



Research Article

Diagnosis of *Sarcocystis* spp. in Slaughtered Cattle by Molecular Technique in Babylon Province in Iraq

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Abstract | This study considered the first study in Iraq to determine the *Sarcocystis hominis* by molecular diagnosis in slaughtered cattle. It is the most important species between humans and animals, causing diseases in humans. This is also determined the *Sarcocystis heydorni* which is zoonosis disease, and *S. cruzi* *S. hirsute*. With the use of (120 slaughtered cattle meat (esophagus) samples and 10 samples from imported meat were compared. In addition, the effect of some certain factors i.e. (age, gender, and months) was undertaken. Samples were collected from July into December 2022. PCR analysis was conducted to confirm presence of *Sarcocystis* for all samples, and to compare with traditional techniques, the results recorded 106/120 (88.33%) in slaughtered cattle and 7/10 (70%) and an imported beef non-significant difference between male 73/84 (86.9%) and female 33/36 (91.7%) were found. Highly significant differences registered according to age group and the highest infection conducted with age groups than 4 years and above was 59/61 (96.72%). Finally, the study have results of non-significant differences, the infection registered at all months of study and have high rate of infection in December.

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Keywords | *Sarcocystis hominis*, Molecular diagnosis, Cattle, Zoonosis infection, Babylon Province, Iraq



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Introduction

A particular intracellular parasitic protozoan of the phylum Apicomplexa and family Sarcocystidae is the cause of sarcocystosis (Abdel-Gaber *et al.*, 2020). It is a zoonotic illness that is present all throughout the world (Rosenthal, 2021). When Miescher found white thread-like cysts in a house mouse's striated muscle in 1843, it was the first time that *Sarcocystis* had been described scientifically (Dubey *et al.*, 2016). According to Rudaitytė-Lukošienė *et al.* (2018),

Sarcocystis spp. parasite infections are primarily host specific. Hosts may carry many species of the parasite. According to Dubey and Lindsay (2006). The clinical signs and symptoms of acute Sarcocystosis in intermediate hosts include encephalitis, meningitis bleeding diathesis and inflammation of the brain and spinal cord. In most cases, *Sarcocystis* does not infect the final hosts. Humans can serve as both an intermediate and final host due to their propensity to get intestinal and muscle parasite infections (Fayer, 2004). Sarcocystosis is detected by a variety

of diagnostic techniques, including pepsin digestion, muscle squashing, and histological techniques (Metwally *et al.*, 2020). Sarcocystis is diagnosed using molecular biology methods, which is highly beneficial for species identification. These are some of the sophisticated techniques used to find and recognize *Sarcocystis* species and the genetic diversity of this parasite may be evaluated in relation to various hosts and populations. Sequence analysis is performed to differentiate *Sarcocystis* spp. using molecular markers based on ribosomal DNA (Chauhan *et al.*, 2020). The purpose of this study was to identify the specific species of Sarcocystis parasites that infect slaughterhouse cow and to ascertain the current prevalence of these parasites in cattle throughout the province of Babylon.

Materials and Methods

Collection of samples

120 sample slaughtered cattle meat and 10 sample from imported meat beef weight 250 gram in labeled clean plastic bags, and carried by cool box to the laboratory of the parasitology Al-Bayati (1993), were collected from different stores, and slaughter house in diverse area of Babylon city, at the start of July 2022 to the end of December 2022.

DNA extraction

Using a tissue DNA extraction kit from Gene aid/ USA, the genomic DNA of bradyzoites that were stored and separated from butchered calf flesh isolate was obtained. the extraction made by fellow manufacturers from the digesting test's tissue cell extraction. Spectrophotometer with nanodrop (ActGene/USA) used to measure the DNA purity and concentration.

PCR amplification

Based on the ITS1 gene on our study was designed as shown in Table 1. Act gene (USA) was supplied the lyophilized primers, which almost amplify at 174 bp

segment (SAR), Preparation of primers is performed as described.

