# Molecular and Phylogenetic Study of Sarcocystis Gigantia from Different Slaughterhouse Regions in Karbala province

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## Abstract

Sarcocystis diseases caused a serious illness in sheep flock which inducing decrease of growth conversion rates and complete or partial loss of carcasses at the abattoirs, This article which described an prevalence of parasites *Sarcocystis gigantea* in sheep slaughtered in a farm in kerbala governerate, Iraq, and performed a phylogenetic analysis by using the Euclidean distances calculation from the *Sarcosystis gigntea* nucleotide frequencies around the world sarcosystis genomes. Fifty sheep showed multiple nodules in the esophagus that were microscopically characterized as encapsulated cysts filled with elongated, nucleated structures morphologically consistent with *S. gigantea* bradyzoites. The preverlance and species of *Sarcocystis gigantia* were revealed as 10% (5 out f 50) for the macroscopic sarcosystis , the shape of these cysts were resembled to oval and pear shape, the sensitivity of Polymerase chain reaction was 75% with 95% of confidence intervals 19.41% to 99.37% and the specificity was 95.65% with 95% of confidence intervals 85.16% to 99.47%. Our results suggest that oligonucleotide sequences were useful for estimation of *Sarcocystis gigntea* phylogenetic relationships for closely related species.

***Key words:*** *Sarcocystis gigntea, Phylogenetic analysis, PCR, sensitivity and specificity.*

## Introduction

Sarcocyst is intracellular cells parasites infect a wide range of domestic animal, some genus of Sarcocystes are pathogenic for animal like cattle and sheep that cause huge economic losses [1], cyst forming coccidiosis parasites with a mandatory two host life cycle which involved Herbivorous as intermediate hosts and carnivores as definitive hosts, an intermediate and final host may harbor more than one species of sarcocysts [2], Sheep are infected by swallowing sporulated cysts in food or water [3]. They are numerous Studies in different city of the world suggested that the spread of parasites infestation in slaughtered cattle, sheep were recorded between 60 % to 90% [4-6]. They are four species of Sarcocystis that have infected sheep which include *Sarcocystis arieticanis, Sarcocystis tenella, S. medosiformis* and *S. gigantean*, *S. gigantea* are responsible for the formation of cysts generally, in the muscles of the tongue and esophagus, or in the bowels of carcasses of slaughterhouses [7]. In the official health control of slaughterhouses, the bodies of infected cysts are macroscopic examination and sometime this cysts are trimmed or removed by veterinarians and doesn't depended on microscopic examination, these cysts are pathogenic in sheep if we leave without clinical and diagnostic tests, in this sense our study decided to conduct molecular identify macroscopic cysts of Sarcosytis parasites in sheep and study of the evolutionary relatedness among some closed related species around the world.

## Materials and Methods

### Sample collection:

The study was conducted by collection samples from sheep aged in different slaughterhouse regions of Karbala province, multiple nodules covered with intact mucosa were present and showed in the esophagus of sheep slaughter, the microscopic characterize as multiple cysts have encapsulated and filled with elongated fingers of small, basophilic, nucleated structures which morphologically consistent with *S. gigantea* bradyzoites.

Macroscopic sacs which visible in the esophagus organ and skeletal muscles were collected by sterile scapel from carcasses , and the samples were keep in sterile petri dish, and sent to the lab as soon as possible. The course of this study was beginning from February 2018 to January 2019, the age of this animals were estimated depending on permanent incisor of their teeth and the gender of each animal was documented [8]. the cysts were excised in the same time the macrocysts samples were detached from the tissues and organs.

**Macrocysts DNA extraction procedure:**

Genomic pellet DNA was extracted from macrocysts samples by using (Geneaid™ DNA Isolation Kit (Tissue). The DNA extraction of macrocysts cells was done according to manufacture company, generally, the extracted DNA was calculated by Nanodrop-spectrophotometer device, and then the extracts were store in -30C at freezing for PCR analysis.

**Polymerase chain reaction assays:**

PCR assays has done for diagnosis sarcosystis spp. Depended on small subunit ribosomal RNA gene by using specific dual primers that used in this study (Bahari et al., 2014). These primers were prepared and made by (Bioneercom.Korea) as following table (1).

**Table(1): The primer used in this study with amplicon product size**

| Primer | Sequence | Amplicon |
|---|---|---|
| small subunit ribosomal RNA gene | 5-GCACTTGATGAATTCTGGCA-3 | 580bp |
| | 5-CACCACCCATAGAATCAAG-3 | |

The PCR solution mix was prepared by the company called (Bioneer). The PCR solution kept at small tube containing freeze-dried pellet of DNA and the PCR premix mixture products was done according to manufacture instructions then filled to the top by the PCR premix tube by deionized water into 20µl and used vortex centrifuge for good mixing . The reaction was done in a Thermal cycler system (Mygene Bioneer) by orders we can sit it on thecontrolpanel the following thermocycler conditions; The products were examined by electrophoresis on a 1% agarose gel under ultraviolet light.

