



Molecular Identification of *Sarcocystis grueneri* in Wild Korean Water Deer (*Hydropotes inermis argyropus*)

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Abstract: The cysts of *Sarcocystis grueneri* were detected and characterized from the cardiac muscles of the Korean water deer (*Hydropotes inermis argyropus*). Of the 38 heart muscle samples examined by light microscopy, 10 were found infected with the cysts of *Sarcocystis* sp. The cysts appeared oval to spherical shape and measured 110-380 µm in length and 90-170 µm in width. A phylogenetic tree of the 18S rRNA sequences (1.5 kb) revealed a close relationship of the infected cysts to genus *Sarcocystis*. The 18S rRNA sequence of the infected cysts showed 100% identity to *S. grueneri* and 97% to *S. capracanis*. Here, we first report the *S. grueneri* infections in the Korean water deer.

Key words: *Sarcocystis grueneri*, Korean water deer, 18S rRNA

INTRODUCTION

The genus *Sarcocystis* is recognized in the phylum Apicomplexa that can cause mortality of many animals and impact animal community diversity. These parasites typically infect both intermediate and definitive host animals. Intermediate host animals include herbivores and omnivorous animals that can be infected when ingesting oocysts in the feces of the definitive host animals. In turn, carnivorous animals of definitive hosts are infected when they consume the intermediate host prey infected by *Sarcocystis* [1-3].

The cysts of *Sarcocystis* species were prevalent in the cervid and in the bovid. These mammals play as the intermediate hosts in life cycle of the parasites. Recently, several species of *Sarcocystis* has been found in diverse cervid species [3-10]. Thus, it has been suggested that the host range of *Sarcocystis* parasites could be broader than the known animal species [11].

The infection surveys of the *Sarcocystis* parasites among a do-

mestic cattle population in the Republic of Korea (ROK) reveal the considerably higher infection prevalence of the parasites, especially in the old aged cattle population and also the studies found that dog is the main definitive host animal of the parasite [12-16]. In addition, *Sarcocystis grueneri* were found in a red deer and *Sarcocystis tenella* in Korean native goats [17,18]. The water deer (*Hydropotes inermis*) is a small deer seemingly more similar to a musk deer than a true deer and is the only species of genus *Hydropotes* which belongs to family Cervidae. There are 2 subspecies: The Chinese Water Deer (*H. inermis inermis*) and the Korean Water Deer (*H. inermis argyropus*) [19,20]. In the ROK, the Korean water deer is one of the widely distributed animals and overpopulated in some regions of the ROK [21]. There is no report on *Sarcocystis* infection in wild Korean water deer in the ROK. Our study first detected and characterized the infection of *S. grueneri* in wild Korean water deer in the ROK.

MATERIALS AND METHODS

Sample collection and histological study

The cardiac muscle samples were collected from 38 wild Korean water deer (male 23, female 15) at the wild animal rescue centers in Gangwon-do, Gyeonggi-do, Chungcheongbuk-do, and Daejeon city (Table 1) in 2004-2012. The divisions of heart

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were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μ m, and stained with hematoxylin and

eosin for screening by light microscopy (LM).

Table 1. Prevalence of *Sarcocystis* from Korean water deer in the Republic of Korea

Locality and no. of samples	No. positive (%)
Gangwon-do (n = 21)	6 (28.6)
Daejeon-si (city) (n = 14)	4 (28.6)
Chungcheongbuk-do (n = 2)	0 (0.0)
Gyeonggi-do (n = 1)	0 (0.0)
Total (n = 38)	10 (26.3)

DNA extraction and PCR

Fresh positive myocardium tissues were examined in order that genomic DNA was extracted with frozen after collection and stored at -20°C before examination immediately upon thawing. With tissue cutting of 0.05 g to 0.25 g, both 10 positive samples and 1 negative sample (confirmed by LM) were placed in 1.5 ml microcentrifuge tubes containing with PBS (pH 7.4).

Genomic DNA extraction was carried out using DNeasy Blood

Table 2. Sequence analyzed of the *Sarcocystis* species used in 18S rRNA genes and GenBank accession no.