Results and Discussion

PCR analyses

The ITS1 internal transcribed spacer gene (PCR amplification results) of *Sarcocystis* sp.. isolates isolated from slaughtered cattle meat. The bands produced by PCR have a molecular weight of 174 bp are visible in the agarose gel image. PCR was analyzed by electrophoresis of agarose gel (1.5%), stained with ethidium bromide stain and using voltage at 100 volts for 1.5 hours. The positive DNA bands were 174bp shown in (Figure 1).

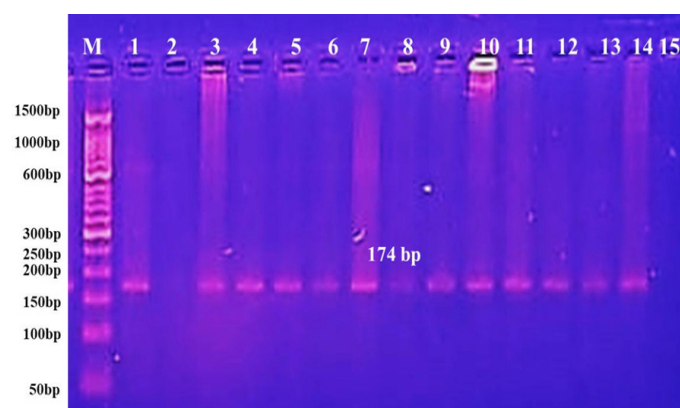


Figure 1: Results of PCR amplification of the internal transcribed spacer 1 gene (ITS1) from isolates of *Sarcocystis* sp. obtained from cattle are shown. The agarose gel picture displays the 174 bp molecular weight bands of the PCR result. 1500 base pairs (M) indicates the DNA ladder, (1) positive control. Negative oversight (2). A few tissue sample PCR results (3–15).

PCR Technique analyses to determine the *Sarcocystis* sp.

The results of PCR detection 18S rRNA gene for four different species of *Sarcocystis* isolated from slaughtered cattle meat. A image of The bands of PCR products with various molecular weights are seen on an agarose gel. 500 bp DNA ladder. *Sarcocystis* *hirsute* (158 bp), *Sarcocystis* *cruzi* (105 bp), *Sarcocystis* *Heydorni* (220 bp), and *Sarcocystis* *hominis* (190 bp) (Figure 2).

Table 1: The primers names and sequences with product size (ITS1 gene).

No	(SAR) Primer sequence 5' to 3'	Amplicon size	Annealing TM	Genbank
F	ATGGCTAATACATGCGCAAAT	174 bp	49 C	ON209179.1
R	AACTTGAATGATCTATCGCCA			

Amplification of 18s rRNA region by Primer Sar by Using PCR: The elements of the PCR master mix reaction used by Sar to detect the 18s rRNA gene (Table 2).

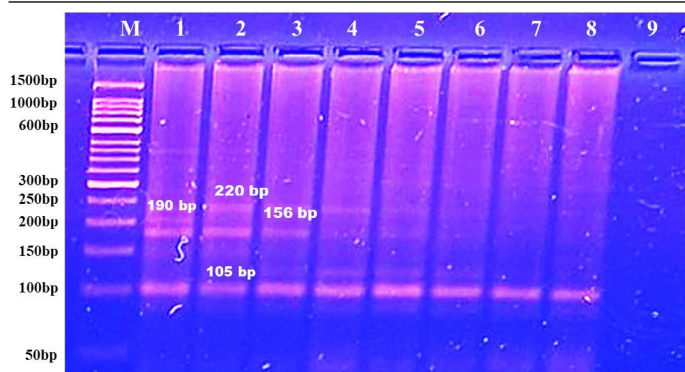


Figure 2: It shows the results of PCR detection 18S rRNA gene for four different species of *Sarcocystis*. isolated from cattle. Agarose gel pictures appear in the PCR product bands with various molecular weights. (M) refers to (500 bp) DNA ladder, (1) (190 bp) *Sarcocystis hominis*. (2) (105 bp) *Sarcocystis cruzi*. (3) (158 bp) *Sarcocystis hirsuta*. (4) (220 bp) *Sarcocystis heydorni*.

Table 2: Elements of PCR Master Mix Reaction for 18s rRNA gene.

