

# Molecular Identification of Sarcocystis Species Infection in Sheep in Karbala Governorate – Iraq

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## Abstract

Sarcocystis spp. are causative agents of ovine muscular stiffness, myopathies and/or inflammations of the mucosa of the intestine, diaphragm Heart and skeletal muscles, which are generate important economic loss when causing clinical and subclinical cases, ovine Sarcocystis spp. are a protozoan parasite of worldwide distribution transmitted by canids, most commonly associated with subclinical infection in sheep, our study were described in unorganized five different localities in Karbala governorate for sheep Sarcocystis spp. investigation and classified in to two ages groups, less than 18 months-old and over 18 months-old, Macroscopic and microscopic and certain biochemical tests were used for parasite observation, Molecular assay PCR assay was used to confirm this protozoan, Currently, PCR assay based on certain regions of the cytochrome oxidase 1 sequences is considered and used widely as an inexpensive, rapid and precise molecular approach to identify the Sarcocystis spp.

**Keyword:** Sheep Sarcocystis, PCR, Macrocyysts and microcyysts.

## Introduction

Sarcocystis is anobligatory intracellular protozoan that belongs to the phylum apicomplexan and has the ability to infect a broad spectrum ofvertebrates' poultry, livestock, and humans with more than 150 knownspecies<sup>16</sup> and<sup>7</sup>. It is a heteroxenous parasite that has anobligatory two host's life cycle in order to thrive, one host,being an intermediate onewhich is usually herbivores or omnivores in which the parasite will have its merogonystage and results in cyst formation inside the skeletal muscles of these hosts which are commonly referred to as (sarcocysts). The other host is the definitive (final) host which in turn would usually be a carnivores or omnivores in which the parasite undergoes it's sporogony and gamogony stages of development that leads to shedding of infective sporocysts and oocysts in the stool of these animals<sup>14</sup>. The same animal species can be infected with more than one species of Sarcocystis, for example, sheep as intermediate hosts, can be infected with more than four different species of Sarcocystis and are intermediate hosts these are *S. gigantea*(*S. ovifelis*), *S. tenella* (*S. ovicanis*), *S. arieticanis*, and *S.medusiformis*<sup>1</sup>. The prevalence of Sarcocystis varies with respect to its species were *S.gigantea* is known

to have a global distribution whereas *S.medusiformis* on the other hand has been only reported in only a few parts of the world like Australia, New Zealand<sup>23</sup>. The pathogenicity of Sarcocystis varies with respect to its species. The inspected sheep meat which turns to be heavily infested with macrocysts in Iraqi abattoir most of the time will be condemned as unfit for human consumption .Another point of concern involves the fact that nearly all investigations on ovine Sarcocystis infection are limited to just inspecting the carcasses at the slaughterhouse without paying attention to determine the exact species of Sarcocystis involved which may return harmless and fit for human consumption and thus reducing the economic losses<sup>29</sup> and<sup>19</sup>.

## Materials and Method

**Animals:** During the course of this study which extended from December 2018 to May 2019, tissue samples involving the esophagus were randomly collected from 412 (289 male and 123 female) sheep slaughtered at five different localities in Karbala. Careful examination was conducted prior to slaughtering to make sure that all included sheep were healthy before being slaughtered. The estimation of sheep age was made on the basis of teeth examination to calculate the number of

temporary teeth as well as the erupted permanent incisor count <sup>26</sup>. The gender of each sheep was recorded. The animals were categorized into two groups according to their age (Table 1).

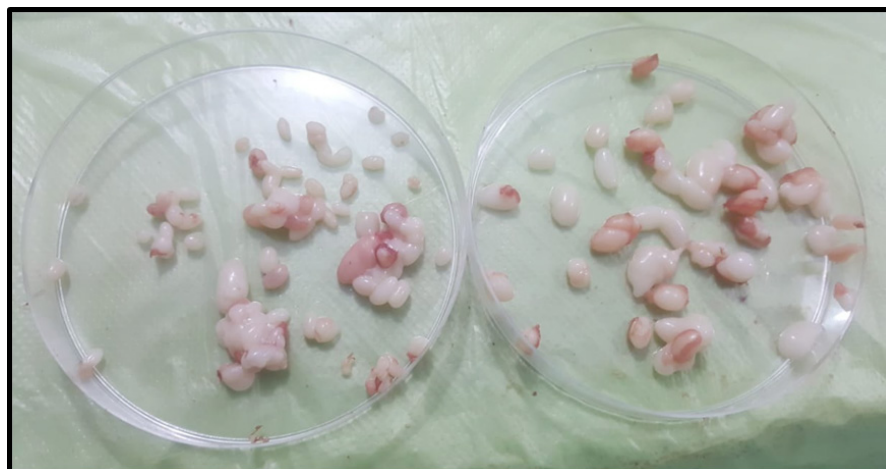
**1- Group A:** this group included all sheep 18-month of age irrespective to their sex. It included (211) sheep with (155) male and (56) female. The ages of this group of male members ranged from 10-18 month ( $16.8 \pm 1.61$ ) and that of the female group member ranged from 14-18 ( $15.38 \pm 2.27$ ) month.

