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Research Brief

First molecular identification of *Sarcocystis miescheriana* (Protozoa, Apicomplexa) from wild boar (*Sus scrofa*) in Iran

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ABSTRACT

Sarcocystis isolate obtained from the thigh muscle of a wild boar (*Sus scrofa*), captured from Gilan Province, northern Iran, was subjected to molecular analysis. Genomic DNA was obtained using a DNA extraction tissue kit and Polymerase chain reaction (PCR) for amplification of the 18S ribosomal DNA region yielded an 842 bp DNA band on agarose gel. Analysis of DNA sequencing by BLAST confirmed the isolate as *Sarcocystis miescheriana* and the sequence was deposited in GenBank by Accession No. GU395554. This is the first molecular identification of an isolate of *S. miescheriana* in Iran.

1. Introduction

Sarcocystis is a genus of cyst-forming coccidia belonging to the phylum Apicomplexa. This genus is comprised of more than 200 species (Frenkel and Smith, 2003). The organism has an obligatory heteroxenous life cycle, with a sexual stage in enteroepithelial cells of the definitive host, and asexual generation in the tissues of the intermediate host (Dubey et al., 1989). Usually herbivores and omnivores serve as intermediate hosts while omnivores and carnivores are definitive hosts (Lindsay et al. 1995). Intermediate and definitive hosts can harbor various *Sarcocystis* spp (Mehlhorn and Heydorn, 1978). Species in the family Suidae generally harbor tissue cysts of two species of *Sarcocystis,* namely *Sarcocystis miescheriana* (Kühn, 1865) Labbé, 1899 (synonym: *Sarcocystis suicanis*) (Heydorn et al., 1975; Erber, 1977; Mehlhorn and Heydorn, 1978) and *S. suihominis* (Tadros & Laarman, 1976) (Heydorn, 1977). The validity of third species, *Sarcocystis porcifelis* (Dubey, 1976) is still unclear (Dubey et al., 1989).

Species identification in *Sarcocystis* is usually performed by morphological characterization of sarcocyst especially cyst wall structure and sporocysts under light or transmission and scanning electron microscopy. Since the appearance may change depending on the location and developmental stage of sarcocyst and other conditions of parasitized cell, molecular studies have been suggested to confirm morphological species identification (Levine, 1986; Dahlgren and Gjerde, 2007). Gene sequence data analysis is also very useful tool to clarify whether morphologically similar sarcocysts in intermediate hosts are the same or different species (Yang et al., 2001).

In Iran, some species of livestock are commonly parasitized by species of *Sarcocystis*. There have been several studies on prevalence of infection in cattle and sheep from different parts of the country, but a few have identified the species involved (Naghibi et al., 2002; Dalimi et al., 2008). In the present study, the first molecular identification of *S. miescheriana* from a wild boar in northern Iran is documented.

2. Materials and methods

The source of parasite was a portion of a frozen thigh muscle of a wild boar hunted in April 2007 from Javaher Dasht Forest, Siahkal, Gilan Province, a temperate area in northern Iran, bordering the Caspian Sea.

A portion of the muscle tissue was prepared for direct examination by light microscope. Gross inspection of unstained tissue smears revealed the presence of *Sarcocystis* cysts. A piece of muscle was preserved in 10% formalin and after tissue processing using conventional histological methods, and 5 μm sections were prepared and stained with H&E (haematoxylin-eosin).

For molecular identification, small pieces of muscles were preserved in 80% ethanol alcohol until further use. Total genomic DNA was extracted from a small piece of muscle stored in ethanol, employing DNeasy blood & tissue (Qiagen, Hilden, Germany)

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according to the manufacturer's instructions for animal tissue extraction. A fragment of the 18S ribosomal DNA gene was amplified by PCR reaction using forward (SarcoF: 5′-CCA TAT TTT ATG GTG GTG GTG A-3′) and reverse (SarcoR: 5′-AGT CTT TGG CAA TGC TTT C-3′) primers, designated in the present study, to amplifying a 842 bp fragment. PCR reactions was performed in a final volume of 50 μl containing 2 μl genomic DNA, 50 pmol of each primer and 10 μl of commercial 2X master mix (Rovalab, Teltow, Germany). The reactions were conducted in a thermocycler (Applied Biosystems, GeneAMP® PCR System 2700, Singapore) under the following PCR program: 5 min at 95 °C for one cycle (primary denaturation) followed by 30 cycles of 45 s at 94 °C (denaturation), 1 min at 55 °C (annealing) and 1 min at 72 °C (extension), and 7 min at 72 °C as a final extension. 5 μl of amplicon was run through 1.5% (W/V) agarose gel in TBE (90 mM Tris–HCl, 90 mM boric acid, 2 mM EDTA, pH 8.3) containing 0.5 μg/ml ethidium bromide and visualized by UV transilluminator (UVitec, Cambridge, UK). A 100 bp ladder was used as a DNA size marker. The PCR product was subjected to automated sequencing by Source BioScience Company (Cambridge, UK) with the Illumina Genome Analysis system in both directions, employing the same primers used in the primary PCR. Nucleotide sequence was aligned with other *Sarcocystis* sequences deposited in the GenBank database (via the National Center for Biology Information, http://www.ncbi.nlm.nih.gov/), using BLAST software. For better understanding of relationship among *S. miescheriana* and other species in the genus *Sarcocystis* a phylogenetic analysis of 18S rDNA was performed employing Bayesian Inference method (BI). BI conducted using MrBayes v.3.1.2 softeware (http://mrbayes.csit.fsu.edu/index.php). *Neospora caninum* was employed as the outgroup.