PCR conditions	Amount
Master mix	10 µl
Primers	Forward 1µl Reversed 1 µl
PCR water	3 µl
DNA template	5 µl
Total volume	20 µl

An amplification mixture of PCR tubes which were put inside Thermal Cycler, then started the amplification program of Sar according Table 3.

Table 3: PCR program for detection of 18s rRNA region by primer Sar1.

Step	Temperature (°C)	Time	No. of cycles
Initial denaturation	92	5 Minutes	1
Denaturation	92	30Minutes	40
Annealing	49	25 Minutes	40
Extension	72	45 Minutes	40
Final extension	72	5 Minutes	1
Hold	4	Forever	-

Table 4: Infection rate of *Sarcocystis* in slaughtered cattle and imported beef samples by PCR technique.

Host	No. of sam- ples examined	PCR technique
Slaughtered cattle meat	120	No. of positive Percentage (%) 106 88.33
Imported meat	10	7 70
Total	130	113 86.92
X ²	11.598667	
P value	0.001 **	

NS: Non-significant differences at ($P \leq 0.05$).

Infection rate of *Sarcocystis* in slaughtered cattle and imported beef samples by PCR technique

After the traditional diagnostic examination, all samples, which were 120 samples from slaughtered cattle and 10 samples from imported meat, were taken to confirm the presence of infection. The results were (88.33%) in slaughtered cattle, while the infection rate in imported meat was (70%). There are no significant differences observed in the rate of infection between both slaughtered cattle and imported beef by PCR technique (Table 4).

These similar (Latif et al., 1999) *Sarcocystis* infected 97.8% of Iraqi cattle. When researchers in Dila province examined the muscular tissue samples of 179 cattle, they discovered the spread of both macrocystis and microcystis was 2.23% and 81%, respectively and agreement with (Kamil and Faraj, 2020), which, according to a genetic analysis, was found in 72.5% of slaughtered cows and 69% of imported beef samples in Baghdad. Variations in *Sarcocystis* spp. prevalence rates are caused by a variety of factors, including management practices, the presence of dogs and cats in the vicinity, and the quantity of sporocysts that they spread. Another element influencing the occurrence of *Sarcocystis* that is influenced by meteorological factors like temperature, humidity, and rainfall is the sarcocysts' capacity to survive in the environment (El-Dakhly et al., 2011).

Rate of *Sarcocystis* spp. infection. in slaughtered cattle by PCR technique according to the sex

Comparative between female and male infections showed a non-significant difference ($P \leq 0.05$). The rate of infection in females was 91.7%, while in male were 86.9% (Table 5).

Table 5: Infection rate of *Sarcocystis* in slaughtered cattle by PCR technique according to the sex.

Gen- der	No. of samples examined	PCR technique
Male	84	No. of positive Percentage (%) 73 86.9
Female	36	33 91.7
X ²	0.554486	
P value	0.456491 NS	

NS: Non-significant differences at ($P \leq 0.05$).

This was similar as recorded by (Mousa et al., 2012) which showed in females 62.73 % more than in males (0.73 %). For the detection of *Sarcocystis* followed by

molecular detection and identification by PCR and Sequencing of *18S rRNA* gene.

Infection rate of Sarcocystis in slaughtered cattle by PCR technique according to age

The samples were collected into four age groups to find out the effect of age on the infection rate above four years old; the infection rate in this group will reach 96.72%. While ≤ 2 years, infection rate was (54.54%). Age groups show a highly significant difference ($P \leq 0.05$) between them, (Table 6).

Table 6: The infection rate of *Sarcocystis* spp. in slaughtered cattle by PCR technique according to age.

Age (year)	No. of the exam. samples	Positive samples by PCR technique	
		No.	%
≤ 2 years	11	6	54.54
$2 \leq 3$ years	12	7	58.33
$3 \leq 4$ years	36	34	94.44
More than 4 years	61	59	96.72
X ²	28.134458**		
P value	0.000003		

*: significant differences at ($P \leq 0.05$).

