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Molecular Identification and phylogeny of Microscopic *Sarcocystis* Sheep in Baghdad Province

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Abstract

Sarcocystis is an obligatory intracellular protozoan parasite which can infect humans and animals. Sheep are intermediate hosts of four *Sarcocystis s*pecies: *Sarcocystis tenella, Sarcocystis gigantea*, *Sarcocystis arieticanis* and *Sarcocystis medusiformis.* The purpose of this study was to perform a molecular identification of the macroscopic and microscopic cysts of *Sarcocystis*in sheep. In this investigation, the microscopic cysts of *Sarcocystis* were assessed in slaughtered sheep. The digestion method was used for bradyzoites observation in , esophagus and inter costal muscle samples($P < 0.01$). PCR analysis was conducted on microscopic cysts and also all other samples. Sequencing was performed for ten PCR products. Genotypes were identified by BLAST search and homology analysis. Digestion method and PCR analysis revealed positive results in all samples taken from esophagus and inter costal muscle. Genotyping of 5 tissue samples proved that the genotype of macroscopic belonged to microscopic cysts to *Sarcocystis tenella*. Microscopic cysts are more prevalent thanmacroscopic cysts and they can cause enormous economic losses.

Keywords: *Sarcocystis*, virulence factors, polymerase chain reaction (PCR), Iraq.

Introduction

Species are intracellular protozoan parasites infecting a wide range of livestocks. Some of *Sarcocystis* genus are pathogenic for animals such as sheep and cattle which cause enormous economic losses (1). Studies in different regions of the world indicate that the prevalence of *Sarcocystis* infection in slaughtered cattle and sheep are between 70% to 100% (2 ; 3). Additionally, studies in Iran showed that the prevalence of this parasite in the animal was between 85% to 100%(4; 5). For example, studies in Kerman and Ahwaz provinces indicated that 100% of animals were infected with *Sarcocystis* (5 ;6). Different species of *Sarcocystis* have been isolated from animals worldwide. *Sarcocystis tenella* was isolated from sheep in Iran and Brazil (7; 8). In another study, *Sarcocystis moulei* was reported from reindeer (9).

Also, Nourani et al. isolated *Sarcocystis hominis* from cattle (10) while Kalantari et al. separated *S. cruzi* from cattle (11). Dalimi et al. determined *S. gigantean* and *S. arieticanis* in sheep (12).

Diagnosis

The diagnosis is usually made *post mortem* by examination of the skeletal muscle. In some species the cysts may be visible to the naked eye (ducks, mice, rabbits and sheep) but in most microscopic examination is required. *Ante mortem* diagnosis may be made with the use of dermal sensitivity testing or complement fixation tests. Muscle biopsy is also diagnostic but this is much less commonly used(13). .

Oocysts with two sporocysts or individual sporocysts in human feces are diagnostic of intestinal infection.
The conventional tools for species diagnosis of *Sarcocystis* spp. were based on transmission electron microscopy, structure of the cyst wall in the striated muscles of the intermediate host or information about the lifecycle of the parasite (Gasbarre L.C. et al., 1984). However, because of showing the morphologic variations in these procedures they are not exactly reliable at the species-specific identification. On the other hand, electron microscopy is not a choice for wide and extensive detective studies (13).

In recent times, various molecular techniques such as PCR and its variants based on sequence changes have been used regarding the sensitivity and rapidity to determine genetic diversity among many parasites, phylogenetic and taxonomic studies and in epidemiological mapping (15).

Thus, definitive diagnosis of sarcocystosis requires identification of sporocysts in feces. However, the sporocysts of different species are similar in size and shape, making species identification almost impossible by microscopy. Therefore, sequencing of the small subunit ribosomal RNA (18S rRNA) gene was introduced as an ideal means forspecies-specific detection .In fact, this gene contains hypervariable regions interspersed within highly conserved DNA sequences, making it ideal for differentiation between species.

Materials and Methods

Sarcocystis spp. strains were isolated from organs samples(esophagus and skeleton muscle) from 100 sheep selected randomly from botier at different localities of Baghdad, Iraq between January 2016 to march 2016 . Isolation and identification of the strains were made by conventional methods (**13**) conventional methods.

