Polish Journal of Veterinary Sciences Vol. 15, No. 3 (2012), 589-596

DOI 10.2478/v10181-012-0090-7

Review

Molecular diagnostics of *Sarcocystis* spp. infections

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Abstract

Protozoa of the genus *Sarcocystis* (phylum Apicomplexa, family *Sarcocystidae*) is one of the most common parasites affecting animals. Interspecies diagnostic of *Sarcocystis* genus was based on electron microscopy for many years. Because of absence of visible differences between species with reachable magnifications, light microscopy is useless. In many cases serological diagnostic method have lack of sensitivity. A variety of molecular methods have been developed and used to detect and identify *Sarcocystis* spp. and to assess the genetic diversity among this protozoan from different population/hosts. Nowadays, molecular diagnostic is the common, time/cost effective method used all over the world to interspecies differentiation.

Key words: sarcocystis, sarcocystiosis, molecular diagnostics, PCR

Introduction

Protozoa of the genus *Sarcocystis* (phylum Apicomplexa), is one of the most common parasites affecting animals. Until now, about 130 species have been recognized in this genus (Dubey et al. 1989b, Tenter 1995).

The intermediate host (herbivores or omnivores) becomes infected by ingestion of sporulated oocysts or sporocysts. Each sporocyst contains four sporozoites, which are released in the digestive tract. These forms penetrate into blood vessels and multiply as merozoites (Dubey et al. 1989b). Afterwards, merozoites get into the muscles where numerous asexual divisions occur. Finally, mature sarcocyst containing numerous bradyzoites are developed. The specific definitive host becomes infected by ingestion of muscles with mature *Sarcocystis* spp. cyst. Bradyzoites are released in host intestine and penetrate into enetrocytes where sexual reproduction (gametogony) and oocyst formation occurs. Disintegration of oocysts can occur, and sporocysts may be found in the feces (Fayer 2004).

Sarcocystiosis (sarcocystosis expression is used by many researchers) is a common and cosmopolitan infection among mammals (Table 1), birds, lower vertebrates and humans.

Acute sarcocystiosis in animal intermediate hosts is characterized by encephalitis, inflammation of the brain and spinal cord, bleeding diathesis. It may cause fetal death, premature delivery, abortions in pregnant animals (Tenter 1995, Caspari et al. 2010). Mild and chronic sarcocystiosis leads to a decrease in body weight and amount of fur (Tenter 1995). Moreover, major changes were also observed in the animals behavior (Reiner et al. 2009).

The WHO reported (1981) that, about 50% of parasitic cysts found in muscles of cattle and pigs belong to the species *S. hominis* and *S. suihominis,* respectively (Acha and Szyfres 2003). High prevalence

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Table 1. *Sarcocystis* spp. in selected mammals. According to Tenter (1995) with modification of Elsheikha and Mansfield (2007) and Olias et al. (2009).

Intermediate host	Species of parasite	Definitive host
Cattle (*Bos taurus*)	*Sarcocystis cruzi*	Canidae, Raccoon (*Procyon lotor*)
	Sarcocystis hirsuta	Felidae
	Sarcocystis hominis	Primates
Yak (*Bos grunniens*)	*Sarcocystis poephagi*	Unknown
	Sarcocystis poephagicanis	Canidae
Goat (*Capra aegagrus hircus*)	*Sarcocystis hircicanis*	Dog (*Canis familiaris*)
	Sarcocystis capracanis	Canidae
	Sarcocystis moulei	Cat (*Felis catus*)
	Sarcocystis cuprifelis	Cat (*Felis catus*)
Sheep (*Ovis aries*)	*Sarcocystis arieticanis*	Dog (*Canis familiaris*)
	Sarcocystis tenella	Canidae
	Sarcocystis medusiformis	Cat (*Felis catus*)
	Sarcocystis gigantea	Cat (*Felis catus*)
Reindeer (*Rangifer tarandus*)	*Sarcocystis hardangeri*	Unknown
	Sarcocystis tarandivulpes	Canidae, Raccoon (*Procyon lotor*)
	Sarcocystis tarandi	Unknown
	Sarcocystis rangiferi	Unknown
	Sarcocystis rangi	Fox (*Vulpes vulpes*)
	Sarcocystis grueneri	Canidae, Raccoon (*Procyon lotor*)
Dromedary (*Camelus dromedarius*)	*Sarcocystis cameli*	Dog (*Canis familiaris*)
Lama (*Lama glama*)	*Sarcocystis aucheniae*	Dog (*Canis familiaris*)
Horse (*Equus ferus caballus*) and Donkey (*Equus africanus asinus*)	*Sarcocystis bertrami*	Dog (*Canis familiaris*)
	Sarcocystis neurona	Opossum (*Monodelphis domestica*) (Elsheikha and Mansfield 2007)
	Sarcocystis equicanis	Dog (*Canis familiaris*)
	Sarcocystis fayeri	Dog (*Canis familiaris*)
Rabbit (*Oryctolagus cuniculus*)	*Sarcocystis cuniculi*	Cat (*Felis catus*)
Chicken (*Gallus gallus domesticus*)	*Sarcocystis horvathi*	Dog (*Canis familiaris*) (Olias et al. 2009)
Mallard duck (*Anas platyrhynchos*)	*Sarcocystis rileyi*	Striped skunks (*Mephitis mephitis*)
Domestic pig (*Sus scrofa* f. *domestica*)	*Sarcocystis miescheriana*	Canidae, Raccoon (*Procyon lotor*)
	Sarcocystis suihominis	Primates
	Ssrcocystis porcifelis	Cat (*Felis catus*)
Dog (*Canis familiaris*)	*Sarcocystis canis*	Unknown

