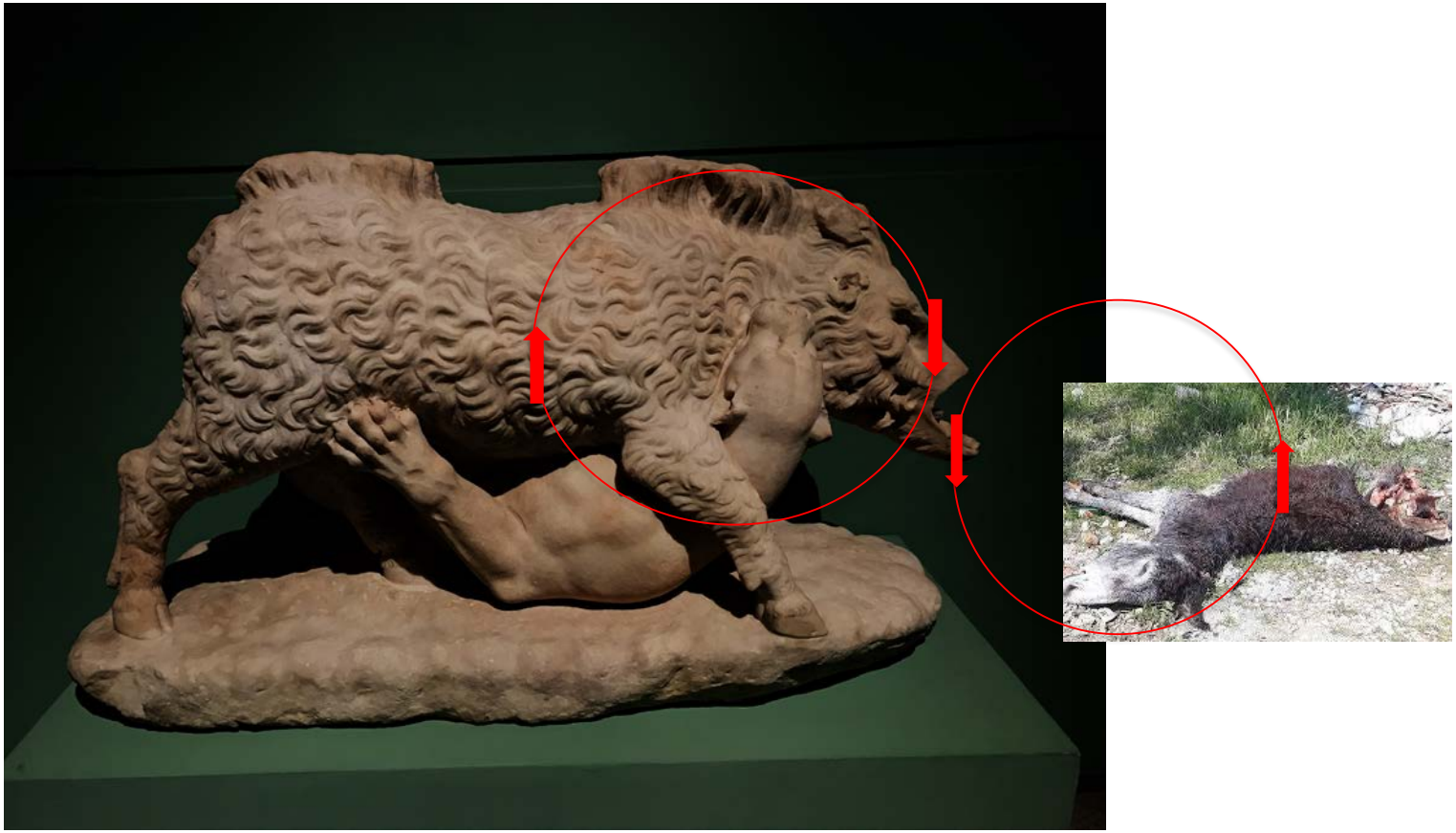
Sarcocystis

Intermediate hosts

- Many herbivorous mammals
- Birds
- Reptiles
- Primates
- Humans

Definitive hosts

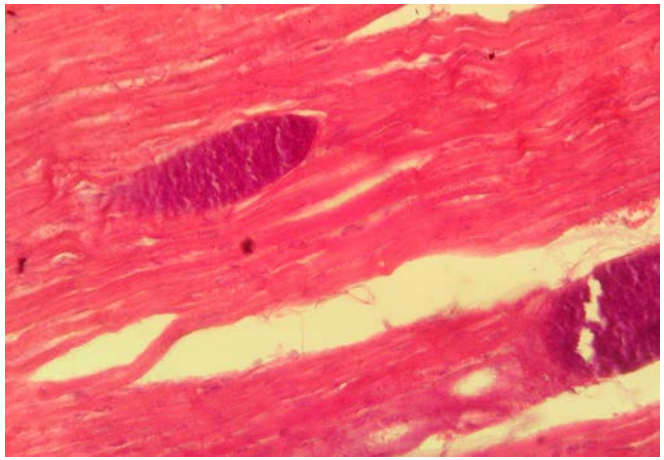
- Carnivorous and omnivorous mammals
- Raptorial birds
- Reptiles
- Humans

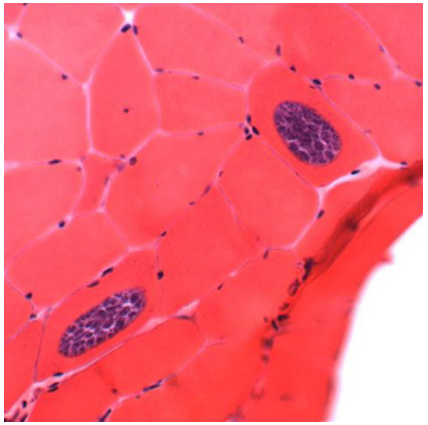


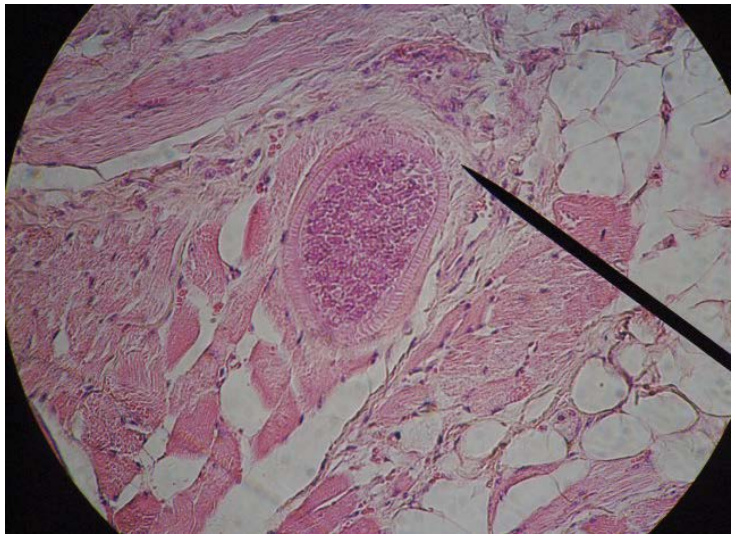
Predator-Prey Cycle

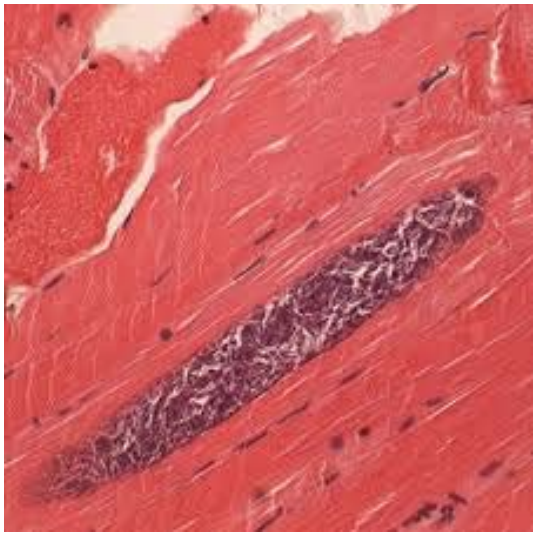
Omnivorous animals can play both roles: intermediated or definitive host.