Digestion method

Tissue digestion method was used for observing bradyzoites in the organ samples. Seventy grams of each tissue were ground and digested in 1.5% HCL acid and 0.5% pepsin at 29 °C overnight. The digested samples were filtered through mesh and centrifuged 1500 RPM for 10 min. Then, the supernatant fluid was discarded and sediment was stained with Gimsa and examined microscopically for detecting bradyzoites of *Sarcocystis*.

Processing of the samples for PCR assay:-

A volume of 1.5 ml of the post-enriched sample was centrifuged at 14,000g for 1 min, DNA was extracted using Presto Mini g DNA Tissue Kit according to manufacturer's instructions (Geneaid, Korea). The extracted DNA was stored −20 ◦C until use .The extracted DNA then quantified through measurement of its OD260 by ND‑2000 spectrophotometer (Thermo Scientific Inc., USA)

PCR amplification analysis:-

The virulence determinants investigated using the oligonucleotide primers included the gene *SAR1*. For all the gene , The polymerase chain reaction (PCR) amplification was performed in a final volume of 20µl containing 10 Intron- Master Mix (KOBA) which contains (Taqplymerase, PCR buffer, MgCl2 and dNTPs) , 200 ng of DNA template added 1µl of 10 pmol each primer, and 6µl of nuclease free water, in the present study , the amplification parameters and primer sequence were used in (table1). The amplification of gene was carried out with Master cycler (Eppendrof , Germany). Amplified products were separated by agarose gel electrophoresis (2% agarosecontaining 0. 5 mg ethidium bromide in 0.5 × Tris - EDTA electrophoresis buffer) at 5V/cm for 2h and photographed under UV illumination.

Results and Discussion

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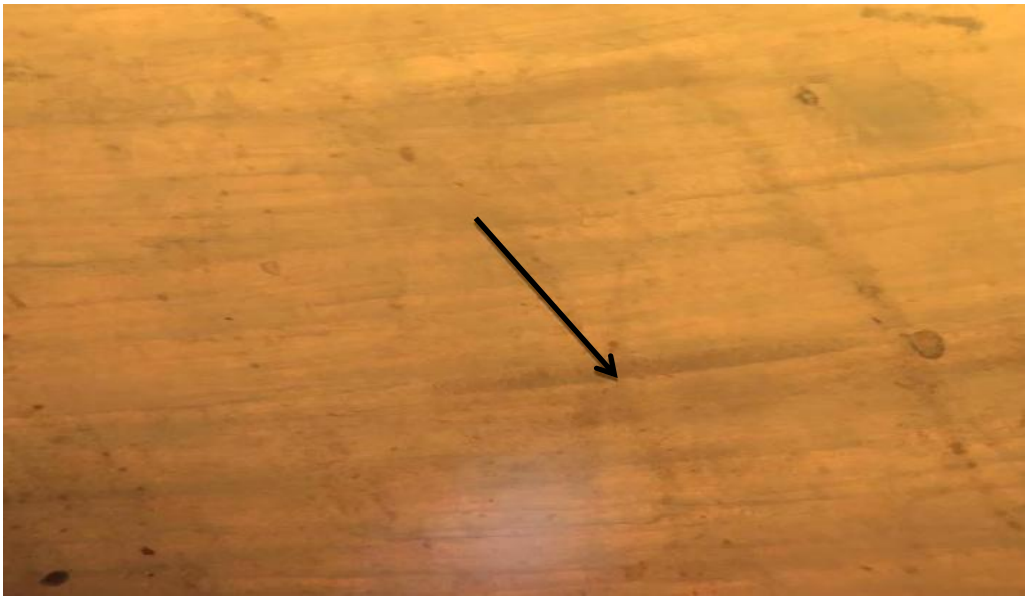

Figure (1) show one types of *sarcocystis* in sheep in different organ X40

Table 1. Number of microscopic cysts in sheep's tissues and used

Methods:-

organ	Microscopic cyst	Digestion method	Molecular method	negative
esophagus	40	40	40	0
muscles	20	20	20	0