**DNA sequencing method:**

Genetic sequencing of subunit ribosomal RNA gene by using analysis of phylogenetic relationship and study level of alignment by mega multiple sequence software alignment programs. a product was purified from the gel by using (QIAquick Gel Extraction/ Qiagen). The purified PCR product was sent to korea for high quality DNA Sequencing service by Macrogen providing techniques (https://dna.macrogen.com/eng/support/ces/guide/ces_sample_submission.jsp ).

## Data Analysis

The nucleotides' Sequences were truncated and aligned at both ends using ApE software (A plasmid editor Version 2.0.51); therefore the sequences majority were started and ended at the homologous nucleotide positions. The sequences target was submitted to BLAST (http://www.blast.ncbi.nlm.nih.gov). the nucleotides with diversity index as well as the Euclidean distances calculated were uploaded to the UPGMA (clustering) tree and the Maximum Pairsmony phylogenetic tree by using the software MEGA 6 (Molecular Evolutionary Genetics Analysis Version 6.0), Specificity and sensitivity were used in this study to compare between PCR assay with macroscopic examination according to (Altman et al., 2013).

**Results and Discussion:**

The preverlance and species of *Sarcocystis gigantia* were revealed as 10% (5 out f 50) for the macroscopic sarcosystis , the shape of these cysts were resembled to oval and pear shape as large rice grain ranged between 9 to 11 mm in length (Pereira& Bermejo (1988) this cysts were consist of white capsule with gelatinous translucent substances.

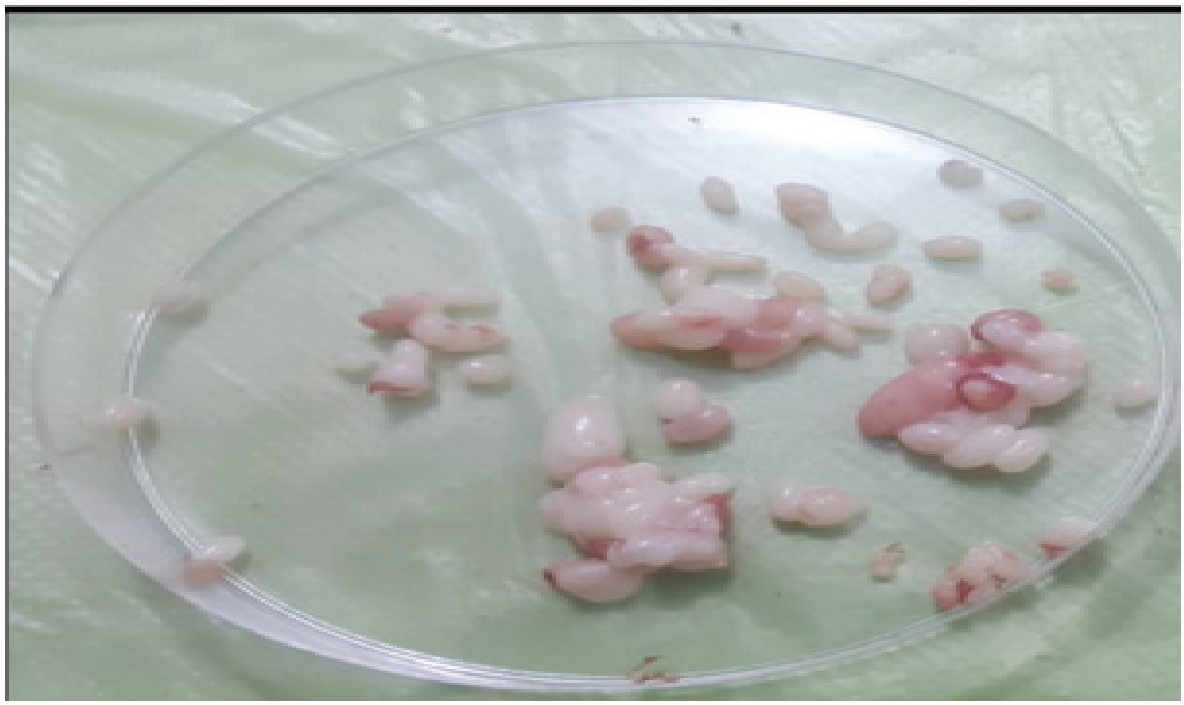

**Figure (1): macroscopic cysts observation of *Sarcocystis spp.***

Our current study was limited to macroscopic examination and sequences analysis of *Sarcocystis gigantea* , otherwise, there are many methods should be consider to confirm this strain by epidemiologic, microscopic findings [9]. The certain risk factors and clinical indicators for infection were the final host connection as well as supply raw meat from sheep to cats, all of them which contribute significantly to the spread of S. gigantea, as well as others types of sarcoecysts [10]. Our finding is in agreement with previous studies reporting an epidemiological association with adults sheep [11]; nevertheless Bertero et al. (1980) did not find macrocysts in their epidemiological study in lambs because S. gigantean macrocysts are found mainly in adult sheep [11].