Intermediated hosts develop Intramuscular cysts



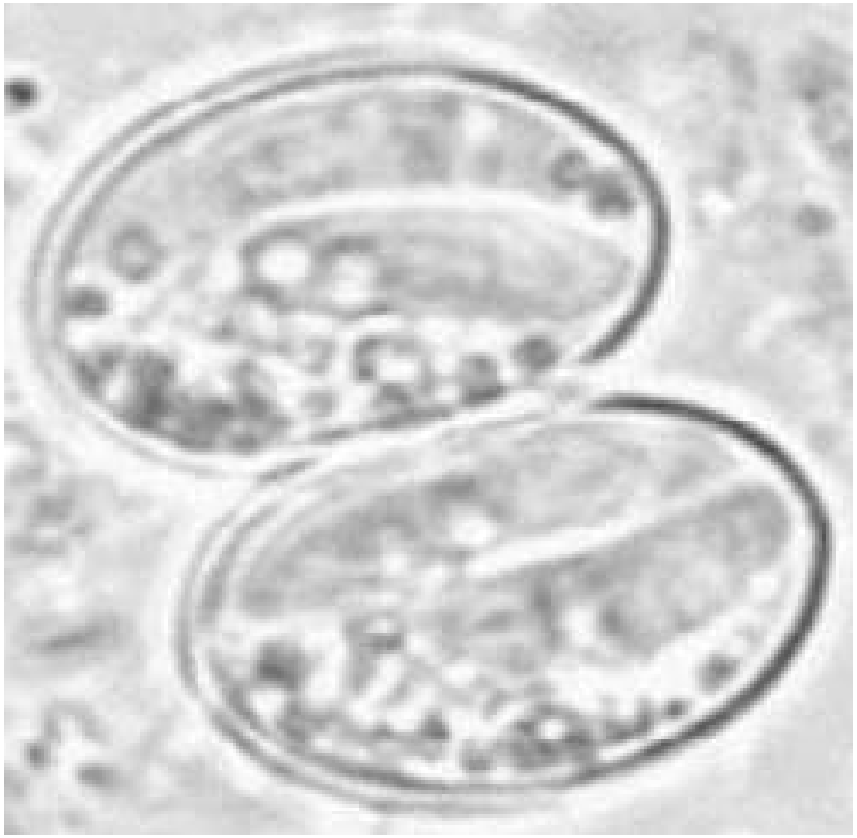


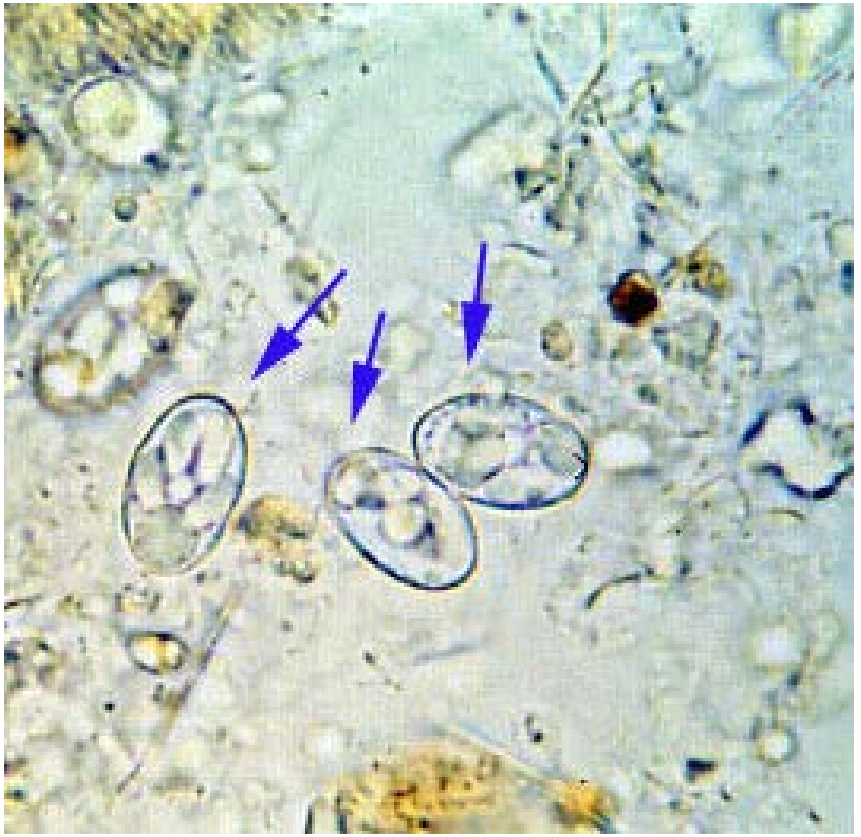




Definitive hosts: sexual cycle in the small intestine up to the excretion of new sporoocysts and oocysts in stool

Sporocystis





Sarcocystis stages in tissues of intermediate hosts (A to F) and definitive hosts (G to I).

IM-1: Schizont in an endothelial cell (artery)

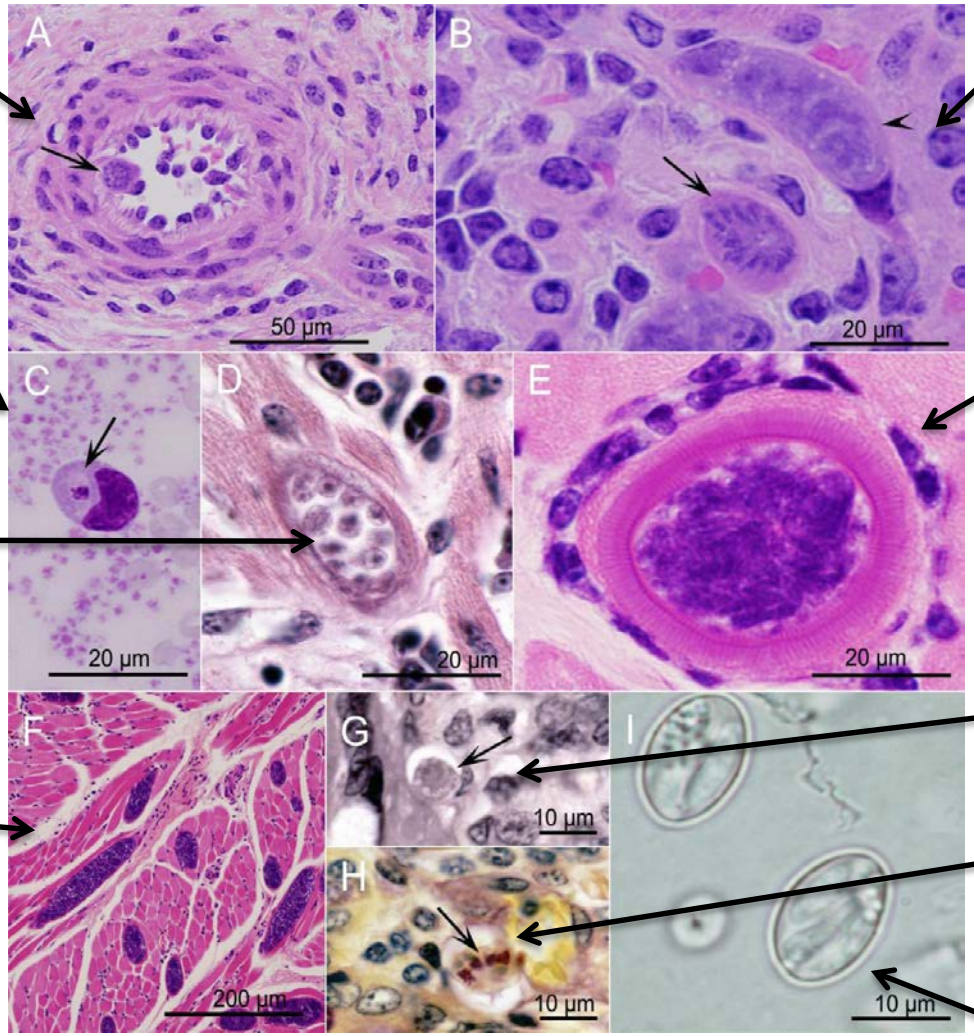
IM-2: Second generation schizont in glomerulus (kidney)

IM-3: Merozoite in a monocyte (blood)

IM-5: Mature sarcocyst in skeletal muscle. Thick wall with mononuclear infiltrate (inflammatory response)

IM-4: Immature sacocyst with metrocytes (Heart)

IM-5 bis: Sarcocyst in skeletal muscle. (no inflammatory response)



Fin-1: Macrogametocyte in lamina propria (small intestine)

Fin-2: Sporulated sporocysts (small intestine)

