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Original Article

Molecular Analysis of *Sarcocystis* Spp. Isolated from Sheep (*Ovis aries*) in Babol Area, Mazandaran Province, Northern Iran

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Abstract

Background: To differentiate *Sarcocystis* macro-cyst-forming species in slaughtered sheep in Babol area, Mazandaran Province, sequence analysis of 18S rRNA gene was performed.

Methods: Overall, 150 slaughtered sheep were examined macroscopically in slaughterhouse, Babol and intra-abdominal and diaphragm muscles tissues infected with macro-cyst of *Sarcocystis* spp. were collected in 2013. One macro-cyst was isolated from the infected muscles of each sheep. The partial 18S rRNA gene was amplified by PCR and sequenced afterward.

Results: The rate of infection with macro-cyst producing *Sarcocystis* spp. was 33.3% (50 / 150). The partial 18S rRNA gene of *Sarcocystis* species was amplified at the expected PCR product size (~1100 bp) from all 50 macroscopic cysts samples. From 30 sequences DNA samples, 20 samples (66.7%), six (20%) and four (13.3%) isolates were identified as *S. gigantea*, *S. moulei* and *Sarcocystis* spp., respectively. Eight and thirty-four variations in nucleotide position were seen in partial sequence of the18S rRNA gene of *S. gigantea* and *S. moulei*.

Conclusion: Sheep can be considered as an alternative intermediate host for *S. moulei*. Furthermore, multiple alignments showed some variations in the consensus sequences of the isolates obtained in the current study compared with previously published isolates. To understand better the genetic diversity among *Sarcocystis* species complete sequences of the18S rRNA gene or sequence analysis of other genetic loci would be beneficial.

Introduction

Sarcocystis species are an obligate intracellular protozoan parasites belong to Apicomplexan phylum. They need two hosts based on prey-predators relationship (intermediate-definitive) to complete their life cycle. These species have been recognized as a common parasite in the muscle of herbivores as intermediate hosts (1). They form muscular cysts varying from microscopic to macroscopic size (2). Sheep can be infected by four species of *Sarcocystis* including *S. tenella, S. arieticanis*, *S. gigantea* and *S. medusiformis* (3). *S. tenella* and *S. arieticanis* are known as pathogenic parasites, which form muscular micro-cysts transferred by dogs. *S. gigantea* and *S. medusiformis* are non-pathogenic and form macro-cysts transferred by cats (4). *Sarcocystis* prevalence in sheep varies between 0-100% in different geographical areas of the world (1). In Iran, prevalence of *Sarcocystis* infection has been reported 57.7% in Fars Province (5), 71% in Kurdistan Province (6), 100% in Khorasan Razavi (7) and Qazvin Province (8), 36.83% in Urmia (9), 96.8% in Karaj (10) and 6.9 % in Hamedan Province (11).

There are no reports on the prevalence of *Sarcocystis* species in sheep in Babol area, Mazandaran Province. This study was aimed to determine *Sarcocystis* species-forming macro-cysts in slaughtered sheep in Babol area, using PCR and sequence analysis methods.

Methods and Materials

Sample collection

From 10 Sep to 10 Oct 2013, 150 slaughtered sheep were examined macroscopically in slaughterhouse, Babol and intra-abdominal and diaphragm muscles tissues infected with macro-cyst of *Sarcocystis* spp. were collected. The samples were transferred to the Parasitology Laboratory at the Para-Medical Faculty, Babol University of Medical Sciences, Babol, Iran.

All muscle samples were carefully examined and individual *Sarcocysts* were removed from the muscle using a fine scalpel blade. The scalpel was washed with distilled water and disinfected with ethanol between each cyst isolated from new sample. One cyst randomly selected from each muscular sample and transferred to a clean and sterile tube. The cyst was washed three times with sterile phosphate buffer solution (PBS). Then, the cyst was dissected and cyst wall was removed. The zoites were washed two times with sterile PBS and the pellet re-suspend in 500 µl of PBS. The washed fresh zoites were aliquoted and stored at -20 ˚C until DNA extraction.

DNA extraction and amplification

Sarcocystis spp. DNA was extracted from 200-µl aliquot of the washed zoites from the excised macro-cyst obtained from the infected sheep by boiling method (12). Briefly, the aliquot was placed in 100 ˚C water for 10 min and kept it in room temperature (RT) until cool. Quality of DNA was checked by gel electrophoresis. The DNA sample with good quality was stored at -80 ˚C.