Agreement with (Zeng *et al.*, 2021). Which showed effect of age was not significant in male meat ($p = 0.872$), while the odds of finding sarcocystis significantly increased with age ($p = 0.003$) within both types of female meat. Disagreement with (Mirzaei-Dehaghi *et al.*, 2013) which recorded 6% and 2.88%, respectively in the oesophagus and diaphragm muscle.

Rate of infection with Sarcocystis in slaughtered cattle by PCR technique according months of study

Samples were collected in six months and the highest infection was in November. That was the infection (95%) From the total number of samples collected, which is 120 samples. While lowest infection was in a October that was the infection (80%). The study showed non-significant difference ($P \leq 0.05$) between the months of infection (Table 7).

Disagreement with (Kamil and Faraj, 2020), which showed a high rate of infection registered in both July and September (85%) and the lowest one was in December. The reason is that the infection does not need a specific environment, although there is a varying percentage of infection from one month to another. *Sarcocystis* spp. differs from the rest of

the protozoa because the definitive host sheds the infected stage directly (Fayer, 2004).

Table 7: Infection rate with *Sarcocystis* in slaughtered cattle and imported beef by PCR technique according month of study.

Month	No. of the exam. samples	No. of positive samples by PCR technique	Percentage (%)
July	20	18	90
August	20	17	85
September	20	17	85
October	20	16	80
November	20	19	95
December	30	26	86.7
X ²	2.289606		
P value	0.807792 NS		

NS: Non-significant differences at ($P \leq 0.05$).

Rate of infection between Sarcocystis spp. in slaughtered cattle and imported meat by PCR technique in all samples
The samples were divided into four groups, and each group was examined in a special primer into four types. This study included the diagnosis of the most important type that causes disease to humans, and the results were as shown in the (Table 8). The study showed a highly significant difference ($P < 0.05$) between *Sarcocystis* spp. infection.

Table 8: The infection rate with *Sarcocystis* spp. in slaughtered cattle and imported beef by PCR technique.

No. of the exam. samples	<i>Sarcocystis</i> spp.	Positive samples PCR technique	
		No.	%
130	<i>Sarcocystis cruzi</i>	113	86.9
130	<i>Sarcocystis heydorni</i>	67	51.5
130	<i>Sarcocystis hominis</i>	90	69.2
130	<i>Sarcocystis Hirsuta</i>	2	1.53
X ²	71.594734		
P value	0.000**		

Similar results by (Eslami *et al.*, 2015), which recorded highly significant differences in Yazd, Iran.

Rate of infection of Sarcocystis hominis in slaughtered cattle and imported meat by PCR technique according month of study

The diagnosed groups were divided. The date of sample collection was known to compare and know the effect of any month that has an effect on the spread of *Sarcocystis* spp. The study showed non-

significant difference ($P \leq 0.05$) between *Sarcocystis* spp infection. According to the month of study, the results were as shown in (Table 9).

Table 9: Rate of infection of *Sarcocystis hominis* in slaughtered cattle and imported meat by PCR technique according month of study.

Month	No. of the exam. samples <i>Sarcocystis hominis</i>	No. of positive samples <i>Sarcocystis hominis</i>	Percentage (%)
July	20	13	65
August	20	14	70
September	20	13	65
October	20	14	70
November	20	13	65
December	30	23	76.5
X ²	1.293981		
	0.935550 NS		

NS: Non-significant differences at ($P \leq 0.05$).