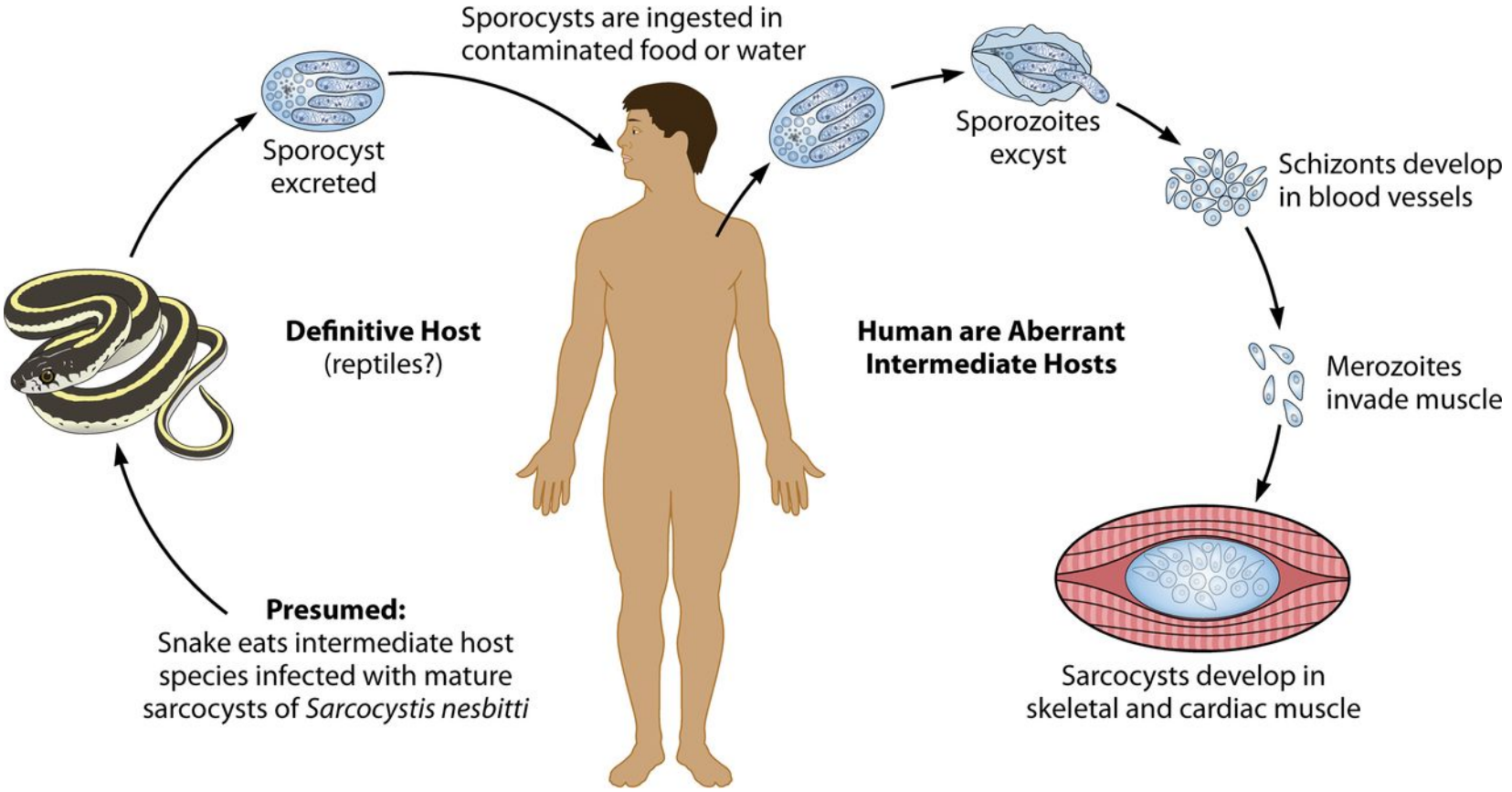
Fin-3: Sporocysts in a fecal smear

Ronald Fayer et al. Clin. Microbiol. Rev. 2015; doi:10.1128/CMR.00113-14

Sarcocystis spp. in Humans

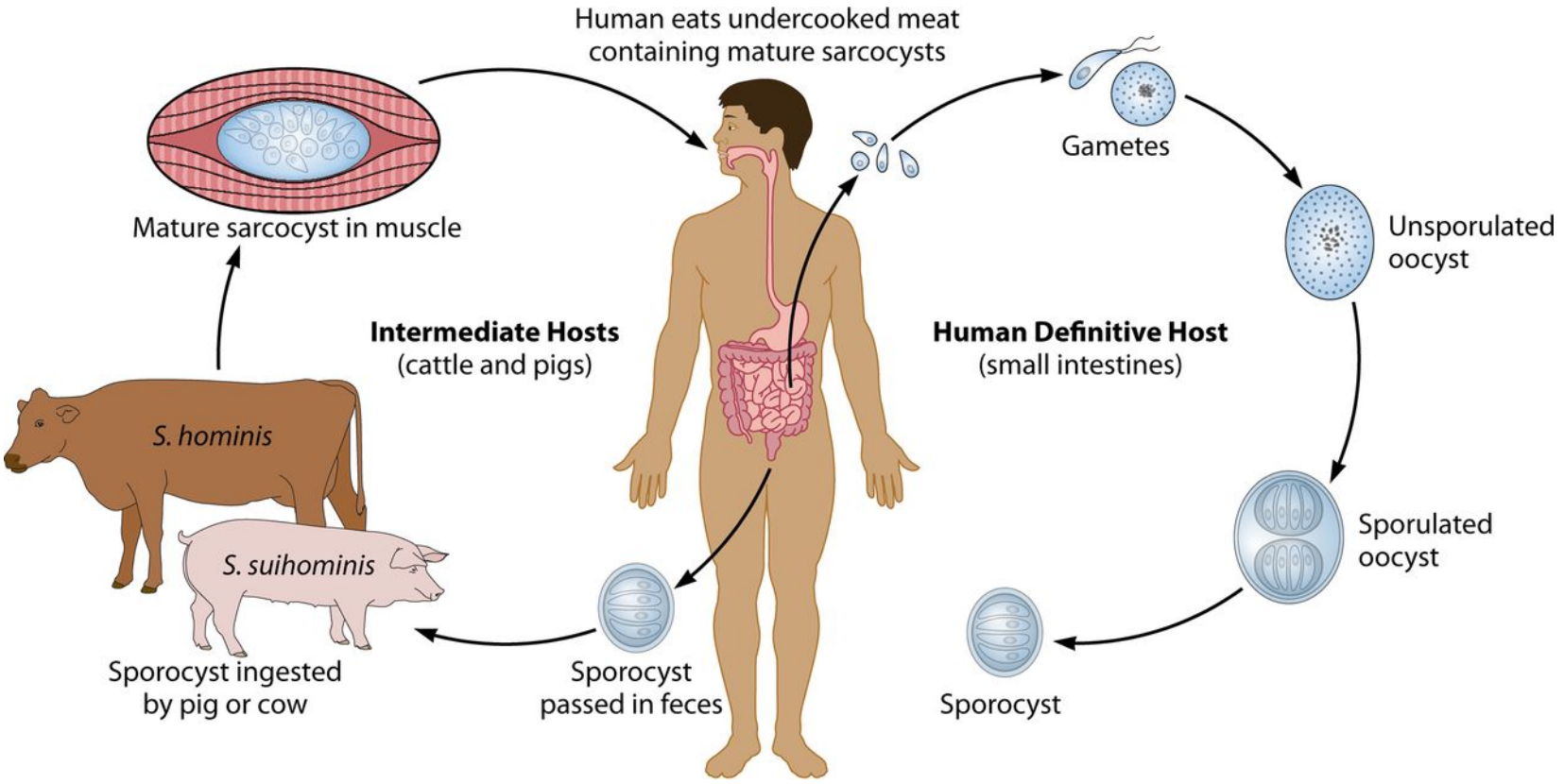
Man as intermediated and definitive host

Humans as aberrant intermediate hosts for Sarcocystis species.



Ronald Fayer et al. Clin. Microbiol. Rev. 2015; doi:10.1128/CMR.00113-14

Humans as definitive (final) hosts for Sarcocystis species.



Ronald Fayer et al. Clin. Microbiol. Rev. 2015;
doi:10.1128/CMR.00113-14

Sarcocystis: a problematic Taxonomy

Based on intermediated-definitive hosts

- S. capreolicanis = roe deer (intermediated)-dog (definitive)
- S. suishominis = pig (intermediated)-human(definitive)

Limits:

- Often one of the host is not known
- The same species can infect different intermediated as well definitive hosts

Based on morphological parameters

- Aspect of sporocysts and/oocysts (definitive host)
- Shape and size of tissue cysts (intermediated host)

Limits:

- Morphology of the sporocysts/oocysts is very similar
- Morphology of the cysts is influenced by various factors: (host, tissue, age, etc.)

Molecular Methods for Detection and Identification of Sarcocystis species

- Establishing an unequivocal genetic identity
- Discrimination of species with similar morphology
- Determination of undefined cycles: intermediate and definitive hosts of a given species
- Molecular epidemiology of sarcocystosis among animals and humans
- Unveiling of possible hidden hosts