of muscles sarcocystiosis in pigs was confirmed by data from India, where investigators found that *S. suihominis* and *S. meischeriana* have been determined in 47% and 43% of pigs, respectively (Saleque and Bhatia 1991).

Sarcosystiosis in definitive hosts (Table 1) is most often asymptomatic, however self-limiting mild diarrhea has been observed (Dubey et al. 1989b).

As a result of *Sarcocystis* spp. zoonotic transmission, there are two known infection scenarios. First: one can act as definitive host (gastrointestinal infection). Second: one can act as intermediate host (muscle infection).

Humans are definitive hosts of two *Sarcocystis* species: *S. hominis* and *S. suihominis* (Dubey et al. 1989b). Some investigators suspect that this type of infection can be connected with abdominal pain, nausea, diarrhea, eosinophilia, inflammatory bowel disease, dyspnoea, increased pulse, and decreased appetite in some patients (Fayer 2004). Lack of hygiene and raw meat consumption are heightening risk factor of gastrointestinal infection for these protozoa

(Wilairatana et al. 1996). Sarcocystiosis connected with gastrointestinal symptoms is present worldwide, as it was confirmed by numerous researches (Dubey et al. 1989b, Fayer 2004). The prevalence of infection was 10% of the adult Laos citizens (Giboda et al. 1991). Among Tibetan citizens, 21.8% were *S. hominis*-positive and 0.06-7% were *S. suihominis*-positive. Also in Malaysia high levels of *Sarcocystis* spp. prevalence (21%) were found (Wong and Pathmanathan 1992). *Sarcocystis* infection was detected in 23.2% of stool samples originated from Thai workers (Wilairatana et al. 1996).

Human muscle sarcocystiosis is seldom detected however, this type of infection has been reported in India, Thailand, Egypt and Malaysia, so far (Acha and Szyfres 2003). In Egypt, 46% among 42 people with rheumatoid disorders examined by western blot, were *Sarcocystis*-positive. Trichinoscopic investigations carried out in 112 Danes showed *Sarcocystis* spp. in muscles of four persons (Greve 1985). The problem of *Sarcocystis* spp. in human muscles remains still unclear. Researchers suspect that this parasite can cause inflammation of muscles, heart diseases, rheumatic pains and abortion (Greve 1985, Pamphlett and O'Donoghue 1990, Habeeb et al. 1996). Moreover, it can be an opportunistic infection in patients with AIDS or immunodeficiency disorders. The species affinity of *Sarcocystis* parasites found in human muscles is not well identified so far. (Arness et al. 1999).

Basic diagnostic methods of *Sarcocystis* spp. are simple and inexpensive. One can use naked eye examination, light microscopy or histology (hematoxylin eosin staining) (Jehle et al. 2009), however, there are some more sophisticated methods, such as: immunofluorescence antibody test (IFAT) (Moré et al. 2011), ELISA (Heckeroth and Tenter 1999), Western Blot (Abdul-Rahman et al. 2002). More sensitive serological tests, which could distinguish *S. hominis* from *S. suihominis*, have not yet been developed (Tenter et al. 1991).