**2- Group B:** this group included all sheep with an age of equal to or more than 18-month. It included (201) sheep with (134) male and (67) female. The ages of this group male members ranged from 18-32 ( $27.26 \pm 2.03$ )

month and that of the female group member ranged from 18-38 ( $28.35 \pm 2.46$ ) month.

### Macroscopic examinations.

After washing and removing of tunica adventitia, each collected esophagus underwent careful and thorough inspection in the search for macrocysts of the *Sarcocystis* parasite. Any specimens found to have macrocysts was isolated, its cysts were excised and liberated out of the esophageal tissue to be collected in a petri dish (Fig. 1), and underwent subsequent classification based on the physical features like shape, color, and size. The cysts ranged (3 -14 mm) in length ( $11.2 \pm 1.3$  mm). Their colors varied from opalescent white to pale yellow and their shapes included both round and oval forms.



**Figure 1: Macrocysts of different sizes and shapes isolated from infected esophagi.**

### Microscopic examination:

Three samples were taken from each esophagus with an approximate weight of at least 25 g in order to be used for *Sarcocystis* bradyzoites that are harbored in the microsarcocysts which are typically banana shaped (Fig. 3) with a length of about 3.08 to 7.45  $\mu\text{m}$  that were estimated using ocular micrometer. All parts were labeled with information representing the age, gender, and date of slaughtering. Each collected esophagus was examined with the following three methods:

### Peptic digestion method.

For performing this procedure, a modified technique commonly used by similar studies was adopted <sup>10</sup> were 2 grams of pooled muscular tissue obtained from the esophagus after removing fat and tunica adventitia. The

extracted piece was in turn cut into smaller and smaller pieces using a scalpel after that it was placed into 10 ml test tube followed by adding 7 ml of peptic digestion solution and incubated at 40 C° for 30 minutes. The digestate was filtered through 3 layers of medical gauze into 10 ml centrifuge tube and centrifuged for 7 minutes at 2000 rpm. The supernatant was discarded and 0.5 ml of 0.9 % physiological saline was added to the precipitant, mixed thoroughly and a drop was taken and placed over a glass slide in order to be examined by microscope for the presence of bradyzoites under the power x 40 <sup>17</sup>.

### 2- Meat mincing by garlic presser:

As described by <sup>21</sup>, 4 grams esophageal muscle sample placed in a metallic garlic presser and firmly squeezed to extract a liquid from the meat from which a drop was taken via Pasture pipette and placed on a

glass slide, covered with a cover-slip and examined by microscope at force 40 X.

**3- Slide squash method (Trichnoscopy):**

A meat sample was cut into as small pieces as possible using a scalpel in a direction parallel to that of muscle fibers. 2 x 2 mm piece placed over a glass slide and covered with two coverslips. After placing the slide on a firm ground, it was pressed by thumb as hard as possible to crush the sample then the upper glass slide was removed and the sample examined at 40 X <sup>27</sup>.

**Molecular study**

For molecular analysis, soft cysts of the macrocysts were dissected, washed several times in 0.01 M phosphate-buffered saline (pH 7.2), and stored at -20 °C until DNA extraction.

**DNA extraction**

G-spin™ Total DNA Extraction Mini Kit, Korea was used for DNA extraction where according to provider’s protocol and after collecting extracted macrocysts in a Petri dish, 20 mg of macrocysts placed in 1.5 ml Eppendorf tube smashed into liquid state using a sterile wooden stick and mixed up with 200 µl of tissue lysis solution followed by adding 20 µl proteinase-K. The

mixture incubated at 60 °C in a water bath for 30 minutes with constant shaking using vortex every 5 minutes. 200 µl binding buffer added and mixture incubated at 60 °C for 10 min then 100 µl of absolute alcohol added. The mixture was transferred into a collection tube for centrifugation at 13000 rpm for 1 minute. 700 µl of washing buffer 1 solution added and another run of centrifugation at 13000 rpm was conducted. After discarding the supernatant, washing buffer 2 added and the mixture was replaced in 2 ml tube together with 200 µl of buffer and let dry at room temperature for 5 minutes. The tube was centrifuged at 13000 rpm for 1 minute then the DNA containing fluid was stored at -20 °C for further evaluation.