The partial 18S rRNA gene was amplified by PCR reaction with a single set of primers, Primer 1L and Primer 3H (Forward, CCA TGC ATG TCTAAG TAT AAG C; Reverse, GGC AAA TGC TTT CGC AGT AG (BIONEER, Korea) (13). One reaction mixture includes 3 µl of the DNA solution, 20 µl of dH2O, 3 µl of 10x PCR buffer (HT Bioscience, England), 0.5 µl of dNTP (HT Bioscience, England), 0.5 µl of Tag polymrase (HT Bioscience, England), 0.5 µl of each primer (20 pmol), 0.5 µl of MgCl 2 and RNase-free water to make a final volume of 30 µl. The thermal profiles used for all reactions were similar to our previous work (14). Three µl of PCR products were evaluated by electrophoresis in 2% agarose, stained with Ethidium bromide and visualized under UV light. DNA

extracted from macro-cysts of *S. moulei* was used as positive control and water as a negative control.

DNA sequencing and phylogeny

Thirty DNA samples were subjected to direct sequencing of the partial 18S rRNA gene. The sequencing was carried out by an automated sequencer (Perkin Elmer ABI 3130, USA; MacrogeneLTD, South Chorea) using 10 µl of the same forward and reverse primer as used for the PCR reaction. Nearly all sequences were truncated slightly at both ends and therefore the majority of sequences started and ended at the same homologous nucleotide positions. The sequences were subjected to BLAST (http://www.blast.ncbi.nlm.nih.gov). A multiple sequence alignment was also generated with a gap-opening penalty of 10 and a gap extension penalty of 1 for the pair wise and multiple alignments, respectively.

Phylogenetic analysis was carried out by the programme Mega v.6. Using maximum likelihood method and bootstrap of 1000 replicates (15). *Cryptosporidium parvum* was used as outgroup to anchor the tree.

Nucleotide sequence accession numbers

The partial sequence of the 18S rRNA gene of three *S. gigantea* and one *S. moulei* isolates have been deposited in the NCBI database under the accession no. KP053892, KP053893, and KP053894 and KP053891, respectively.

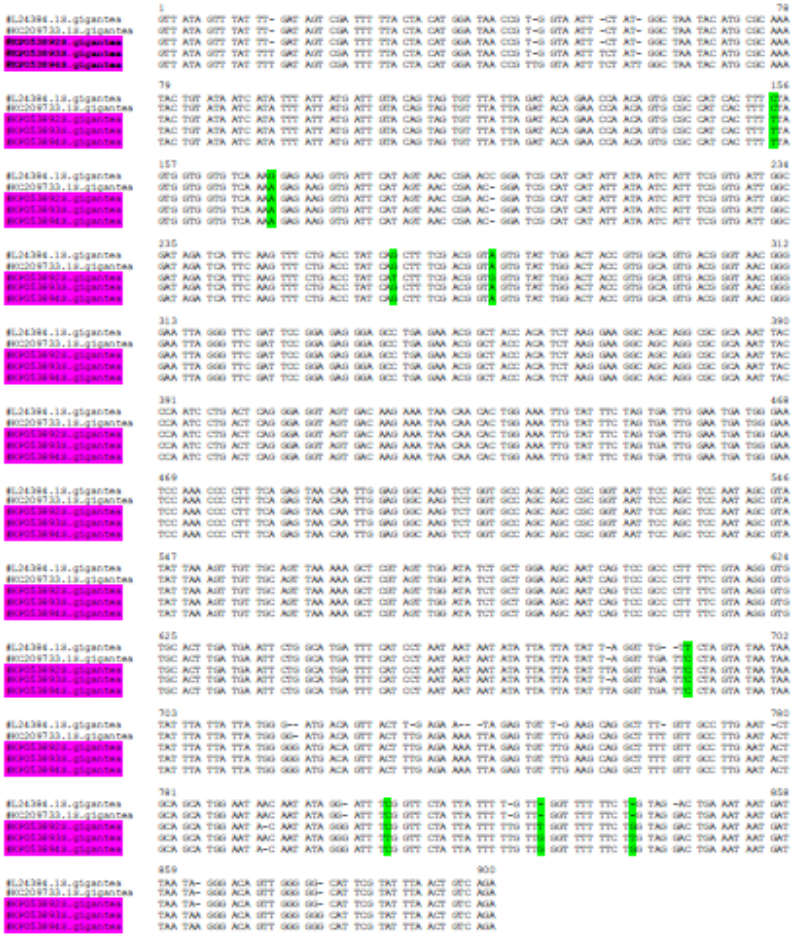

Fig .1: Multiple sequence alignment of the partial 18S rRNA gene derived from the comparative analysis of the Iranian *S. gigantea* isolated from slaughtered sheep and previously *S. gigantea* strains deposited in GenBank