AIMS

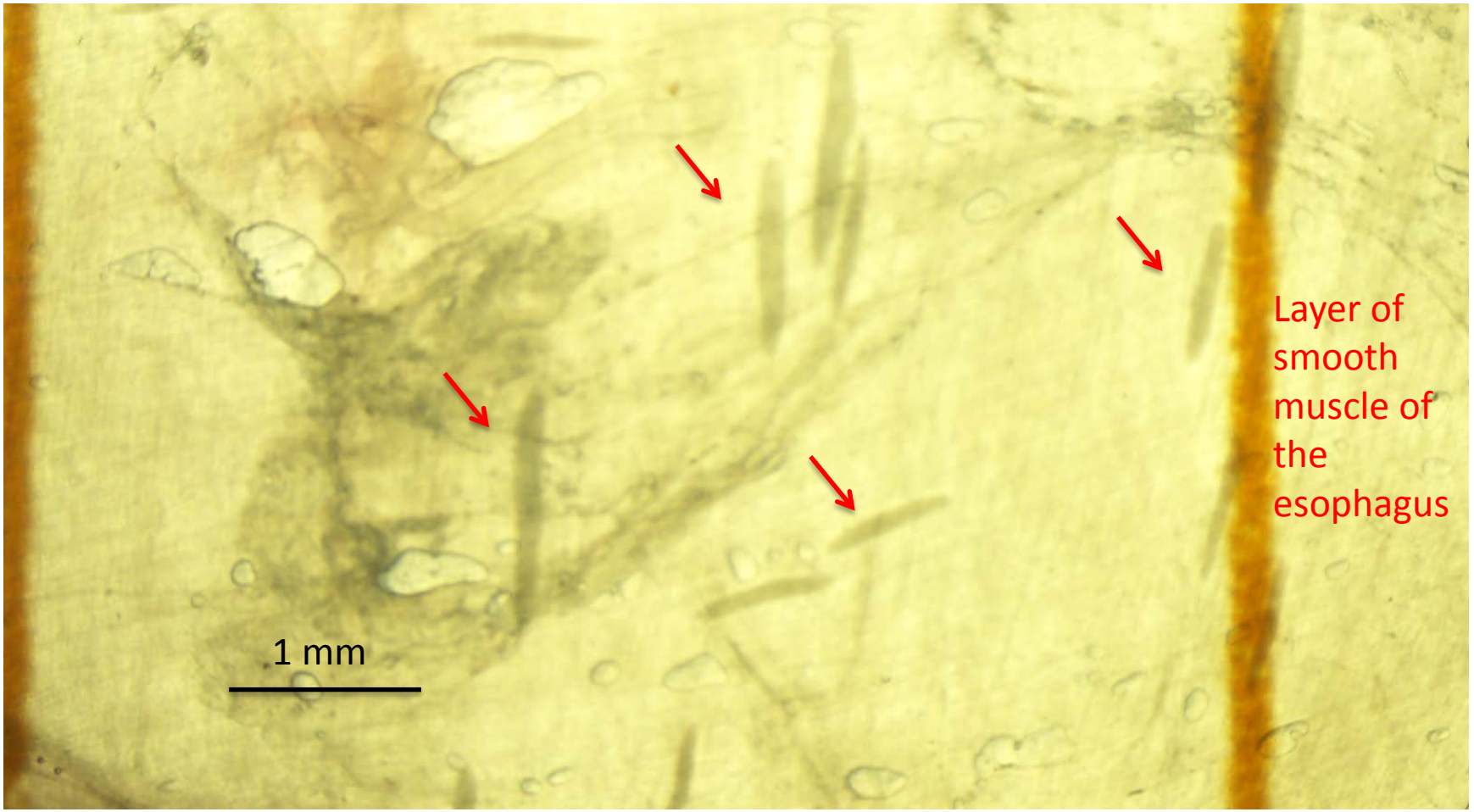
- Development of a standard procedure for the molecular identification of Sarcocystis species in meat for human consumption

- Detection of zoonotic species by PCR and sequencing

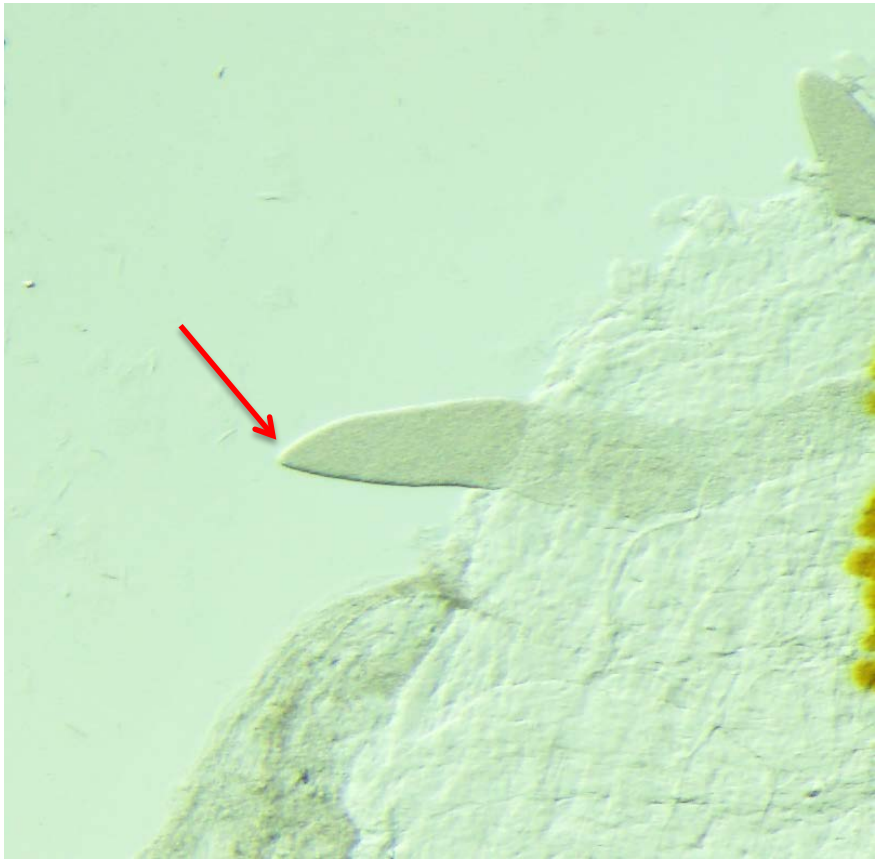
To Develop a Simple Procedure Based on PCR and Sequencing

- Collection of samples of tissue with sarcocysts
- Collection of DNA samples from identified species
- DNA extraction from cysts or infected tissue
- Selection of reference DNAs to use as controls for PCR assays
- Development of a specific PCR assay
- Sequencing of amplification products
- Identification of species by homology with referenced DNA sequences

Sarcocystis from animal muscles

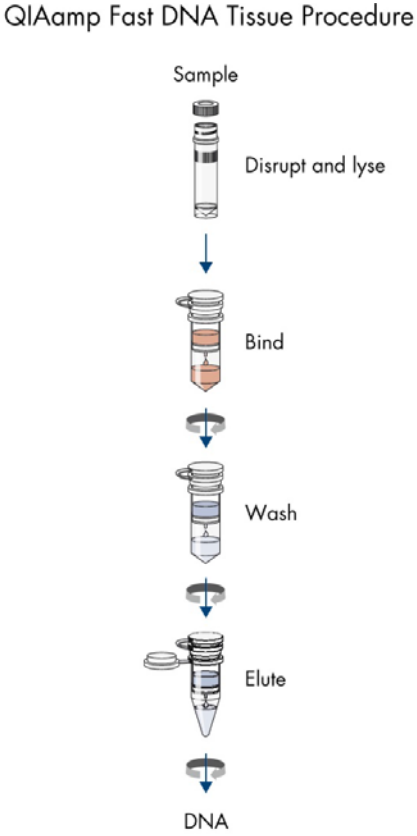


Isolated Sarcocyst in esophagus

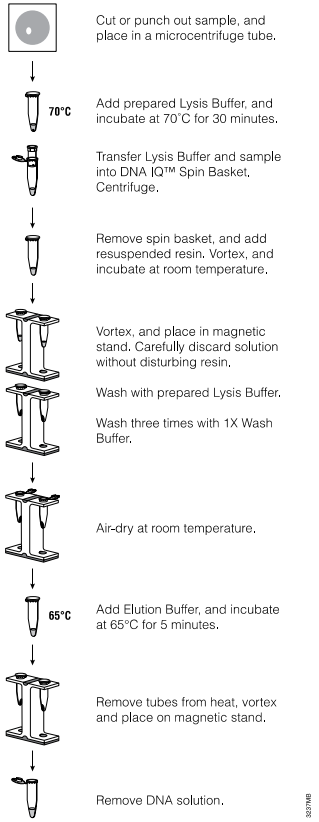


Two different methods for the DNA extraction

QIAmp technology based on silica gel affinity (more suitable for direct extraction from meat)