Taxon name/Strain	GenBank accession no.	Intermediate host
<i>S. alces</i> , SaAa1N	EU282018	Moose (<i>Alces alces</i>)
<i>S. alceslatrans</i> , SaAa1C	EU282033	Moose (<i>Alces alces</i>)
<i>S. arieticanis</i>	L24382	Sheep (<i>Ovis aries</i>)
<i>S. buffalonis</i>	AF017121	Water buffalo (<i>Bubalus bubalis</i>)
<i>S. capracanis</i>	L76472	Goat (<i>Capra aegagrus hircus</i>)
<i>S. cornixi</i> , V1	EU553478	Hooded crow (<i>Corvus cornix</i>)
<i>S. cruzi</i> , NSMT: Pr319	AB682782	Water buffalo (<i>Bubalus bubalis</i>), Cattle (<i>Bos taurus</i>)
<i>S. dispersa</i>	AF120115	Mouse (<i>Mus musculus</i>)
<i>S. fusiformis</i> , 3f5	AF176927	Water buffalo (<i>Bubalus bubalis</i>)
<i>S. fusiformis</i>	U03071	Water buffalo (<i>Bubalus bubalis</i>)
<i>S. gallotiae</i>	AY015112	Lizard (<i>Gallotia galloti</i>)
<i>S. grueneri</i> , Sg1RtN	EF056010	Reindeer (<i>Rangifer tarandus</i>)
<i>S. hardangeri</i> , ShRt11	EF467654	Moose (<i>Alces alces</i>)
<i>S. hirsuta</i>	AF017122	Cattle (<i>Bos taurus</i>)
<i>S. hjorti</i> , Sd1CeN	GQ250990	Red deer (<i>Cervus elaphus</i>)
<i>S. lacertae</i>	AY015113	Common wall Lizard (<i>Podarcis muralis</i>)
<i>S. mucosa</i>	AF109679	Rat (<i>Rattus norvegicus</i>)
<i>S. muris</i>	M64244	Mouse (<i>Mus musculus</i>)
<i>S. neurona</i> , SN5	U07812	Brown-headed cowbirds (<i>Molothrus ater</i>)
<i>S. ovalis</i> , SoAa1N	EU282019	Moose (<i>Alces alces</i>)
<i>S. rangi</i> , Sr1RtI	EF467655	Reindeer (<i>Rangifer tarandus</i>)
<i>S. rangiferi</i> , Srf1CeN clone 2	GQ251022	Red deer (<i>Cervus elaphus</i>)
<i>S. rileyi</i> , Europe	HM185742	Mallard duck (<i>Anas platyrhynchos</i>)
<i>S. rodentifelis</i>	AY015111	Reindeer (<i>Rangifer tarandus</i>)
<i>S. silva</i> , NoCc11.3	JN226125	Roe deer (<i>Capreolus capreolus</i>)
<i>S. sinensis</i> , kxmo	AF266959	Water buffalo (<i>Bubalus bubalis</i>), Cattle (<i>Bos taurus</i>)
<i>S. tarandi</i> , St1RtN	EF056017	Red deer (<i>Cervus elaphus</i>)
<i>S. tarandi</i> , St4RtN clone 3	GQ250974	Red deer (<i>Cervus elaphus</i>)
<i>S. tarandi</i> , St1CeN clone 3	GQ251013	Red deer (<i>Cervus elaphus</i>)
<i>S. tarandi</i> , St4RtN clone 1	GQ250972	Red deer (<i>Cervus elaphus</i>)
<i>S. tarandi</i> , St3RtN clone 4	GQ250970	Red deer (<i>Cervus elaphus</i>)
<i>Sarcocystis</i> sp. TO-2012	AB691780	Ryukyu Rat (<i>Diplothrix legata</i>)
<i>Sarcocystis</i> sp. cyst type I ex) <i>Anser albifrons</i>	EU502869	Mallard duck (<i>Anas platyrhynchos</i>)
<i>Sarcocystis</i> sp. cyst type III ex) <i>Anser albifrons</i>	EU502868	Hooded crow (<i>Corvus cornix</i>)
<i>Sarcocystis</i> sp. ex) <i>Larus marinus</i>	JQ733508	Black-backed gull (<i>Larus marinus</i>)
<i>Sarcocystis</i> sp. HM050622	AB257156	Sika deer (<i>Cervus nippon yesoensis</i>)
<i>Sarcocystis</i> sp. SspEaA1N	EU282017	Moose (<i>Alces alces</i>)
<i>Neospora caninum</i>	U17345	Cattle (<i>Bos taurus</i>)
<i>Toxoplasma gondii</i>	U00458	Vertebrates