PCR used to determine species of sarcosystosis is (4) out from (50) as (8%) strain isolates, the sensitivity of Polymerase chain reaction was 75% with 95% of confidence intervals 19.41% to 99.37% and the specificity was 95.65% with 95% of confidence intervals 85.16% to 99.47% table 1.

**Table (2): Sensitivity and specificity of polymerase chain reaction with Macroscopic examination.**

| Method | Polymerase chain reaction | | Total | Sensitivity 95% CI | Specificity 95% CI |
|---|---|---|---|---|---|
| Macroscopic examination | 3<br>True + | 2<br>False + | 5 | 75%<br>19.41% to 99.37% | 95.65%<br>85.16% to 99.47% |
| | 1<br>False - | 44<br>True - | 45 | | |
| Total | 4 | 46 | 50 | | |

**The specificity and yielding can be readily determined by running a gel electrophoresis that separated DNA molecules (Fig 1), So this specificity would generate one product of the correct size through using specific double primers (Ghazaei, (2018).**

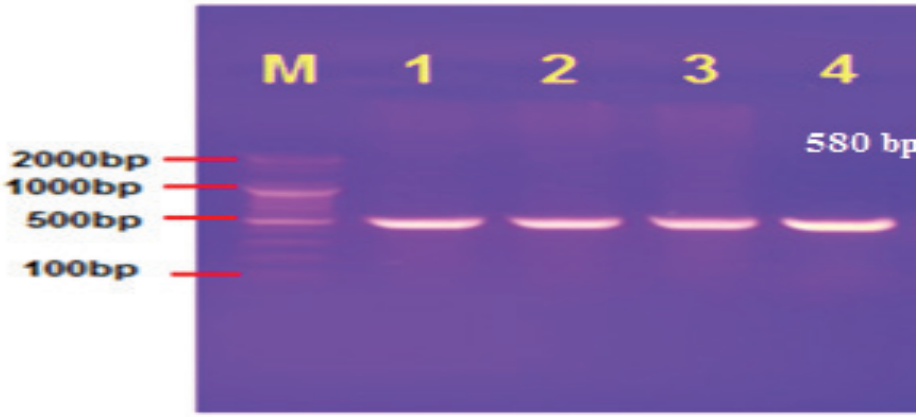


**Figure (2): Agarose gel under UV light that shows the PCR product analysis of small subunit ribosomal RNA gene in Sarcocystis spp. isolates. M: marker ( range between 100 to 2000bp), lane (1-4) some of positive Sarcocystis spp.**

Many studies revealed that Sheep are vector and harbor of parasite to reach definitive host for four Sarcocystis species which include: *S.gigantea, S. tenella , S. medusiformis* and *S. arieticanis*, both species. *S. tenella* and *S. gigantea* have worldwide distribution phylogenic of *Sarcocystis spp.* has been making Homology sequence identity by use rRNA gene according to NCBI-BLAST site show table (2) highly sensitivity to accuracy of molecular detection.

**Table (4): NCBI-Blast Homology sequence identity for rRNA gene in Trichophyton sp. isolate-1 with NCBI-BLAST Trichophyton spp.:**

**Sequences producing significant alignments:**

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

| Description | Max Score | Total Score | Query Cover | E value | Per. Ident | Accession |
|---|---|---|---|---|---|---|
| Sarcocystis gigantea isolate SC small subunit ribosomal RNA gene, partial sequence | 40.1 | 40.1 | 100% | 0.001 | 100.00% | MN398408.1 |
| Sarcocystis gigantea isolate GA small subunit ribosomal RNA gene, partial sequence | 40.1 | 40.1 | 100% | 0.001 | 100.00% | MN398402.1 |
| Sarcocystis hominis isolate HRF115 18S small subunit ribosomal RNA gene, partial sequence | 40.1 | 40.1 | 100% | 0.001 | 100.00% | MK482391.1 |
| Sarcocystis hominis isolate S073 small subunit ribosomal RNA gene, partial sequence | 40.1 | 40.1 | 100% | 0.001 | 100.00% | MK981208.1 |
| Sarcocystis hominis isolate S011 small subunit ribosomal RNA gene, partial sequence | 40.1 | 40.1 | 100% | 0.001 | 100.00% | MK981207.1 |
| Sarcocystis heydorni isolate S093 small subunit ribosomal RNA gene, partial sequence | 40.1 | 40.1 | 100% | 0.001 | 100.00% | MK981205.1 |
| Sarcocystis cruzi isolate S089 small subunit ribosomal RNA gene, partial sequence | 40.1 | 40.1 | 100% | 0.001 | 100.00% | MK981204.1 |
| Sarcocystis cruzi isolate S103 small subunit ribosomal RNA gene, partial sequence | 40.1 | 40.1 | 100% | 0.001 | 100.00% | MK981203.1 |
| Sarcocystis cruzi isolate S100 small subunit ribosomal RNA gene, partial sequence | 40.1 | 40.1 | 100% | 0.001 | 100.00% | MK981202.1 |
| Sarcocystis cruzi isolate D170269 small subunit ribosomal RNA gene, partial sequence | 40.1 | 40.1 | 100% | 0.001 | 100.00% | MK981201.1 |
| Sarcocystis bovifelis isolate S056 small subunit ribosomal RNA gene, partial sequence | 40.1 | 40.1 | 100% | 0.001 | 100.00% | MK981199.1 |

