Molecular identification of Microscopic Cysts of *Sarcocystis* in Sheep in Baghdad Province

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Abstract

Sarcocystis is an obligatory intracellular protozoan parasite which can infect humans and animals. This study was aimed to identify the macroscopic and microscopic cysts of *Sarcocystis* in sheep by using PCR. One hundred random samples taken from sheep at abattoir. The digestion method was used for bradyzoites observation in, esophagus and inter costal muscle samples. PCR analysis was conducted on microscopic cysts and the 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.

Keywords: *Sarcocystis*, PCR, sheep, esophagus.

Introduction

Sarcocystis species are intracellular protozoan parasites infecting a wide range of livestock. Some of *Sarcocystis* genus are pathogenic for animals such as sheep and cattle which cause enormous economic losses (Heckerroth and Tenter, 1998). Studies in different regions of the world indicate that the prevalence of *Sarcocystis* infection in slaughtered cattle and sheep are between 70% to 100% (Pereira and Bermejo, 1988 ; Woldemeskel and Gebreab, 1996). Additionally, studies in Iran showed that the prevalence of this parasite in the animal was between 85% to 100% (Oryan *et al.*, 2010; Hamidinejat *et al.*, 2010). For example, studies in Kerman and Ahwaz provinces indicated that 100% of animals were infected with *Sarcocystis* (Hamidinejat *et al.*, 2010; Fard *et al.*, 2009). Different species of *Sarcocystis* have been isolated from animals worldwide. *Sarcocystis tenella* was isolated from sheep in Iran and Brazil (da Silva *et al.*, 2009; Shahbazi *et al.*, 2013).

In another study, *Sarcocystis moulei* was reported from reindeer (Gjerde, 1985). Also, Nourani *et al.* isolated *Sarcocystis hominis* from cattle (Nourani *et al.*, 2010) while Kalantari *et al.* (2013) isolated *S. cruzi* from cattle. Dalimi *et al.* (2008) determined *S. gigantean* and *S. arieticanis* in sheep.

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 (Van den *et al.*, 1995).

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 (Dalimi *et al.*, 2008).

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 (Gasbarre *et al.*, 1984).

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 (La Perle *et al.*, 1999).

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 for species-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

One hundred sheep organs samples (esophagus and skeleton muscle) were used to isolate *Sarcocystis* spp. strains taken from abattoir at different locations of Baghdad, Iraq, between January 2016 to March 2016. Isolation and identification of the strains were made by conventional methods (Van den *et al.*, 1995).

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 Giemsa 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 20 µl containing 10 Intron- Master Mix (KOBA) which contains (Taq polymerase, PCR buffer, MgCl₂ and dNTPs), 200 ng of DNA template added 1 µl of 10 pmol each primer, and 6 µl of nuclease free water, in the present study, the amplification parameters and primer sequence were used in (table 1). The amplification of gene was carried out with Master cycler (Eppendorf, Germany). Amplified products were separated by agarose gel electrophoresis (2% agarose containing 0.5 mg ethidium bromide in 0.5 × Tris - EDTA electrophoresis buffer) at 5V/cm for 2h and photographed under UV illumination.

Results and Discussions

In this study, 60 samples of muscle and esophagus had microscopic cysts (table 1). In addition, these organs were found to be infected with microscopic cysts by digestion method. The results of digestion method showed that all samples of muscle and esophagus were infected with bradyzoites of *Sarcocystis*. PCR analysis of microcysts as well as all samples showed a specific 600 bp band on the agarose gel (Figures 2). The results obtained from sequencing of five samples (3 muscles and 2 esophagus) showed that the genotype of microscopic cysts to *Sarcocystis tenella*.

Microscopic examination by trichinoscopy Technique direct between two slides to appear diameter (13-15)*(7-0.5) like figure (1).

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

The studies in Baghdad and other parts of the world indicated that live stocks are infected with *Sarcocystis* spp. (Hamidinejat *et al.*, 2013). Other studies in different provinces of Iran showed that 97% of sheep had *Sarcocystis* infection (Shekarforoush *et al.*, 2006). In previous studies throughout the world, species of *Sarcocystis* were isolated from different animals (daSilva *et al.*, 2009; Al-Hoot *et al.*, 2009). daSilva *et al.* (2009) isolated *S. tenella* from the sheep in Brazil, while Al-Hoot *et al.* 2009 isolated *S. moulei* from the sheep in Saudi Arabia. The high prevalence rate of microscopic sarcocysts in sheep at this study indicates the importance of the Infection for the intermediate hosts. The macrosarcocysts species are almost non-pathogenic but are responsible for economic losses because of the complete or partial rejection of the animal carcasses at slaughterhouses (Oryan *et al.*, 1996).

Moreover, Gjerde (1985) isolated and characterized *S. grueneri* from reindeer based on molecular method. In other studies in Iran, *Sarcocystis hominis* and *Sarcocystis cruzi* were studied in cattle (Kia *et al.*, 2011). Also, Fard *et al.* (2009) isolated *S. gigantea* and *S. arieticanis* from the sheep by PCR-RFLP method in Qazvin province, Iran.

Furthermore, other researchers reported that *S. miescheriana* were isolated from boar and *S. tenella* from sheep in Iran (Nourani *et al.*, 2010). Additionally, Mahran (2009) in Egypt used morphometric method for identification *S. gigantea* and *S. tenella*, and showed that *S. gigantea* and *S. tenella* caused macroscopic and microscopic cysts. By Using daub smear method, the results of Bonyadian and Meshki (2006) showed that 91% of cows were infected with microscopic cyst and did not have any macroscopic cysts. Jahromi *et al.* (2012) using digestion method proved that goats had microscopic and macroscopic cysts.

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 (O'Donoghue and Rommel, 1992). *S. tenella* can lead to acute sarcocystosis in uninfected sheep (Heckerthand Tenter, 1998). 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 (Jeffrey, 1993). 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 (Munday, 1984, Dubey, 1988, Neefs *et al.*, 1990). Also, Dubey reported that *S. tenella* caused symptoms such as inflammation, hepatitis and myocarditis in sheep inoculated with *S. tenella* sporocysts from canine feces (Kolenda *et al.*, 2014).

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 (Oryanet *et al.*, 2010). In recent years, molecular diagnostic techniques have been assessed for specific determination of *Sarcocystis* spp. (Yang and Zuo 2000; Yang *et al.*, 2001). Among various genomic targets, the highly conserved 18S ribosomal subunit is used widely for species-specific detection of different protozoa as well as *Sarcocystis* spp. due to presentation of highly variable regions (Yang and Zuo, 2000). On the other hand, many authors confirmed the 18S rRNA for firmly species-specific discriminating of sheep sarcocysts (Tenter *et al.* 1992, 1994; Ellis *et al.* 1995; Yang *et al.* 2001).

Conclusions

In conclusion, the present work has demonstrated that *Sarcocystis* infection was common in sheep in Baghdad. Also, it was concluded that PCR method is a tool with high specificity and susceptibility of discriminating *Sarcocystis* species worldwide.

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