& Tissue Kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was eluted in 30 μ l of buffer (10 mM Tris-Cl, pH 8.5) yielding 33.2 ng of the average concentration were measured by Nano spectrophotometer (Nano Photometer, Implen, Munich, Germany). Loads of overlapping region between members of the Sarcocystidae covering 18S rRNA gene were amplified by the polymerase chain reaction (PCR) using primer 18S 2L (F)-GGATAACCGTGGTAATTCIATG and 18S 1H (R)-TATCCCCATCACGATGCATAC [22]. One reaction mixture contained the genomic DNA of 33.2 ng (1 μ l/reaction), HiPi PCR PreMix (Elpis Biotech. Inc., Daejeon, Korea)-*Taq* polymerase in 250 mM Tris-HCl (pH 9.0) of 1 unit, 80 mM $(\text{NH}_4)_2\text{SO}_4$, 10% DMSO, 8.75 mM MgCl_2 , 0.05% bromophenol blue, 12% glycerol, and the oligonucleotide primers (10 pmol each/ reaction of 1 μ l), RNase-free water to add to final composition. Reactions were started at 95°C for 3 min, followed by 35 cycles of 94°C for 40 sec, 55°C for 45 sec or 1 min, and 72°C for 1 min 30 sec, with final incubation at 72°C for 5 min. The PCR products have been analyzed by electrophoresis of 1.2% agarose gel. The 1,500 bp amplicon of the both species examined proved to be difficult to sequence directly from PCR products; the region was cloned for specific sequences.

Sequencing and phylogenetic analysis

The purified PCR amplicons were cloned into the pGEM®-T

Easy Vector (Promega, Madison, Wisconsin, USA). Plasmid DNA were purified and sequenced using an automatic sequencer (ABI 3730xl DNA Analyzer, Applied Biosystems, Foster City, California, USA). The achieved sequences were compared with phylogenetic analyses on the SSU rRNA gene from *Sarcocystis* species sequenced as well as with other registered sequences of *Sarcocystis* spp. from intermediate hosts, retrieved from GenBank using BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST>). A score of sequences were aligned by the Align IR (Ver 2.0; <http://biosupport.licor.com/support>), Clustal X (Ver 2.0; www.clustal.org), and MEGA 4 [23], respectively. Using the

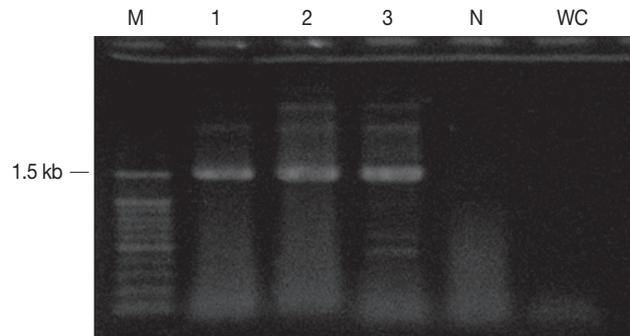


Fig. 2. PCR amplicons (1.5 kb) of sarcocysts inform the cardiac muscle. M, 100 bp DNA ladder; N, negative control; WC, water control (negative control).