3. Results and discussion

In direct examination of the thigh muscle with the aid of light microscope microscopical sarcocysts were found. In the histological sections of the muscle, *Sarcocystis* cysts were observed (Fig. 1) measuring 50–77 μm × 139–396 μm in size. No inflammatory reaction was found around the cysts in the tissue.

The PCR reaction, using specific designed primers, demonstrated an expected band on agarose gel (Fig. 2). The assembling of DNA sequence in both directions (using forward and reverse primer) yielded a fragment containing 783 consensus nucleotides. The obtained DNA sequence was analyzed using BLAST software and the isolate was identified as *S. miescheriana*. The nucleotide sequence of *S. miescheriana* was deposited in the GenBank/EMBL/DDBJ database under Accession No. GU395554.

Importance of species identification in *Sarcocystis* is significant since some species are more pathogen for certain livestock and vary in infectivity for human. The 18S ribosomal DNA region has been extensively used as a suitable target to differentiate the *Sarcocystis* spp. and phylogenetic relationship among them (Alex et al., 1997; Yang et al., 2002; Dahlgren and Gjerde, 2007; da Silva et al., 2009).

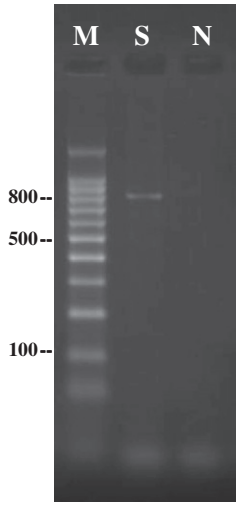

Fig. 2. Electrophoresis of PCR product of *Sarcocystis miescheriana* isolated from wild boar in northern Iran. Lanes: M, 100 bp ladder DNA size marker; S, *Sarcocystis miescheriana*; and N, negative control.

There are several studies on *Sarcocystis* in livestock from different regions in Iran, mostly reporting prevalence of infection; but a few have identified *Sarcocystis* species. *S. cruzi* has been reported in two infected calves based on clinical, morphological and pathological criteria after experimental infection in two puppies and consequently in a calf (Naghibi et al., 2002). Dalimi et al. (2008) studied *Sarcocystis* isolates from slaughtered sheep by PCR-RFLP based on 18S rDNA and reported macroscopic cysts as *S. gigantea* and microscopic cysts as *S. arieticanis*. Current report on occurrence of *S. miescheriana* in a wild boar in Iran constitutes the first molecular characterization of this species in the country. *S. miescheriana* probably has a worldwide distribution; it has been reported from pigs and wild boars of different countries throughout the world (Tadros and Laarman, 1982).

The method used in this study for identification combined 18S rDNA amplification, sequencing and comparing with the key reference sequences of *Sarcocystis* species deposited in the GenBank. The nucleotide sequence of *S. miescheriana* obtained in this study was deposited in the GenBank/EMBL/DDBJ database under Accession No. GU395554. This sequence has 100% and 99% homology compared to *S. miescheriana* from Germany with Accession Nos. EU327974 and DQ839352, respectively. The phylogenetic analysis based on BI (Fig. 3) indicated four distinct clades in the genus *Sarcocystis*. *S. miesheriana* from the present study (Accession No. GU395554) placed in a group with *S. miesheriana* from Germany (Accession No. EU327974) having a strong statistical support (pp = 0.97). These group with a maximal statistical support (pp = 1.00) was adjacent with another group containing two *S. suihominis* sequences (pp = 0.97). This issues indicates that