Results

Macroscopic and Microscopic findings

One-hundred-fifty sheep carcasses aged from three to seven years old were carefully examined. The rate of infection with macro-cyst-forming *Sarcocystis* spp. was 33.3%. Size of the cysts was varied from 3 to 25 mm. One *Sarcocyst* was separated from each infected muscular sample and a careful microscopical examination was carried out on the semi-liquid contents of each cyst. All cysts contained a copious number of half-moon shaped cells with *Sarcocystis* characteristics.

PCR analysis and sequencing

The partial 18S rRNA gene of *Sarcocystis* species was amplified at the expected PCR product size (approximate length 1100 bp) of all 50 macroscopic cysts samples.

Thirty PCR products of the 18S rRNA gene were successfully sequenced. Each PCR product yielded a fragment containing 850 to 1021 consensus nucleotides. Overall, 20 out of 30 samples (66.7%) and six/thirty (20%) isolates had a similarity of more than 98% and 100% coverage to *S. gigantea* accession number KC209733 or L24384 and *S. moulei* accession number L76473 or KC 508513, respectively (NCBI GenBank). 13.3% isolates showed a similarity of more than 89% and 50% coverage to *Sarcocystis* spp. accession number GQ131808. Multiple alignments showed some variation in the consensus sequences of the isolates obtained in the current study compared with previously published *S. gigantea* isolates and with each other (Fig. 2). The same results were observed for *S. moulei* (Fig. 2). A phylogenetic tree of three *S. gigantea,* two *S. moulei* and some published sequences (NCBI GenBank) is shown in Fig. 3.

Fig. 2: Multiple sequence alignment of the partial 18S rRNA gene derived from the comparative analysis of the Iranian *S. moulei* isolated from slaughtered sheep and previously *S. moulei* strains deposited in GenBank