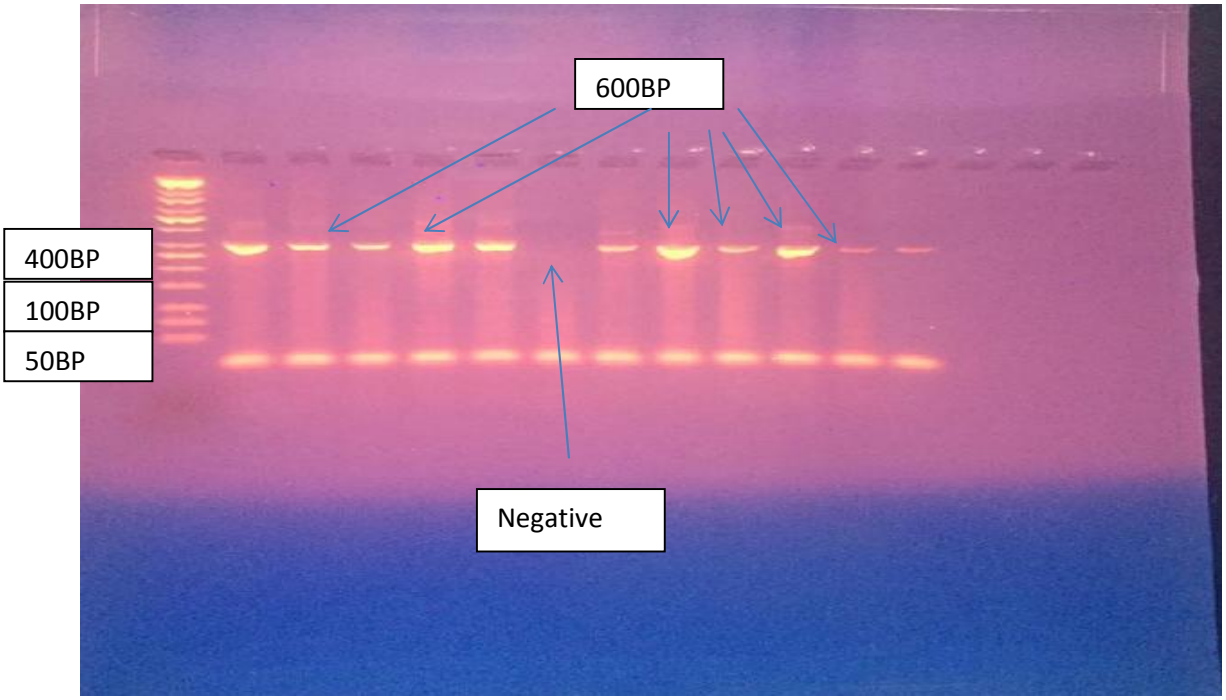

Figure (1) Gel electrophoresis of 1% agarose gel stained with ethidium bromide for DNA extraction of *Sarcocystis*