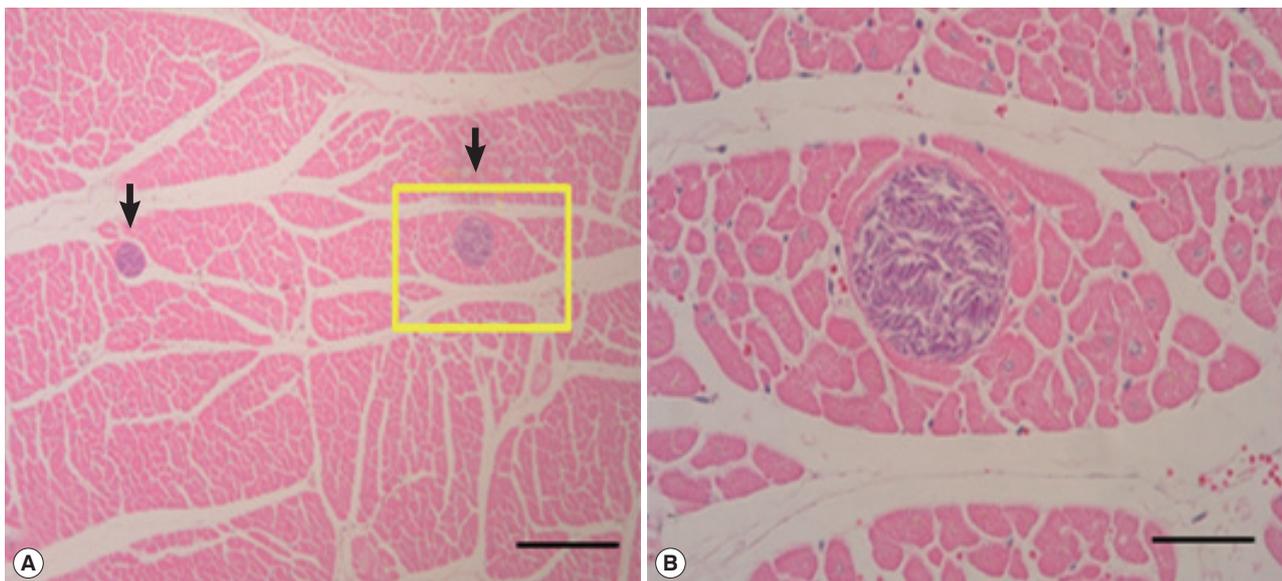


Fig. 1. (A) Photos of sarcocysts (arrows) in the cardiac muscle of the Korean water deer. Tissue reactions by the sarcocysts were not recognizable. Scale bar=200 μ m. (B) Higher magnification of a sarcocyst in A (rectangle), showing many bradyzoites in the cyst. Scale bar=50 μ m. H-E stain.

neighbor-joining (NJ) (Kimura 2-parameter distance model) methods was based on a guide tree as pairwise and multiple alignment parameters. The final alignment was comprised of a score of sequences with 39 taxa (Table 2).

RESULTS

Sarcocysts were not found in the liver or lungs (n=38), but detected from 10 heart muscle samples. All cysts were located