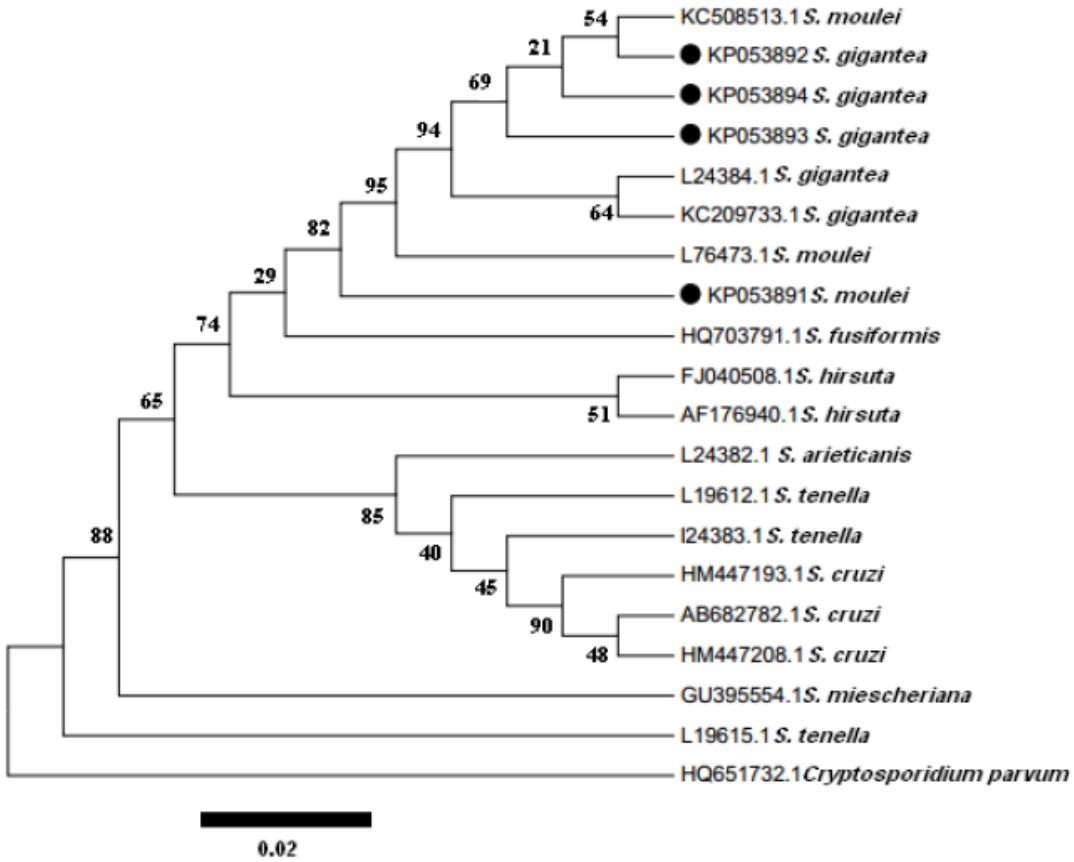

Fig. 3: Phylogeny of *Sarcocystis* spp. isolates by the programme Mega v.6. using maximum likelihood method and bootstrap of 1000 replicates based on 18S rRNA gene. The reference sequences accession numbers are included

Discussion

Of *Sarcocystis* species detected in sheep, macro-cyst forming species are considered non-pathogenic but they can affect the meat quality and marketing and therefore lead to economic loss. In the last two decades, molecular techniques have been developed and used as a diagnostic tool to differentiate *Sarcocystis* species (16). Molecular studies on *Sarcocystis* species are not widely performed on sheep in Iran. Therefore, the current study was undertaken to identify the *Sarcocystis* spp. in slaughtered sheep in Babol area by PCR and sequence analysis methods.

Macroscopic examination of carcasses found that the rate of *Sarcocystis* spp. infection in slaughtered sheep aged between 3-7 yr old was relatively high (33.3%) in comparison with results obtained from other studies in North Khorasan Province that such infection exists in 5% of examined sheep aged between three months to three years (7). Furthermore, macroscopic cysts in 13.5% and 20.53% of studied sheep, was reported, respectively (17-18). In addition, our results were in contrast with another study from Iran, where 66.6% of slaughtered sheep were infected with macro-cysts of *Sarcocystis* spp. (8). These differences can be explained by a variety of risk factors involved in *Sarcocystis* infection such as location and age (19).

On the other hand, the rate of infection with macroscopic cysts obtained here was lower

than microscopic cysts infections reported in Iran. For example, all sampled sheep in North Khorasan Province was infected by *Sarcocystis* spp. (7). This may be caused by a lower probability of pastures being contaminated by cat faeces than dog faeces, as dogs are used to shepherd sheep and goats.

The variable regions of the 18S rRNA gene has been successfully employed as a valuable targets for the identification and characterization of different protozoan parasites as well as *Sarcocystis* species (4, 20-21). Similarities and differences were identified in the infecting *Sarcocystis* species among sampled sheep by molecular analysis of the 18S rRNA gene. *S. gigantea* was more frequently detected in the infected sheep, which is in agreement with results obtained from other studies performed in Iran and other countries (8, 22-23). Furthermore, *S. moulei*, goat-specific specie, was found in six macro-cysts infected sheep. This finding demonstrated that *S. moulei* is able to use the sheep as an intermediate host and is not restricted to goat. This result is in contrast with findings obtained from other reports, which did not find this species from sheep (8, 22-23).

S. gigantea and *S. moulei* are very close species and usually form a sister clade in phylogenetic analysis and therefore cross infection may occur. However, the evidence indicates that some species have a wider intermediate host choice than previously thought (13, 24). Sequence similarity obtained by BLAST may result in some problems concerning their identification due to sequences errors such as missing nucleotides. This possible explanation can be confirmed by another study where most of the differences in sequences were due to sequencing errors as obtained by a closer comparison of the sequences when aligned against each other. To rule out this possibility, PCR and sequence analysis of other genetic loci such as cytochrome c oxidase subunit I gene (cox1) should be examined (23).

However, the *Sarcocystis* species isolated in the current study showed a high level of similarity with other isolates from different geographical areas exhibiting a very low level of genetic diversity. These findings are supported earlier (25-26).

The polymorphisms observed in the partial sequence of 18S rRNA gene of the *S. gigantea* and *S. moulei* strain in comparison with the previously published sequences may be due to their geographic locations.

Conclusion

This study demonstrated high prevalence of *Sarcocystis* infection by macro-cyst forming species. Sheep could be considered as an alternative intermediate host for *S. moulei***.** Furthermore, complete sequences of the18S rRNA gene or sequence analysis of other genetic loci would be beneficial to better understanding of genetic diversity among *Sarcocystis* species isolated through the world.

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