**Figure (2):Basic local sequence alignment analysis of local Sarcocystis gigntea isolate-1with NCBI-BLAST Sarcocystis 100% identity.**

Analysis of Phylogenetic tree has done depend on the clone rRNA, a that used for final detection of *Sarcocystis gigntea* draw atree by Phylogenetic analysis of rRNA gene sequences has become the principal method for knowing parasites phylogeny**.** Ou rresult show the phylogenetic tree has done according to these strains isolates figure (3), the evolution histories of *Sarcocystis gigantea* were inferred using the Neighbor-Joining method (Saitou, & Nei, (1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches, the tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances

used to infer the phylogenetic tree.

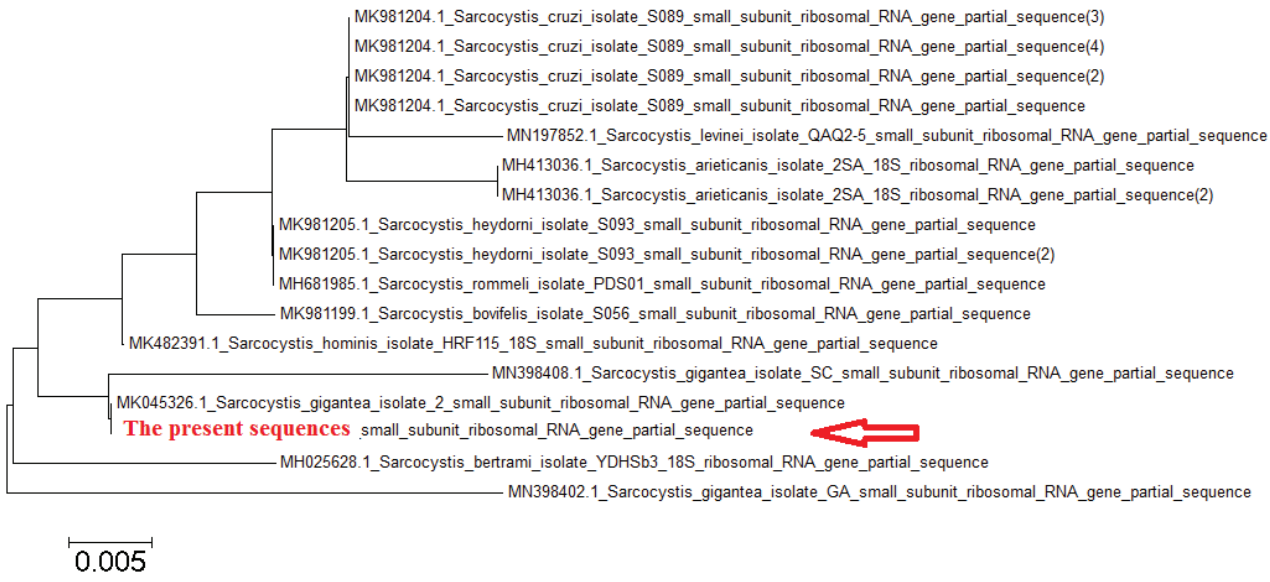


**Figure (3): Phylogenetic tree was created by using method named (UPG) Unweighted Pair Group with in (MEGA software 6.0 version), the red arrow showed the present of our sequences.**

Our results were found that *Sarcocystis gigneata* in the polygenetic tree diagram with an accession number (MK045326), a species identified in sheep, was the next-highest match at 100% homology with our sequence [12].

Finally, The host species; the geographical factors and environmental conditions are main causes to formed contract values phenomones [13]. We also found *Sarcocystis gigneata* genomic sequences showed identical with other species sequences that isolates according to the geographical regions, the present study was found identical nucleotides of *Sarcocystis gigneata* similarity with (MN398408) in sheep Erbil city, Kurdistan region.

**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 and all experiments were carried out in accordance with approved guidelines.

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