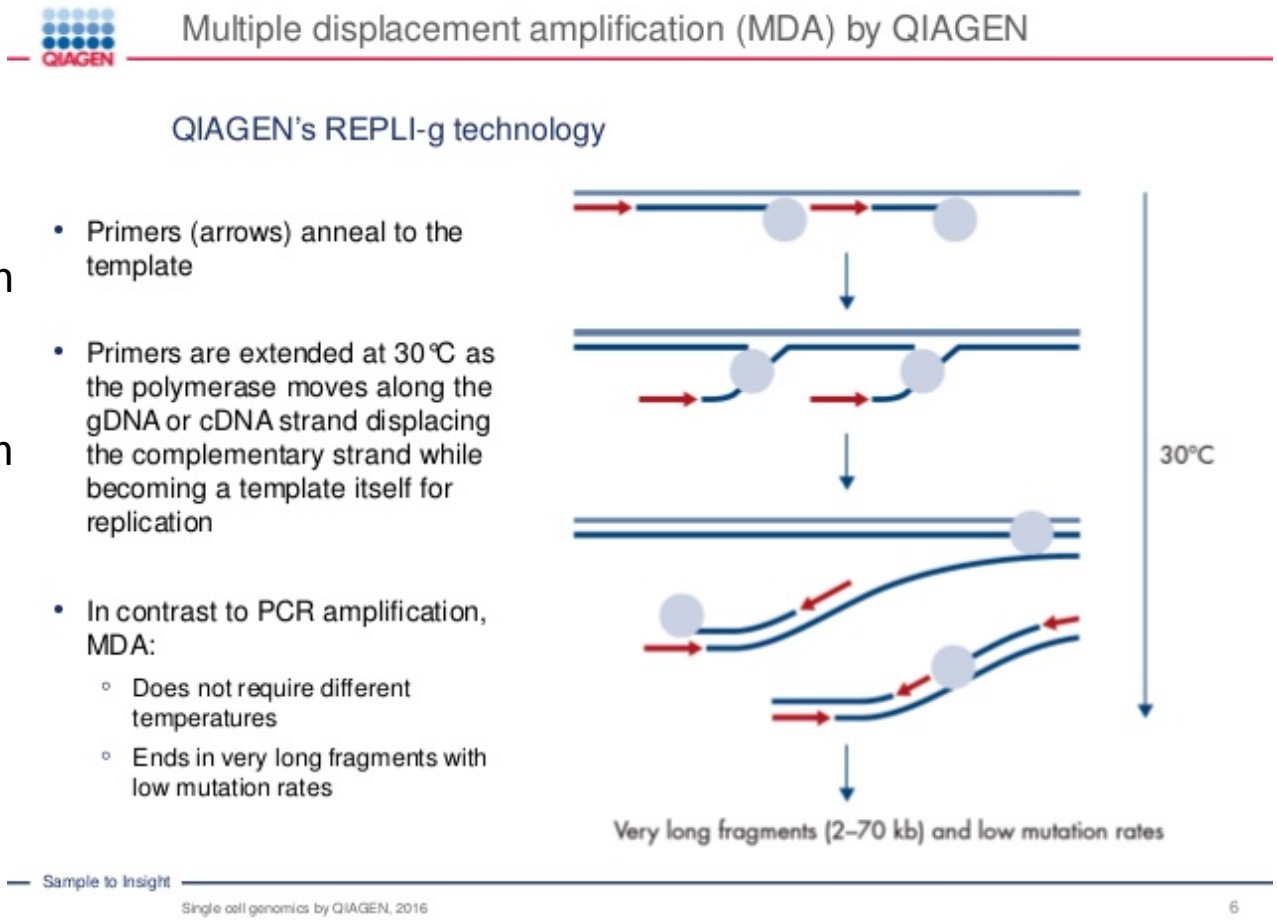


Promega IQ system based on magnetic beads (more suitable for extraction from isolated cysts)



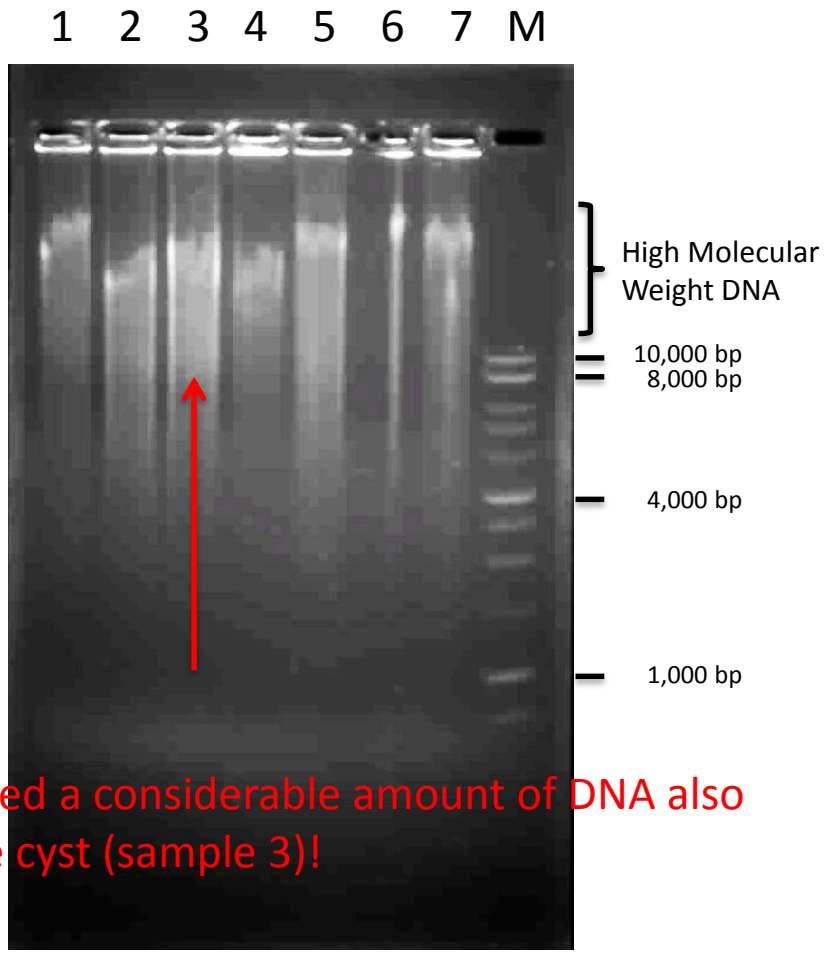
Whole Genome Amplification of positive DNAs for reference samples in PCR assays

MDA technique allows the amplification of an entire genome starting from a small amount of initial DNA.



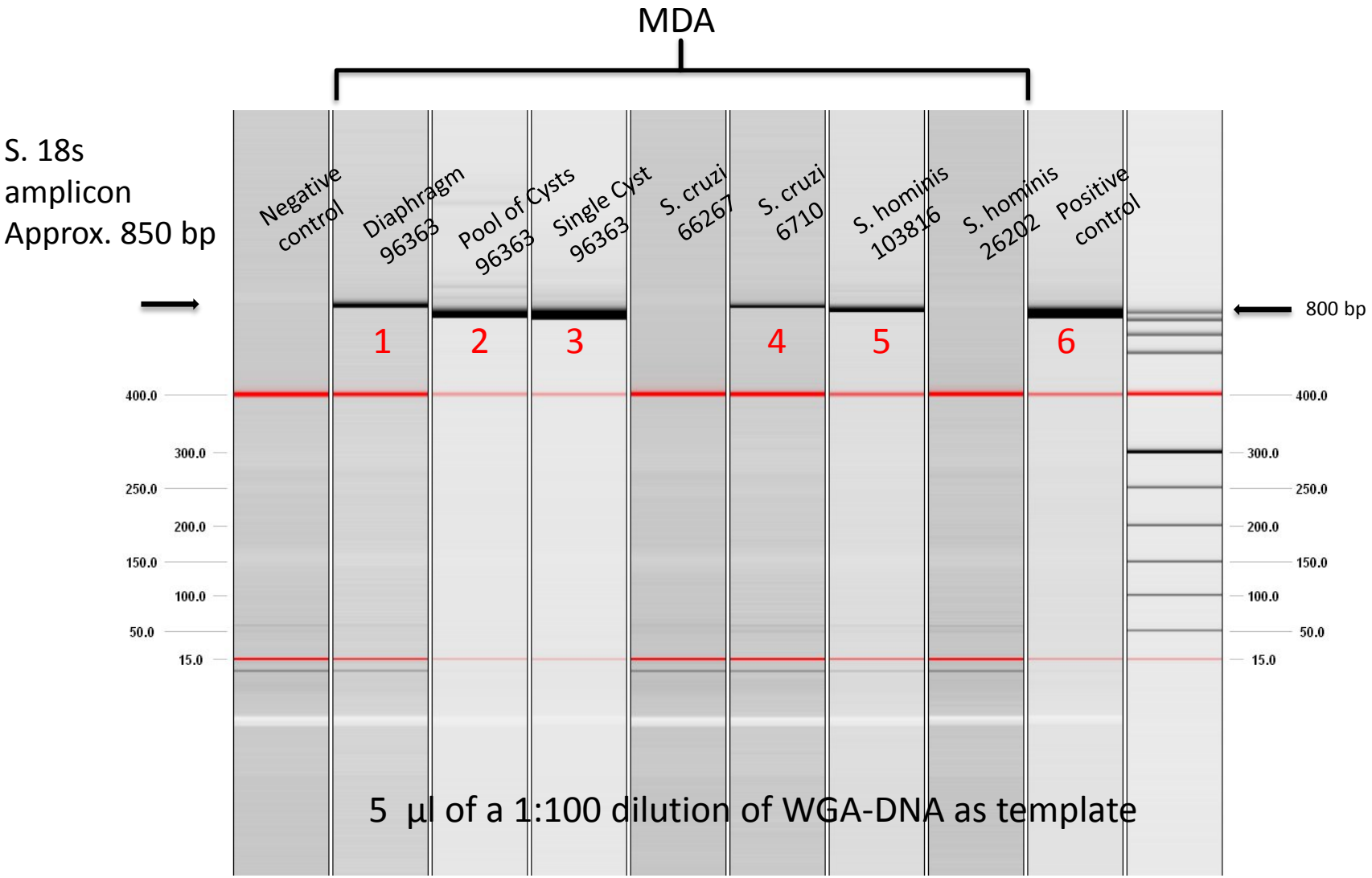
MDA from DNA extracted from isolate cysts and DNA samples of defined species

1) DNA extracted from positive diaphragm (muscle tissue) of roe deer (96363)
2) Isolate Cysts from diaphragm of roe deer (96363)
3) Single Cyst from esophagus of roe deer (96363)
4) S. cruzi 66267
5) S. cruzi 6710
6) S. hominis 103816
7) S. hominis 26202



MDA produced a considerable amount of DNA also from a single cyst (sample 3)!