Interspecies diagnostic of *Sarcocystis* genus has been based on electron microscopy for many years. Light microscopy is useless, because of absence of visible differences between species with reachable magnifications (Dubey et al. 1989a, b), however, electron microscopy is expensive and time-consuming (Pritt et al. 2008). Thus, molecular biology techniques are very helpful in the diagnosis of this protozoan species.

A variety of molecular methods have been developed and used to detect and identify *Sarcocystis* spp. and to assess the genetic diversity among this protozoan from different population/hosts. Ribosomal DNA sequences are the most common molecular markers used in *Sarcocystis* spp. differentiation (Dahlgren and Gjerde 2008, Rosenthal et al. 2008, Xiang et al. 2009). This fragment of nucleic acid consists of small ribosomal subunit DNA (SSU rDNA), large ribosomal subunit DNA (LSU rDNA), two internal transcribed spacer (ITS), and two external transcribed spacer (ETS). From the aforementioned, only ETS fragments have not been used in molecular diagnostic of *Sarcocystis* spp.

Genotyping of SSU rRNA

In 1994, Tenter et. al. created a molecular diagnostic test for *S. tenella, S. arieticanis, S. gigantea*, and *T. gondii* differentiation in sheep. The test was based on ssu rDNA PCR. This type of assay is often used in phylogenetic analysis (Woese et al. 1990, Neefs et al. 1991). This sequence is conservative for related organisms, and has a variable region characteristic for the species (Maidak et al. 1997), which makes it a good diagnostic marker. SSU rDNA sequence was also used in studies based on hybridization techniques with 32P isotope, in order to diversify *S. cruzi, S. tenella, S. fusiformis, S. gigantea,* and *T. gondii* (Holmdahl et al. 1993). Diagnostics of *Sarocystis* spp. based on SSU rRNA was performed in cattle (Fischer et al 1998), horses (Elsheikha and Mansfield 2007), and pigs (Caspari et al. 2010). The investigation of Yang et. al. (2001) is an example of using SSU rRNA in phylogenetic analysis. Investigators have proved that in both bovine and buffalo (*Bubalus bubalis*), the same species of *Sarcocystis* are present. Oryan et al. (2011) used the SSU rDNA fragment to investigate *S. fusiformis* from water buffalo by means of RFLP and sequencing. Heterogeneity between isolates from various geographical localizations was reported. Other protozoa, from *Apicomplexa* phylum, were studied by the same fragment of rDNA (Morrison and Ellis 1997). This type of investigations were performed in numerous wild animals including pigeons (*Columba livia f. domestica*) (Olias et al. 2009), black bears (*Ursus americanus*) (Davies et al. 2011), raccoon dogs (*Nyctereutes procyonoides*) (Kubo et al. 2010), sparrowhawks (*Accipiter nisus*) (Olias et al. 2010), ducks (*Anas platyrhynchos*) (Kutkienl et al. 2011), roe deers (*Capreolus capreolus*) (Dahlgren and Gjerde 2008), moose (*Alces alces*), red foxes (*Vulpes vulpes*) (Dahlgren and Gjerde 2010), wolverines (*Gulo gulo*) (Dubey et al. 2010), vipers (*Atheris nitschei*) (Slapeta et al. 2003), and otters (*Enhydra lutris nereis*) (Miller et al. 2009).

Genotyping of LSU rRNA

Rbotyping, based on SSU rDNA in some cases, did not provide comprehensive results for scientists.

Usage of large ribosomal subunit (LSU rDNA, 28S rDNA) in *Sarcocystis* spp. diagnostics can enhance the sensitivity of investigation. This sequence is defined as a high variable DNA fragment at the interspecies level. Data obtained from LSU rDNA analysis were useful in sophisticated phylogenetic studies, and verified results of other assays (Mugridge et al. 2000). An example of the survey performed with LSU rDNA is Slapeta et. al. (2003) work on the occurrence of *Sarcocystis* in vipers. Investigators used the D2 domain of mentioned nucleic acid fragment, and made phylogenetic parallel. LSU rDNA was used in the diagnosis of *S. rileyi*, found in mallard duck (*Anas platyrhynchos).* It was the first detection of this parasite in Europe (Kutkienl et al. 2011). The same type of sequence has also been used to confirm that *S. wobeseri* is a parasite common in both mallard ducks as well as in Barnacle goose (*Branta leucopsis*) (Kutkienl et al. 2010). LSU rDNA was also used to phenotype *S. cornix* in corvids (Kutkienl et al. 2008). Olias et. al. (2010) applied this tool to examine *Sarcocystis* spp. in pigeons (*Columba livia f. domestica*) and sparrowhawks (*Accipiter nisus*), what resulted in detection of two new *Sarcocystis* species.