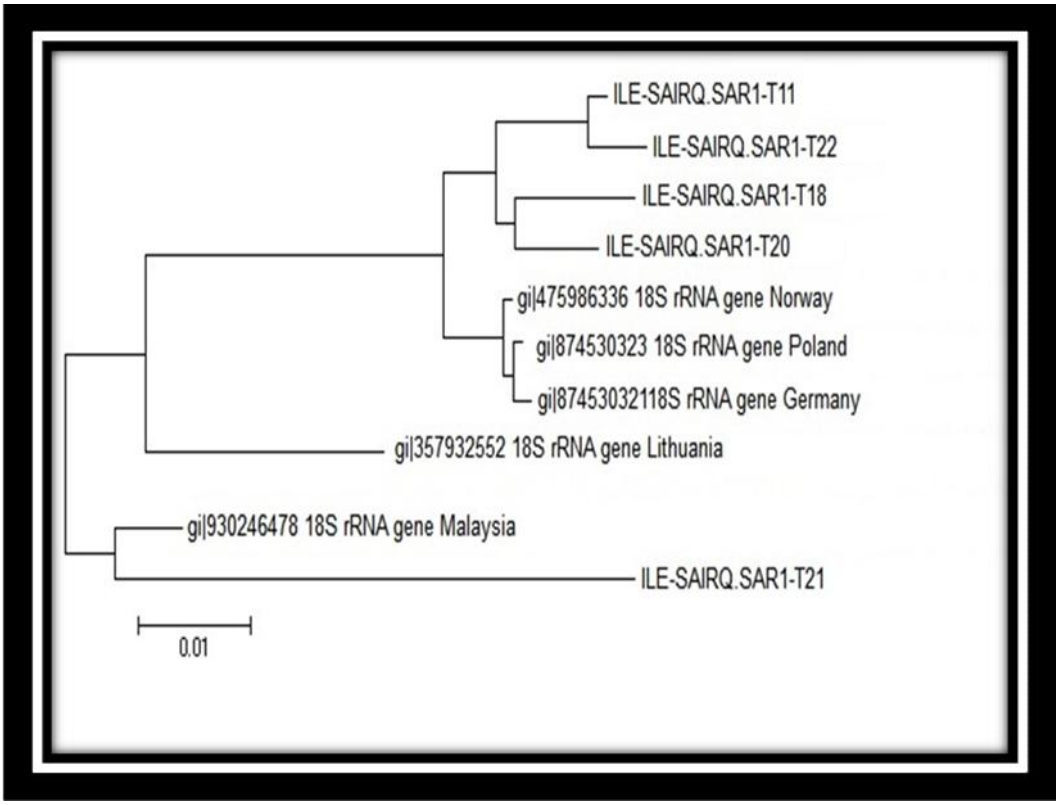

Fig. 3 Showed Phylogeny of *Sarcocystis*spp. isolates by the programme Mega v.6. using maximum likelihood method and bootstrap of 600 replicates based on 18S rRNA gene(Iraq strain ILH – SA IRQ .SAR1 –T) . The reference sequences accession numbers are included between sheep in different country .

Discussion

All samples (muscle and esophagus) were infected with *Sarcocystis*s pp. The studies in Baghdad and other parts of the world indicated that live stocks are infected with *Sarcocystis* spp. (16). Other studies in different provinces of Iran showed that 97% of sheep had *Sarcocystis* infection (17). In previous studies throughout the world, species of *Sacocystis* were isolated from different animals(18 ;19). DaSilva and Langoni isolated *S. tenella* from the sheep in Brazil (18). Al- Hoost et al. reported *S. moulei* from the sheep in Saudi Arabia(20). Moreover, Gjerde isolated and characterized S. grueneri from reindeer based on molecular method (19). In other studies in Iran,*Sarcocystis hominis* and *Sarcocystis cruzi* were studied in cattle . (21)isolated*S.gigantea* and *S. arieticanis* from the sheep by PCR-RFLP method in Qazvin province, Iran (6). Furthermore, other researchers reported *S miescheriana* from boar 21) and *S. tenella* from sheep in Iran (10). Additionally, Mahran in Egypt using morphometric method indicated that *S gigantean* and *S. tenella* caused macroscopic and microscopic cysts (22). Using daub smear method,. showed that 91% of cows were infected with microscopic cyst and did not have any macroscopic cysts (23). KargarJahromi et al. using digestion method proved that goats had microscopic and macroscopic cysts (24). Molecular analysis was not performed in the above studies, but, the use of molecular methods in the present study showed that *S. gigantean* and *S. tenella*can cause macroscopic and microscopic cysts, respectively. The results of this investigation was in accordance with Anja and Astrid's study who reported that *S. gigantean* and *S tenella* can cause macroscopic and microscopic cysts, respectively (1) *S. tenella* is among the pathogenic species and can induce microscopic cysts . The severity of clinical symptoms caused by this species depends on the dose of ingested sporocysts and the immune system of the host (25 -26). *S. tenella* can lead to acute sarcocystosis in uninfected sheep (1). Nonspecific infection symptoms include fever, anorexia, tachycardia and anemia could be observed following infection. In acute sarcocystosis, central nervous system will be involved, and it can cause encephalitis and encephalomyelitis and subsequently death in sheep (27). In pregnant sheep, acute sarcocystosis can cause fetal death or premature birth of offspring. Chronic sarcocystosis can create economic problems due to reduced meat, milk and wool (28, 29 , 30). Also, Dubey reported that *S. tenella* caused symptoms such as inflammation, hepatitis and myocarditis in sheep inoculated with *S. tenella*s porocysts from canine feces (31).

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. tenella* 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).

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 (33).

Sequence similarity obtained by BLAST may result in some problems concerning their identification due to sequences errors such as missing nucleotides (34).

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 (33).

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).

The polymorphisms observed in the partial sequence of 18S rRNA gene of the *S. tenella* strain in comparison with the previously published sequences may be due to their geographic locations (35).

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