PCR amplification of 18S RNA Gene (Moré et al. 2016) and Capillary Electrophoresis of the Amplicons



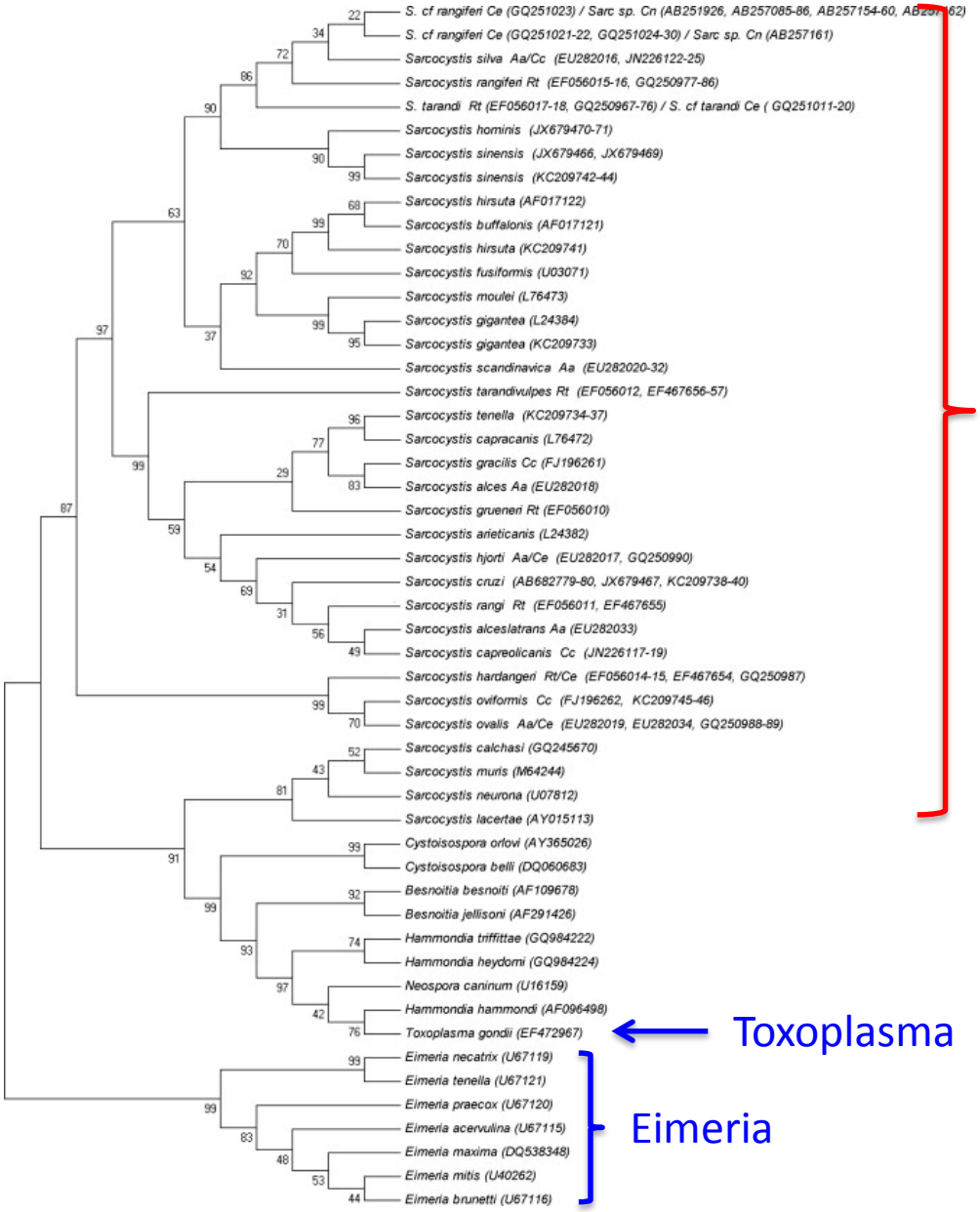
Taxonomy of Sarcocystis spp.

Genetic identification based on the DNA sequences of one or more genetic markers would be the better way to identify the specific species in a given biological sample.

Molecular identification of Sarcocystis species is mainly based on the following genetic loci:

- Ribosomal RNA genes (18s, ITS1)
- Genes of the surface antigen family (SAG 1-4)
- Mitochondrial gene like cytochrome oxidase subunit 1 (cox1)

Recent phylogenetic trees are based on sequences of these loci, however the molecular phylogenesis of Sarcocystis is more complicated than other related parasites like Toxoplasma or Eimeria.



Phylogenetic tree of the Sarcocystidae and related coccidia based on ssrRNA genes. Gjerde et al. 2013

Design of primers to amplify the cytochrome c oxidase subunit I (Cox1)

International Journal for Parasitology 43 (2013) 579–591

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journal homepage: www.elsevier.com/locate/ijpara

Phylogenetic relationships among *Sarcocystis* species in cervids, cattle and sheep inferred from the mitochondrial cytochrome c oxidase subunit I gene ☆

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Cox1 gene has been proposed as the most suitable marker for the identification of the strictly related species of Sarcocystis

- The actual reconstructions of this gene are obtained assembling different sequences
- To overcome the problem of multiple PCR and sequencing, we tried to find one or more couple of primers able to amplify different species, particularly S. hominis and S. suishominis
- Design of primers was based on the of S. cruzi Cox1 and tested “in silico” to maximise the annealing with other Sarcocystis species.

Setting a PCR assay for Sarcocystsis Cox1

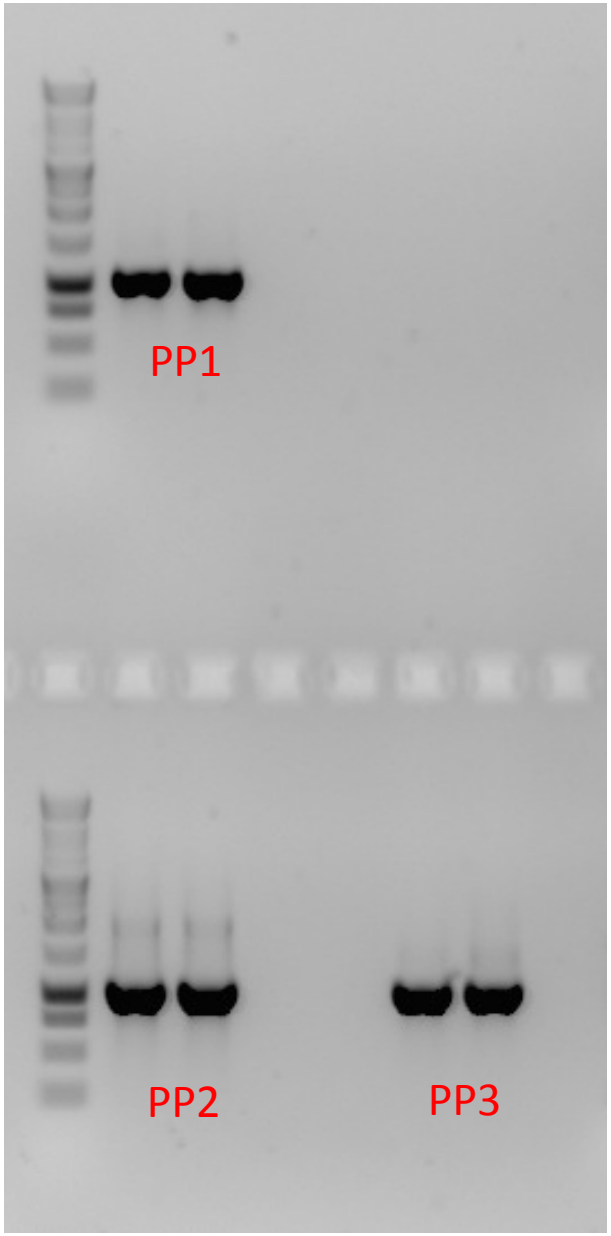
- Identification of primer pairs by Primer-BLAST https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi
- Four primers were selected on the basis of similarity with other Cox1 sequences.
- They were tested in four possible combinations and with different Annealing temperatures
- Three primer pairs (PP) gave optimal results:

PP1 = SarCoxIF1 / SarCoxIR1

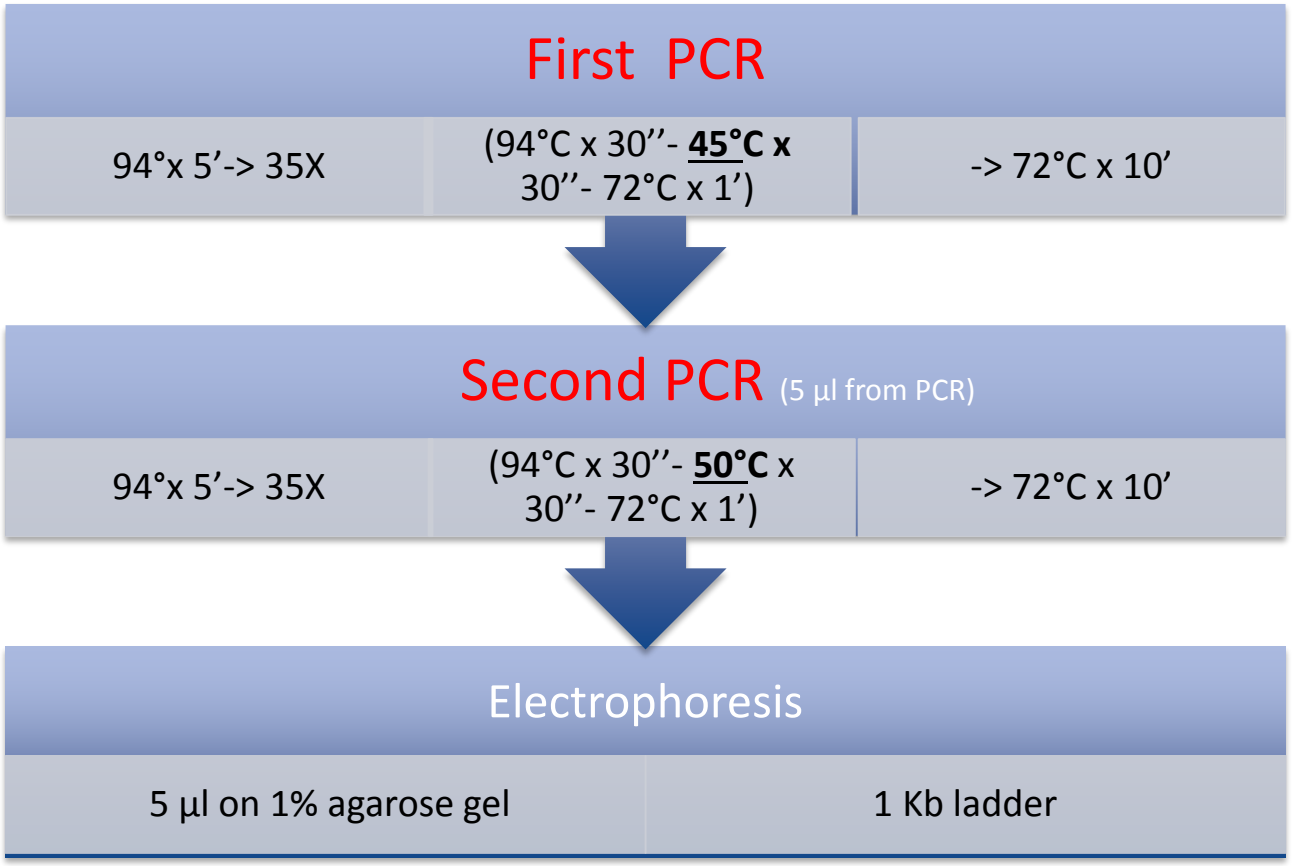
PP2 = SarCoxIF1 / SarCoxIR2

PP3 = SarCoxIF2 / SarCoxIR1

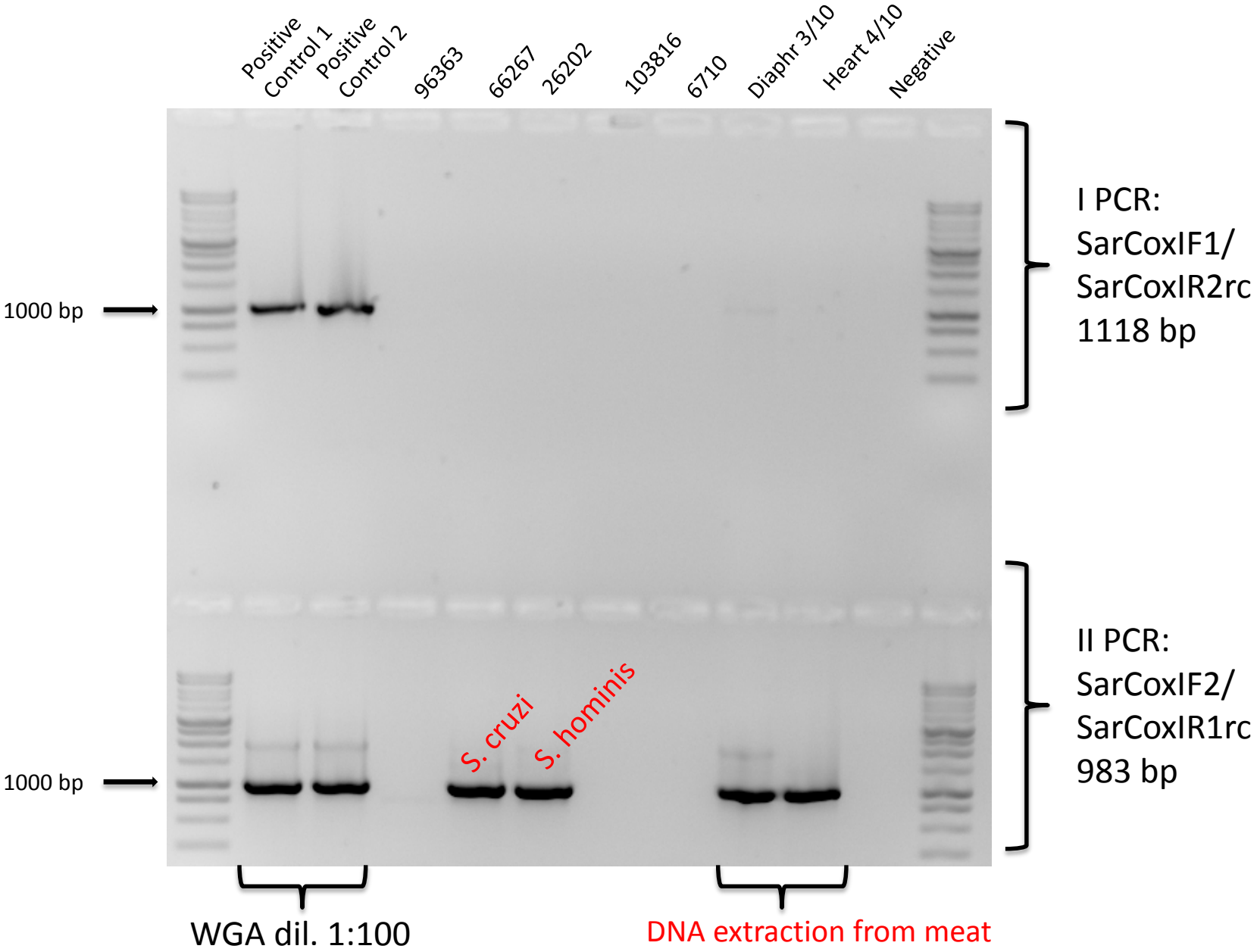
- PP1 and PP3 are suitable for nested PCR