Genotyping of ITS fragments

Both, a ITS-1 and ITS-2 are useful molecular markers in population genetics studies. These fragments of nucleic acids, are adjacent to conservative genes that encode ribosomal RNA. ITS sequences are determined as species/strain variable (McManus and Bowles 1996). These features had an impact on ITS fragments popularity in genotyping of *T. gondii* and *Neospora* spp. (Homan et al. 1997). It was confirmed by Su et al. (2003). The investigators performed sensitive PCR assay for *Eimeria* spp. derived from poultry. ITS fragments were useful in *Sarcocystis* spp. diagnostic as well. With the use of described genom fragment, Rosenthal et al. (2008) compared *S. cruzi* isolates from North America with isolates from South America. They confirmed that both populations were mixed and *S. cruzi* can cross intercontinental borders.

Interspecies differentiation of *Sarcocystis* taxa, based on ITS fragments is a common practice in researches. An example of this type of application is *S. neurona* and *S. faculta* molecular differentiation assay (Marsh et al. 1999). It is also a useful tool in new *Sarcocystis* species/strains searching, because it is a conservative molecular marker (Gozalo et al. 2007). ITS fragments analysis were used in numerous studies concerning wild animals, including pigeons (*Columba livia f. domestica*) (Olias et al. 2009), rhesus macaque (*Macaca mulatta*), ducks (*Anas platyrhynchos*) (Kutkienl et al. 2011), otters (*Enhydra lutris nereis*) (Miller et al. 2009), wolverines (*Gulo gulo*) (Dubey et al. 2010), sparrowhawks (*Accipiter nisus*), (Olias et al. 2010), bald eagles (*Haliaeetus leucocephalus*), and golden eagles (Aquila chrysaetos) (Wunschmann et al. 2010).

In some cases, additional analysis of nucleic acids is required. For this purpose, scientists can use restriction fragment length polymorphism (RFLP-PCR) and single nucleotide polymorphism (SNP) analyses.

Restriction Fragment Length Polymorphism (RFLP)

An important tool used in the diagnosis of *Sarcocystis* spp. is restriction fragment length polymorphism (RFLP) analysis. This method is based on fragmentation of nucleic acids obtained from PCR reaction. In order to carry out RFLP assay, one must digest nucleic acids by restriction enzymes. After digestion, investigators receive a specific pattern of nucleic acids fragments. Comparison between obtained fragments define sequences variation. The described method is inexpensive, easy to use, and provides high sensitivity of measurements (McManus and Bowles 1996). A RFLP assay was used to examine ITS-1 fragments. In this investigation, *S. cruzi* was determined as infective for cattle and buffalo (Li et al. 2002). Tanhauser et al. (1999) performed RFLP based method that allowed for *S. falcatulta* and *S. neurona* differentiation. Opossum feces were investigated in this study. The described method is also a useful to investigate stool samples from humans, cats and dogs (Xiang et al. 2009). Universality of restriction enzymes creates possibilities in diagnostics of both domestic (More et al. 2011) and wild animals (Kia et al. 2011). Based on this type of assay, molecular profile of *S. cameli* has been described (Motamedi et al. 2011). Yang et. al. (2002) summarized the use of RFLP in *Sarcocystis* spp. differentiation in domestic animals. The authors have created a solution of restriction enzymes selection in order to obtain interspecies distinction of parasite.

Single nucleotide polymorphism (SNP)

Nucleotide polymorphism is caused by an evolution – internal agent. Genetic polymorphisms can also be the result of viruses or radiation impact (external agents). SNPs are present in all regions of the non-coding and coding regions of a genomes. Moreover, nucleotide variation may affect amino acid sequence in proteins. SNP analysis is common in mod-

ern evolutionary researches, so that investigators can seek for correlations between different species/strains (Brown 2007). Dahlgren and Gjerde (2008) carried out SNP analysis of six *Sarcocystis* species occurring in moose. *S. hardangeri* was established as genetically most variable among other species.

Whole genome analysis methods and tandem repeats investigations were used in order to receive data about phylogenetic and epidemiological studies of *Sarcocystis* spp.