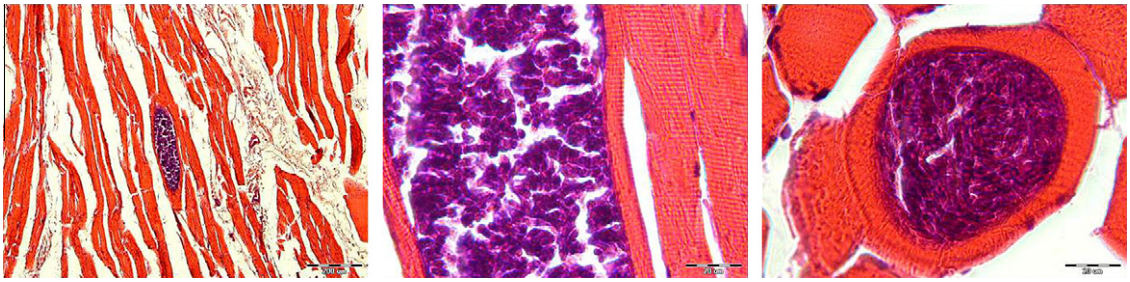

Fig. 1. *Sarcocystis miescheriana* tissue cysts from thigh muscle of wild boar, stained with H&E. (A) and (B) longitudinal sections and (C) cross section. Scale bar indicates 200 μm in (A) and 20 μm in (B) and (C).

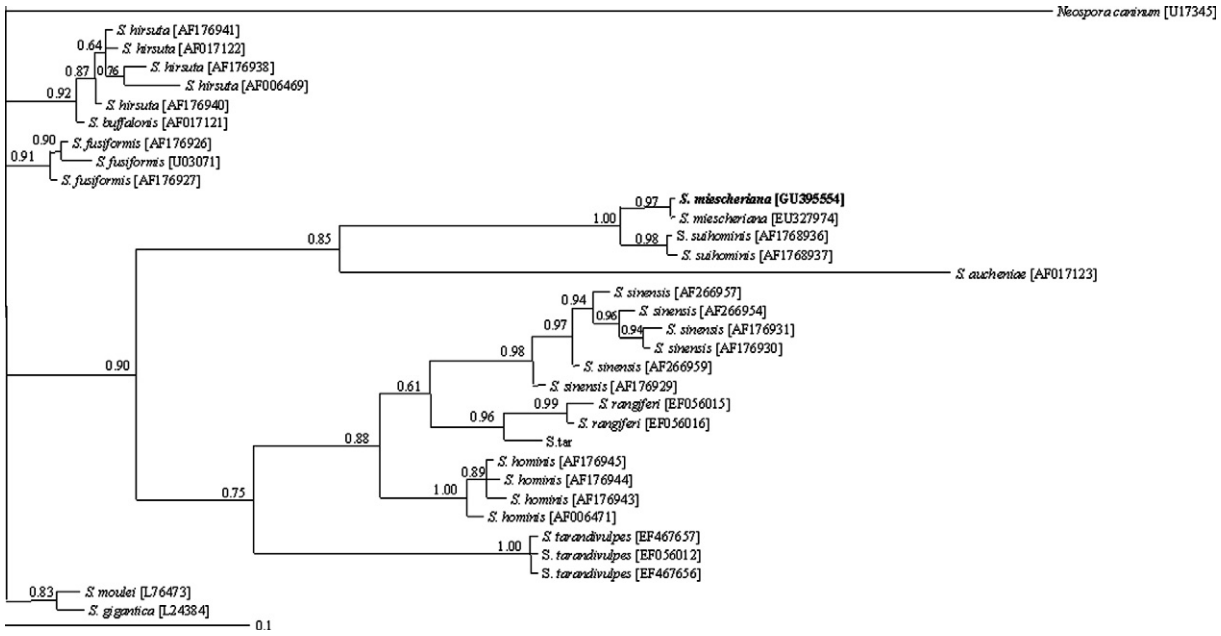

Fig. 3. Genetic relationships of *Sarcocystis miescheriana* isolate from Iran (indicated in Bold) with other *Sarcocystis* species deposited in the GenBank. *Neospora caninum* was used as the outgroup. The relationships were inferred by phylogenetic analysis of partial 18S rDNA sequence data using Bayesian Inference. The accession numbers of sequences are given in the square parentheses. The scale bar indicates distance. Nodal support is given as a pp value.

S. suihominis have the closest relationship with *S. miescheriana* compare with other species in the genus *Sarcocystis*.

Further molecular characterization of the *Sarcocystis* isolates from domestic and wild animals, especially the species without any former accession numbers in the GenBank, in different parts of the world can be helpful for better comparing and understanding of phylogenetic placements of *Sarcocystis* species.

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