Nested PCR For Sarcocystis Cox1 gene

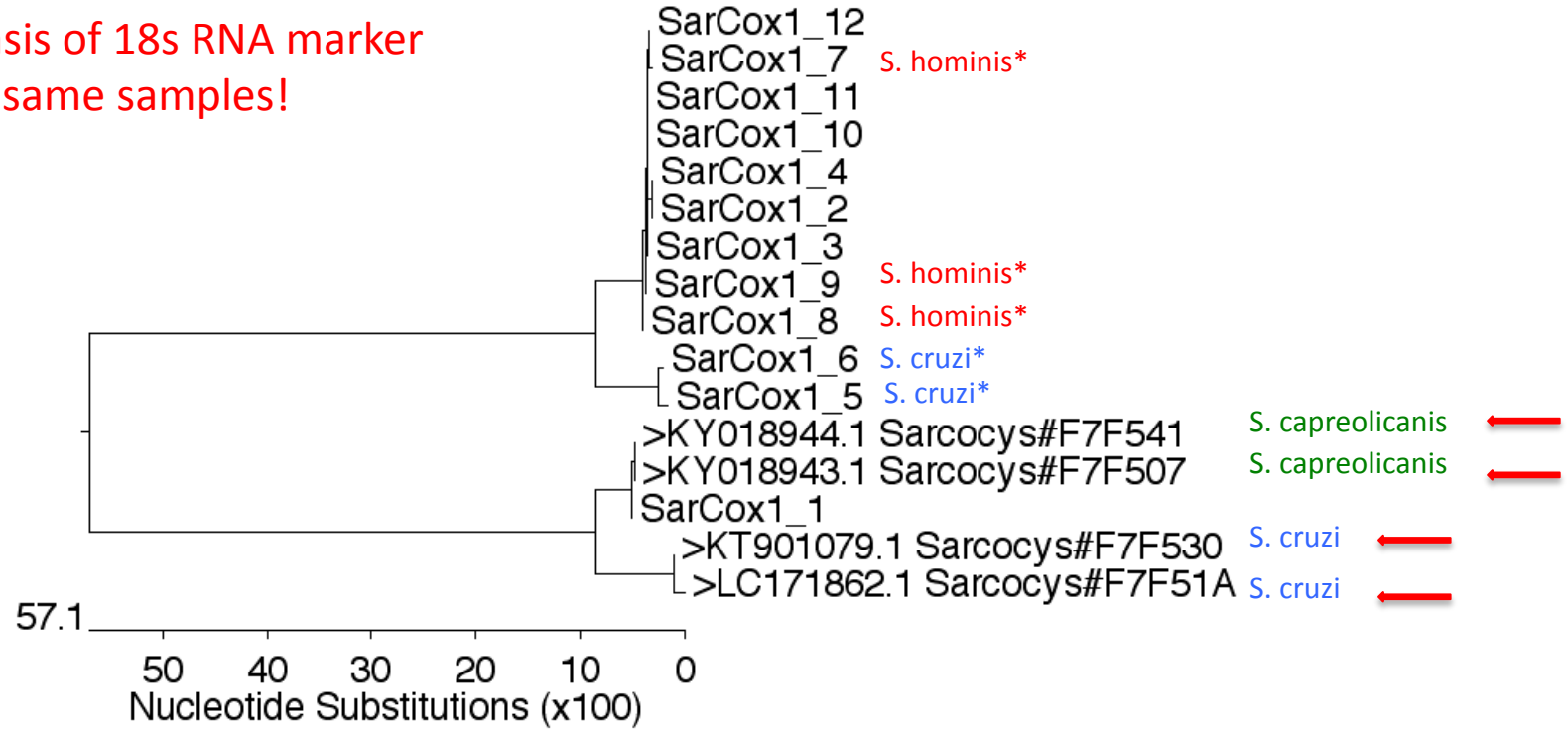


Nested PCR Sarcocystis Cox1 as genetic marker



Phylogenetic Tree based on Cox1 genetic marker

S. gracilis was identified on the basis of 18s RNA marker in the same samples!

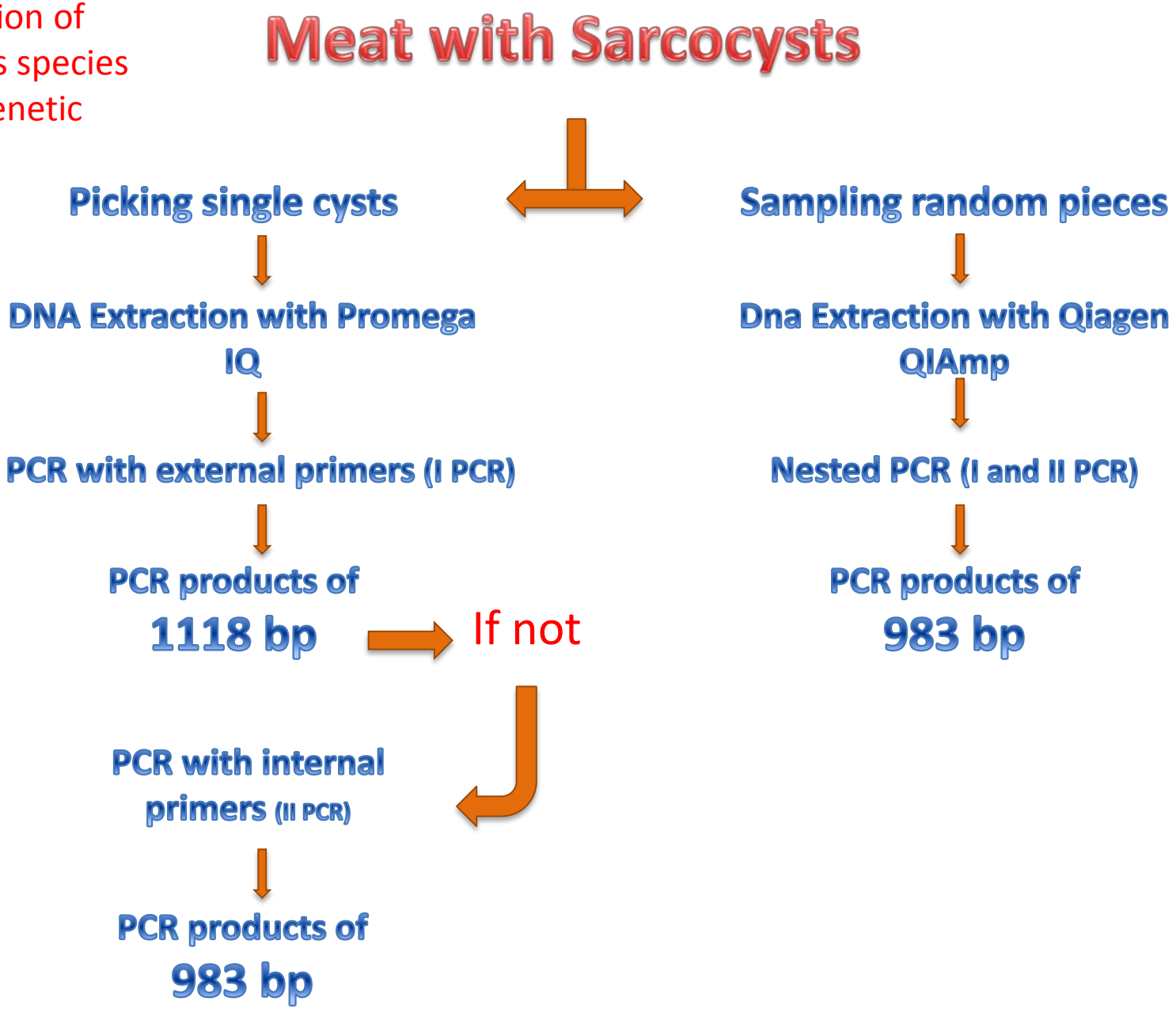


**identification by IZSTO of Turin

Conclusions

- A procedure for DNA extraction from isolated sarcocysts
- A procedure for DNA extraction from meat with sarcocysts
- Application of a PCR assay for 18s RNA genes
- Development of a new Nested-PCR assay targeting Cytochrome-oxidase subunit 1(Cox1)

Flowchart for the identification of Sarcocystis species by Cox1 genetic marker



Aknowledgements

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- Edoardo Pozio ISS Roma - Italy

Muscular sarcocystis in humans (Reports from 1981)

- All the recent cases of muscular sarcocystis come from tropical or subtropical countries
- Prevalently from the south-east Asia regions and particularly from Malaysia

TABLE 2 Summary of reports of muscular sarcocystosis in humans not included in the review by Beaver et al. and presented in chronological order of publication

Country or region	Description of human subject(s) or specimen(s)	Sarcocyst location	Biopsy or autopsy[c]	Reference, yr of publication
Malaysia	58-yr-old M[a]	Tongue	B	9, 1981
India	2 persons	Leg	B	10, 1983
		Gluteus	B	
Denmark?	4 of 112 specimens[b]	Muscle	A?	11, 1985
Malaysia	45-yr-old M[a]	Nasopharynx	B	12, 1987
	53-yr-old M[a]	Nasopharynx	B	
Malaysia	1 person	Skeletal muscle	B	13, 1988
Australia via Thailand	31-yr-old M	Skeletal muscle	B	14, 1990
Egypt	1 person	Skeletal muscle	B	15, 1990
Malaysia	45-yr-old M[a]	Tongue	B	16, 1992
	67-yr-old F[a]	Tongue	B	
	32-yr-old F[a]	Pectoral muscle	B	
Malaysia	21 persons aged 16 to 57 yr	Tongue	A	17, 1992
Belgium via Brazil, Kenya, and Tanzania	31-yr-old M	Thigh	B	18, 1995
India	40-yr-old M	Thigh	B	19, 1996
	14-yr-old F	Arm	B	
	52-yr-old F	Thigh	B	
	23-yr-old F	Arm	B	
Malaysia	44-yr-old F[a]	Thigh	B	20, 1998
	19-yr-old F	Calf	B	
Malaysia	7 adults, M	1 muscle	B	21, 1999
Thailand	66-yr-old M[a]	Larynx	B	22, 2011
India	20-yr-old M	Arm	B	23, 2012
India	50-yr-old M	Neck	B	
India	47-yr-old M	Leg	B	24, 2013
Pangkor Island, Malaysia	89 people	Leg	B	25, 2013 26, 2014 27, 2014
Tioman Island, Malaysia	39 F and 29 M subjects, aged 4 to 72 yr	Skeletal muscle from 15 patients; 1 PCR positive	B	28, 2014 29, 2014
Tioman Island, Malaysia	3 F and 3 M subjects	Febrile myositis syndrome	NTE	30, 2014
Tioman Island, Malaysia	26 cases in Germany reported previously	Symptomatic changes; skeletal muscle	B	31, 2014

[a] Cancer patients.
[b] Tissues examined by trichinoscopy.
[c] NTE, no tissue examined.

Malaysian monkeys are intermediate hosts for S. nesbitti