**PCR-reaction.**

PCR was conducted on all esophageal samples using *Sar* primers including *Sar-F1* Forward 5’GCACTTGATGAATTCTGGCA3’ and *Sar-R1* Reverse 5’CACCACCCATAGAATCAAG3’ (2, 12, 13).

PCR reaction was carried out in 30 µl of Premix (AccuPower PCR PreMix, Korea). Twenty-five microliters of TaqMaster Mix were used with 10 ng template DNA, 0.1 µM of each primer and distilled water. Cycles of PCR are summarized in (Table- 2):

**Table 1: age, sex, and type of infection together with the detection method used for detecting prevalence of microcystic infection in sheep included in the study.**

	Males		Females		Total
	< 18	≥ 18	< 18	≥ 18	
Age group (month)	< 18	≥ 18	< 18	≥ 18	
Number of sheep (N)	155	56	134	67	412
Macroscopic cysts (n1)	2	4	5	12	23 (5.58%)
Microscopic cysts (n2)					
Pepsin digestion	140	126	52	56	374 (90.78%)
Trichnoscopy	138	111	46	49	344 (83.50%)
Mincer	91	82	40	43	256 (62.14%)

N = total number of sheep in any subgroup.  
 n1 = number of sheep infected with macrocysts in any subgroup.  
 n2 = number of sheep infected with microcysts in any subgroup.  
 % = (n/N) x 100.

**Table2: Summary of steps involved in PCR.**

Primer Volume Reference	Amplification program			
	Stage	Temperature (C°)	Time	RPM
Forward:5'GCACTTGATGAATTCTGGCA3' Reverse:5'CACCACCCATAGAATCAAG 3' Product: 600 Bp Bahari, <i>et al.</i> ,2014))	Denaturation	95	5 sec	35
	Denaturation	95	30 sec	
	Annealing	55	45 sec	
	Extension	72	30 sec	
	Terminal	72	10 min	

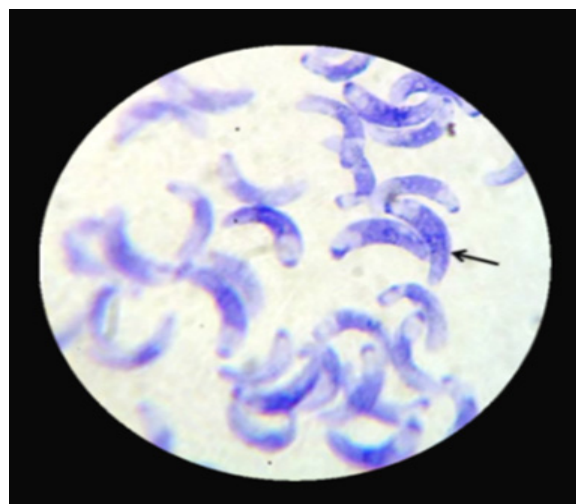
The amplified DNA obtained after PCR procedure was electrophoresed using 2% agarose gel, stained with Ethidium Bromide (0.5 µg/ml) and visualized under the UV light.

### Statistical Analysis

Statistical analysis was done to evaluate the results with a confidence interval of 95% using non-parametric  $\chi^2$  test using SPSS (Version 22; IBM, USA). A probability values of  $p < 0.05$  were regarded as statistically significant.

### Results and Discussion

The results of this study concluded that after examining esophageal muscular tissue samples, sheep had been infected with both macroscopic and microscopic cysts figure (2 &3). With respect to infection with the macroscopic form of the parasite, it was found that among total of (N=412) examined sheep by naked eye, there was (n=23, 5.82 %) confirmed infections with macrocytic form, while the infection rate with the microscopic form of the parasite was (n=374, 90.78%). These finding are consistent with those of similar studies on prevalence of Sarcocystis in livestock in Iraq, were it was found that a prevalence rate for macrocysts of about (4.1 %) was reported in the south of Iraq<sup>21</sup> likewise, a prevalence of (97.5%)for microcysts was reported in north of Iraq<sup>19</sup>.



**Figure 2: Sarcocystis bradyzoites extracted from microcysts showing typical banana shaped appearance after staining with Giemsa stain and examining using x200 power**

Upon comparing the prevalence of infection between both age groups, it was evident that infection rate was significantly higher from the statistical point of view in sheep with older age when compared to those with younger age (7.96% vs. 3.32%,  $P < 0.05$ ) for the first and second groups respectively. It was also noticed that there was a statistically significant difference in infection prevalence between males and females members of both groups ( $P < 0.05$ ). Infection rate was higher in females when compared to males (8.93% vs. 1.29%) respectively for the first group. The same was true with respect to members of second group (17.91% vs. 2.99%) for females and males respectively. It was found the females in both age groups were having the highest prevalence of infection with the macroscopic form when compared to

males ( $P \leq 0.05$ ) as shown in table (1). The results also concluded that older sheep had more prevalent infection were there was a statistically significant difference ( $P \leq 0.05$ ) upon comparing infections in both age groups.