Random Amplification of Polymorphic DNA (RAPD)

RAPD is a PCR reaction variant. In contrast to classical PCR, in the RAPD method, random primers (10-15 nucleotides) are used. The template for the reaction is whole genomic DNA. This results in amplification of nucleic acid fragments, which are various and specific for the organism examined. The reaction is usually visualized on electrophoresis method by different pattern of bands. RAPD is a proper tool for insertions/deletions detection, gene mapping, gene localization, phylogenetic research, the evolutionary markers detection. This method is time effective, easy-to-use, knowledge about the sequence of DNA is not required, and relatively small amounts of DNA are needed to perform the reaction (Bardakci 2001). The RAPD method was used by MacPherson and Gajadhar (1994) in a molecular probe for *S. cruzi* in a cattle investigation. The aforementioned study inspired other investigators to work out a sensitive *S. cruzi*, *S. hirsuta*, *S. hominis* detection method (Guclu et al. 2004). This type of investigation was used for detecting *Sarcocystis* spp. in sheep (Joachim et al. 1996) and differentiation between *S. neurona* and *S. falcatula* (Tanhauser et al. 1999). In 1994, Granstrom et. al. described a sensitive method for *S. neurona* detection. Investigators obtained 550 bp DNA fragment by means of 16 nucleotides primer. This marker was sensitive for *S. neurona* among 8 coccidia species, inter alia other *Sarcocystis* spp., *T. gondii*, and *Eimeria* spp. This method was also used in the diagnosis of *Sarcocystis* in the domestic cat (Gillis et al. 2003) and brown-headed cowbird (*Molothrus ater*) (Mansfield et al. 2008).

Amplified Fragment Length Polymorphism PCR (AFLP)

Methodology of AFLP PCR comprises several steps. First, restriction enzymes digest the whole genomic DNA, followed by ligation of adaptors to the sticky ends of the restriction fragments. Reaction is performed by ligase T4 DNA. Subsequently, two PCR reactions should be performed. Primers for PCR are complementary to adapter sequences. Low specific primers are used in the first PCR, and specific are used in the second reaction. Amplification products are visualized on the electrophoretic gel, usually polyacrylamide. This method is characterized by high resolution and repeatability. The knowledge about the sequence of the template is not required. This type of assay gives investigators a genetic fingerprint, which enables diagnostics and genotyping studies (Vos et al. 1995). AFLP was used to analyze *S. neurona* derived from different hosts. *S. neurona* was characterized by high intraspecies genetic variability, from 82% to 94% of whole genomic DNA. Sixty four primers were examined, nine of which gave a high resolution image in relation to polymorphism and phylogeny of *S. neurona* (Elsheikha et al. 2006).

Tandem Repeats Analysis (Microsatellite sequences)

Microsatellite sequences are common in eukaryotic genomes and consist of many repeated 2 to 6 nucleotides fragments. These sequences are highly variable, which is associated with mutation occurrence. The aforementioned feature gives the possibility to make genetic variations and to conduct phylogenetic studies. Usually, the difference between fragments length is investigated. Changes in the nucleotide sequences composition of studied tandem repeats are not very important. Most of the microsatellites occur at the end of chromosomes. These types of genetic markers are used in genotyping, evolutionary studies, and population studies (Ellegren 2004).

The investigation concerning *Sarcocystis* spp. phylogeography, twelve microsatellite markers were analyzed. Samples were collected from distant geographical locations and were investigated in terms of *S. neurona* and *S. falcatulta* derived from horses, sea otters and opossums. The occurrence of one genotype was frequently established in all examined species of animals. However, genetic variation was estimated at a high level. *S. neurona* from the North American population proved to be genetically homogeneous (Asmundsson et al. 2006).

Conclusions

The development of molecular biology has contributed to improve diagnostic methods and

phylogenetics. *Sarcocystis* spp. has become one of the targets of molecular parasitologists. Many investigations were inspired by *Sarcocystis* spp. morphological similarity and species richness of the aforementioned family. There are many useful tools for differentiation of *Sarcocystis* spp., which allow scientists to carry out epidemiological studies. However, there is no sequenced genome of any species from *Sarcocystis* family so far. Lack of clarity associated with the pathogenicity of this protozoan, the growing number of diagnosed sarcocystiosis cases, and the zoonotic potential of *Sarcocystis* spp. encourage researchers to make further studies on this group of organisms.

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