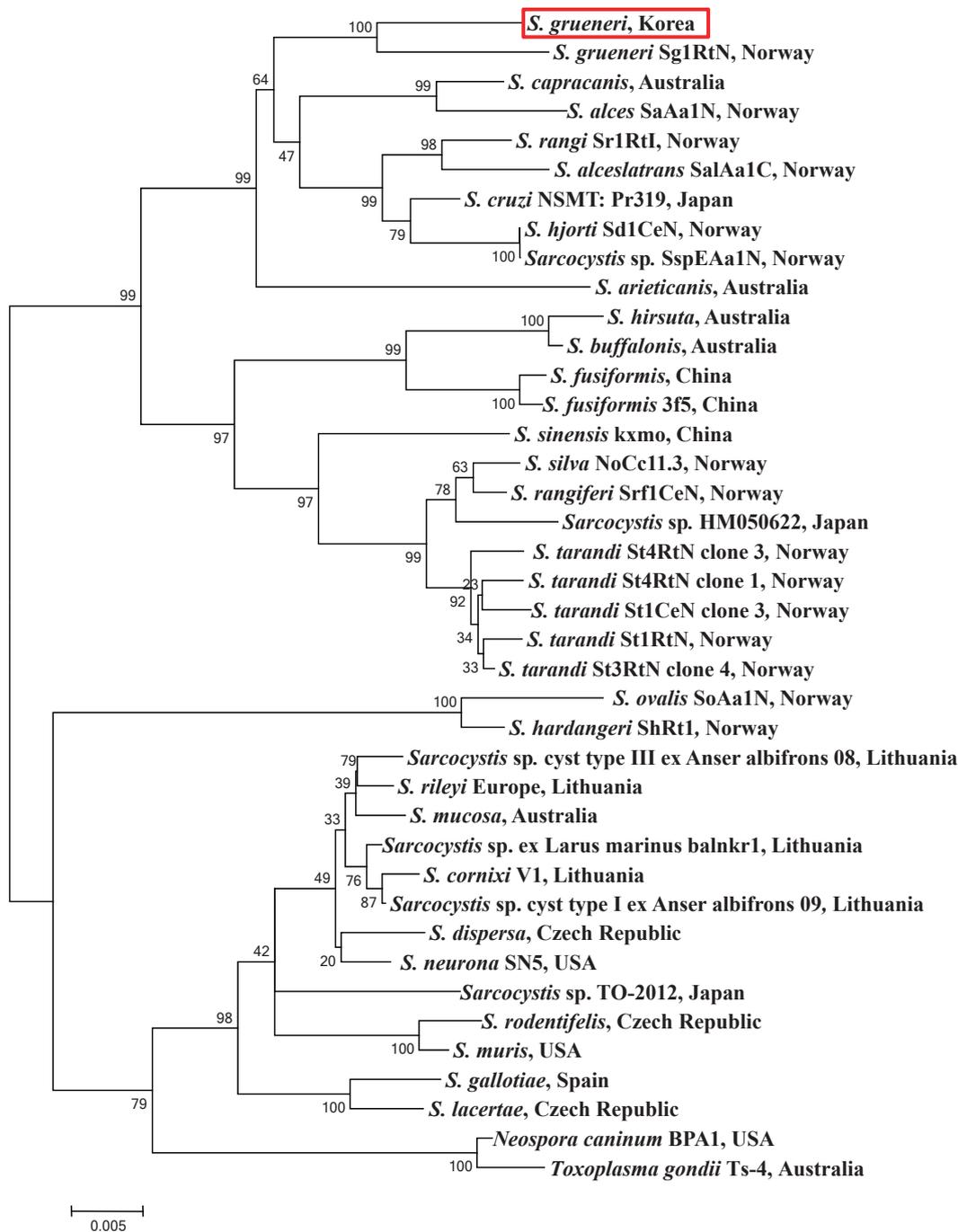


Fig. 3. Phylogram of 18S rRNA sequences from *Sarcocystis* spp. with evolutionary relationships of 39 taxa. Bootstrap values (>50%) based on 1,000 replications are shown. The *S. grueneri* detected in this study is red-boxed.

in sarcoplasm of myocardium surrounded by thin and smooth cyst wall. All of the sarcocysts were filled with bradyzoites (Fig. 1). The cysts appeared oval to spherical shape, 110-380 μm long and 90-170 μm wide under LM. The prevalence of *Sarcocystis* was 26.3% (Table 1). Although it appears that these parasites were the species of *S. grueneri*, the further molecular identification of these parasites is required.

The cyst samples were amplified using the primers of 18S rRNA and revealed 1.5 kb fragment (Fig. 2) which was a close analogue of *S. grueneri* found in reindeer (*Rangifer tarandus tarandus*) [5]. One sequence as a representative of was deposited in GenBank (accession number KC556825). The sample sequences were compared with complete 18S rRNA sequences from other 39 taxa of genus *Sarcocystis* (Table 2). The 18S rRNA sequence is proposed as a standard gene for the phylogenetic analysis among the species within the protozoa phyla, which is hence used for species identification in this study [24]. The sequence identified between the cyst samples showed 100% homology with *S. grueneri* and 97% with *S. capracanis*. In addition, both 53% AT base construction and low level of sequence variations were consistently shown in the phylogenetic analysis (Fig. 3).

DISCUSSION

In previous study, the cysts of *S. grueneri* were found in the heart muscles, which indicate the specific organ tropism of the parasite [10]. Similar to the previous study, a cervid animal may be infected in heart muscle by similar *Sarcocystis* species. The Korean water deer may serve as an intermediate host with a similar level of high specificity and prevalence as observed in other studies [9]. For instance, 98.0% of the sister species of a cervid animal in Germany were infected with *Sarcocystis* parasites [25,26]. Thus, it appears that the infection of *Sarcocystis* species is quite common to many species of a cervid animal. Although the Korean water deer is widely distributed in ROK, it has not been extensively studied until recent years especially in infection study of the parasite of *Sarcocystis* species. In this study, we found that the infection rate of the *Sarcocystis* parasites was 26.3% (10 out of 38 samples of the Korean water deer). Among the 38 collection sites in the ROK, 6 infection muscles (15.8%) were detected in the provinces of Gangwon, Gyeonggi, and Chungcheongbuk-do, and 4 infection samples in Daejeon city (10.5%), respectively. Furthermore, identification of the parasite that is collected from the Korean water

deer was examined by molecular method. In previous study, molecular technique could identify a single species of the parasite from the host animal tissues and distinguish individual species from other parasites of *Sarcocystis* species [5]. For more accurate morphometry for the species identification of the parasites, the electron microscope has been used and successfully reported key morphological features in several parasites. For instance, the cyst of *S. grueneri* collected from reindeer are morphologically similar to the cysts collected from either the host animal roe deer (*Capreolus capreolus*) or red deer (*Cervus elaphus*) in the protrusion of the cyst wall [8,27].