Table 3: age, sex, and type of infection together with the detection method used for detecting prevalence of microcystic infection in sheep included in the study.

	Males		Females		Total
	< 18	$\geq 18$	< 18	$\geq 18$	
Age group (month)	< 18	$\geq 18$	< 18	$\geq 18$	
Number of sheep (N)	155	56	134	67	412
Macroscopic cysts (n1)	2	4	5	12	23 (5.58%)
Microscopic cysts (n2)					
Pepsin digestion	140	126	52	56	374 (90.78%)
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N = total number of sheep in any subgroup.  
n1 = number of sheep infected with macrocysts in any subgroup.  
n2 = number of sheep infected with microcysts in any subgroup.  
% =  $(n/N) \times 100$ .

Regarding infection with the microscopic form of the parasite, there was no seasonal variation in the rate of infection among study months ( $P=0.41$ ). The results of this study which encompassed 412 esophageal samples from slaughtered sheep in Karbala/ Iraq, revealed that macrosarcocysts when compared to microsarcocysts had a lower prevalence in the examined sheep. On the other hand, PCR amplification of Sarcocystis parasite with mentioned primers revealed an electrophoretic bands (Fig. 3) but, in the same time our isolates demonstrated a Sarcocystis specific 600 bp band. We easy used molecular technique for detection of Sarcocystis compare with microscopic examination, it is take long time may be need more expertise for Ophthalmic screening, other wise it is an inexpensive, rapid and precise molecular approach to identify this parasite.

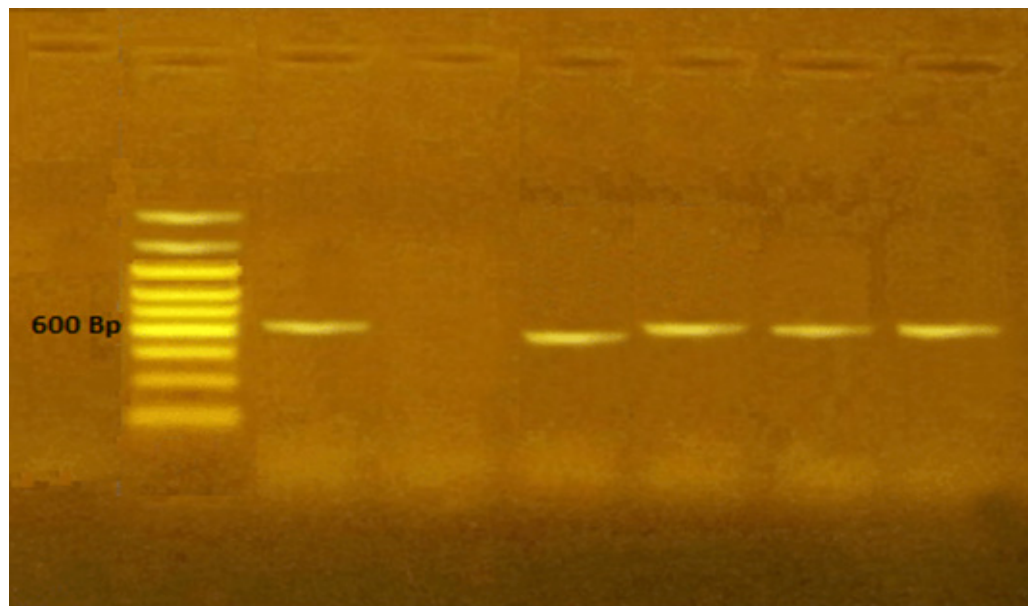


Figure 3: 2% agarose gel electrophoresis of DNA produced by PCR and stained with ethidium bromide showing bands detected at 600 bp region of 1100 Bp ladder.

At yet, special molecular procedures such as cytochrome oxidase 1, agarose gel electrophoresis staining with safety red DNA staining, have been employed for diagnosis of sarcocystosis in meat samples. These methods are genus no specific and just performable on slaughtered carcasses, so it is different with some researcher who have designed some nucleotides primer have ability to form genus-Specific PCR (28, 18).

**Financial Disclosure:** There is no financial disclosure.

**Conflict of Interest:** None to declare.

**Ethical Clearance:** All experimental protocols were approved under the Department of Biology, College of Education For pure Science, University of Kerbala and all experiments were carried out in accordance with approved guidelines.

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