The general phylogenetic trend of Sarcocystinae has already explained other former studies [22,24,28,29]. In our study, the fundamental composition did not change by including *Sarcocystis* species from a similar intermediate host. Phylogenetic topology of bootstraps of 1,000 described the 2 species highly similar and their branch length very close from the sequences of 18S rRNA gene in *S. grueneri* (Genbank accession number EF056010). Thus, the results of phylogenetic analysis indicate that our sample is in the clade of a sister taxa including *S. capracanis*, *S. alces*, *S. rangi*, and *S. alceslatrans*. Furthermore, these species also share the similar intermediate host animal in the family Cervidae except for *S. capracanis* (Fig. 3).

In addition, it has been known that the parasites of *Sarcocystis* species infect both animal hosts such as intermediate host and definitive host, especially in the stage of sporocyst and oocyst. Interestingly, in previous study, it has been shown that humans are susceptible to the infection of sarcocysts [30]. This infection could be caused by water contaminated with feces from the other animal hosts or food washed with unsanitary water [31]. Other unknown animals including human could be a potential target for the infection of *Sarcocystis* parasites. In this context, the DNA sequences of 18S rRNA gene related to *Sarcocystis* parasites presented in this study can promote further development of needed diagnostics to prevent a potential infection of *Sarcocystis* parasite in wild animals and humans.

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CONFLICT OF INTEREST

We have no conflict of interest related to this work.

REFERENCES

- Dubey JP. A review of *Sarcocystis* of domestic animals and of other coccidia of cats and dogs. *J Am Vet Med Assoc* 1976; 169: 1061-1078.
- Fayer R. Production of *Sarcocystis cruzi* sporocysts by dogs fed experimentally infected and naturally infected beef. *J Parasitol* 1977; 63: 1072-1075.
- Dubey JP, Speer CA, Epling GP. Sarcocystosis in newborn calves fed *Sarcocystis cruzi* sporocysts from coyotes. *Am J Vet Res* 1982; 43: 2147-2164.
- Colwell DD, Mahrt JL. Ultrastructure of the cyst wall and merozoites of *Sarcocystis* from moose (*Alces alces*) in Alberta, Canada. *Z Parasitenkd* 1981; 65: 317-329.
- Dahlgren SS, Gjerde B. Genetic characterisation of six *Sarcocystis* species from reindeer (*Rangifer tarandus tarandus*) in Norway based on the small subunit rRNA gene. *Vet Parasitol* 2007; 146: 204-213.
- Dahlgren SS, Gjerde B. *Sarcocystis* in Norwegian roe deer (*Capreolus capreolus*): molecular and morphological identification of *Sarcocystis oviformis* n. sp. and *Sarcocystis gracilis* and their phylogenetic relationship with other *Sarcocystis* species. *Parasitol Res* 2009; 104: 993-1003.
- Dubey JP. Coyote as a final host for *Sarcocystis* species of goats, sheep, cattle, elk, bison and moose in Montana. *Am J Vet Res* 1980; 41: 1227-1229.
- Entzeroth R, Chobotar B, Scholtyseck E. Ultrastructure of *Sarcocystis* sp. from the muscle of a white-tailed deer (*Odocoileus virginianus*). *Z Parasitenkd* 1982; 68: 33-38.
- Entzeroth R, Chobotar B, Scholtyseck E, Neméseri L. Light and electron microscope study of *Sarcocystis* sp. from the fallow deer (*Cervus dama*). *Z Parasitenkd* 1985; 71: 33-39.
- Kutkienė L. Investigations of red deer (*Cervus elaphus*) *Sarcocystis* species composition in Lithuania. *Acta Zool Litu* 2003; 13: 390-395.
- Atkinson CT, Wright SD, Telford SR, McLaughlin GS, Forrester DJ, Roelke ME, McCown JW. Morphology, prevalence, and distribution of *Sarcocystis* spp. in white tailed deer (*Odocoileus virginianus*) from Florida. *J Wildl D* 1993; 29: 73-84.
- Kang YB, Kim SH, Wee SH. A survey of *Sarcocystis* infections in slaughtered cattle in Korea. *Korean J Vet Res* 1988; 28: 387-390 (in Korean).
- Jang H, Kang YB, Wee SH, Choi SH. Survey of *Sarcocystis* infections in cattle in Korea. *Res Rept RDA(V)* 1990; 32: 32-37 (in Korean).
- Yang JH, Kang YB, Wee SH, Lee ST, Kim KS. Prevalence of *Sarcocystis* infections in the slaughtered cattle in Cheju. *Korean J Vet Res* 1990; 30: 507-510 (in Korean).
- Park YJ, Kim JS, Joeung DS, Sin MK, Kim KS, Kim TJ. A survey of *Sarcocystis* infections in the slaughtered cattle and identification of *Sarcocystis cruzi*. *Korean J Vet Publ Hlth* 1994; 18: 251-259 (in Korean).
- Wee SW, Shin SS. Experimental induction of two-host life cycle of *Sarcocystis cruzi* between dogs and Korean native cattle. *Korean J Parasitol* 2001; 39: 227-232. (in Korean)
- Son WY, Kim NS, Ryu SY, Kim HC, Rhee JH, Cho JG, Park BK. Ultrastructure of *Sarcocystis grueneri*-like sarcocysts from cardiac muscle of red deer (*Cervus elaphus*) in Korea. *J Vet Clin* 2009; 26: 595-599.
- Hong EJ, Sim C, Chae JS, Kim HC, Park J, Choi KS, Yu DH, Park CH, Yoo JG, Park BK. Ultrastructural and molecular identification of *Sarcocystis tenella* (Protozoa, Apicomplexa) in naturally infected Korean native goats. *Veterinari Medicina* 2016; 61: 374-381.
- Allen GM. Mammals of China and Mongolia. Natural history of central Asia. American Museum of Natural History, New York. 1940; 11: 1126-1261.
- Valerius G. Deer of the World: Their Evolution, Behaviour, and Ecology. Pennsylvania, USA. Stackpole books. 1998, p 26-28.
- Cooke A, Farrell L. Chinese water deer. Mammal society. 1998, p 30.
- Yang ZQ, Zuo YX, Yao YG, Chen XW, Yang GC, Zhang YP. Analysis of the 18S rRNA genes of *Sarcocystis* species suggests that the morphologically similar organisms from cattle and water buffalo should be considered the same species. *Mol Biochem Parasitol* 2001; 115: 283-288.
- Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* 2007; 24: 1596-1599.
- Morrison DA, Bornstein S, Thebo P, Wernery U, Kinne J, Mattson JG. The current status of the small subunit rRNA phylogeny of the coccidia (Sporozoa). *Int J Parasitol* 2004; 34: 501-514.
- Erber M, Boch J, Barth D. *Sarcosporidia* strains in the roe deer. *Berl Munch Tierarztl Wochenschr* 1978; 91: 482-486 (in German).
- Partenheimer-Hannemann C. Untersuchung zum Vorkommen von Sarkosporidien bei Reh- und Rotwild im Raum Bitburg-Prüm area (Rheinland-Pfalz). [Inaugural-Dissertation]. [Hannover]: Tierärztliche Hochschule Hannover. 1991.
- Speer CA, Dubey JP. *Sarcocystis wapiti* sp. nov. from the North American wapiti (*Cervus elaphus*). *Can J Zool* 1982; 60: 881-888.
- Slapeta JR, Kyselová I, Richardson AO, Modrý D, Lukes J. Phylogeny and sequence variability of the *Sarcocystis singaporensis* Zaman and Colley, (1975) 1976 ssrDNA. *Parasitol Res* 2002; 88: 810-815.
- Tenter AM, Johnson AM. Phylogeny of the tissue cyst-forming coccidia. *Adv Parasitol* 1997; 39: 69-139.
- Heydorn, AO. Sarkosporidieninfiziertes Fleisch als mögliche Krankheitsursache für den Menschen. *Arch Lebensmittelhyg.* 1977; 28: 27-31 (in German).
- Fayer R. *Sarcocystis* spp. in human infections. *Clin Microbiol Rev* 2004; 17: 894-902.