Sequencing analysis 18S rRNA gene sequencing for *Sarcocystis* sample

After running NCBI Blast on these PCR amplicons, the sequencing reactions for the studied samples S1, S2, and S3 revealed the precise locations. Sequence similarities between the sequenced samples and this target have been displayed using the NCBI BLAST engine. *Sarcocystis hominis* genomic DNA sequences have exceptional similarity with the predicted target, covering a section of the 18S rRNA, according to the NCBI BLAST engine. Local samples monitored DNA sequences were compared with the reference DNA sequences (GenBank Access No. QVPWQ1 QVPWQ2, QVPWQ3). Six mutations are the presence of samples revealed as alignment results of the 199bp. *S. cruzi* (S4, S5, S6) Local samples DNA sequences were compared within reference DNA sequences (GenBank acc. QVPWQ4, QVPWQ5, and QVPWQ6), the exact positions, with all details were identified. Results of the samples alignment with 105bp, show four mutations when analyzed, and comparison of some samples with the referring 18S rRNA genetic sequences. Four mutations were found in the examined samples that were distributed differently from the corresponding 18S rRNA genetic sequences Within *Sarcocystis hirsuta* genomic DNA sequences samples (S7, S8, S9). Local samples observed DNA sequences were compared with the reference DNA sequences (GenBank Access No. QVPWQ7 and QVP Q8 QVP W9). Result of the samples alignment with 158bp; show no mutation

found when analyzed, and compared of some samples refereed 18S rRNA genetic sequences. While DNA sequences *Sarcocystis heydorni* genomic samples (S8, S9, S10) Local samples observed DNA sequences were compared with the reference DNA sequences (GenBank Access No. QVPWQ10, QVPWQ11, QVPWQ12), no mutation found. Within *Sarcocystis hirsuta* genomic DNA sequences samples (S7, S8, S9). Local samples observed DNA sequences were compared with the reference DNA sequences (GenBank Access No. QVPWQ7 and QVP Q8 QVP W9). Result of the samples alignment with 158bp; show no mutation found when analyzed, and compared of some samples refereed 18S rRNA genetic sequences. While DNA sequences *Sarcocystis heydorni* genomic samples (S8, S9, S10) Local samples observed DNA sequences were compared with the reference DNA sequences (GenBank Access No. QVPWQ10, QVPWQ11, QVPWQ12), no mutation found.

Phylogenetic tree analysis Phylogenetic tree analysis of samples (S1, S2, S3)

Phylogenetic tree in the present study is comprehensive based on 18S rRNA 199bp amplicons. A nucleic acid sequence that is aligned, for total numbers of the protozoan different, Nine sequences were present in this extensive tree. Investigated S1, S2, S3 *Sarcocystis* spp. samples were distributed into main species of this genus all over the scanned 18S rRNA-based variants sequence-related organisms (Figure 3). It was found that the studied variants were located in distinct positions within this tree. Three samples of *Sarcocystis*, namely S1, S2, S3 was found to be positioned within the *Sarcocystis hominis* phylogenetic portion in the vicinity to accession number (QVC WQ1 QVC WQ2, QVC WQ3). Which belongs to Germany and Netherland *Sarcocystis hominis* strain also from Thailand, Lithuania and Italy *Sarcocystis hominis* strain and China *Sarcocystis hominis* strain.

Phylogenetic tree analysis of samples (S4, S5, S6)

Phylogenetic tree in the present study is comprehensive based on 18S rRNA 105bp PCR amplicons. This phylogenetic tree was incorporated in S4, S5, S6 samples as they have the same PCR primers sequences. An aligned nucleic acid sequence. It was found that the S4, S5, and S6 were located in one distinct position within the tree in the vicinity to strains of *S. cruzi*, namely the Iran *S. cruzi* strain and the Japanese *S. cruzi* strain and turkey strain also

China strain. The reason behind this deviation of S4.S5.S6 is attributed to the exclusive presence of four mutations in this isolate. However, these mutations did not change the species definition of this isolate and it was just a minor modification within the same species (Figure 4).

Phylogenetic tree analysis of samples (S7, S8, S9)

Phylogenetic tree in the present study is comprehensive based on 18S rRNA 158bp amplicons. There were nine nucleic acid sequences that matched in this

extensive tree, regardless of the protozoan variations. Investigated S7, S8, S9 *Sarcocystis hirsuta* samples were distributed into main species of this genus all over the scanned 18S rRNA-based variants sequence-related organisms. It was found that the studied variants were located in distinct positions within this tree. Three sample of *Sarcocystis hirsuta*, namely S7, S8, S9 was found to be positioned within the *S. hirsuta* phylogenetic portion in the vicinity to accession number (QVPWQ7 and QVPWQ8) which belonged to the Iran *S. hirsuta* strain and China *S. hirsuta* strain

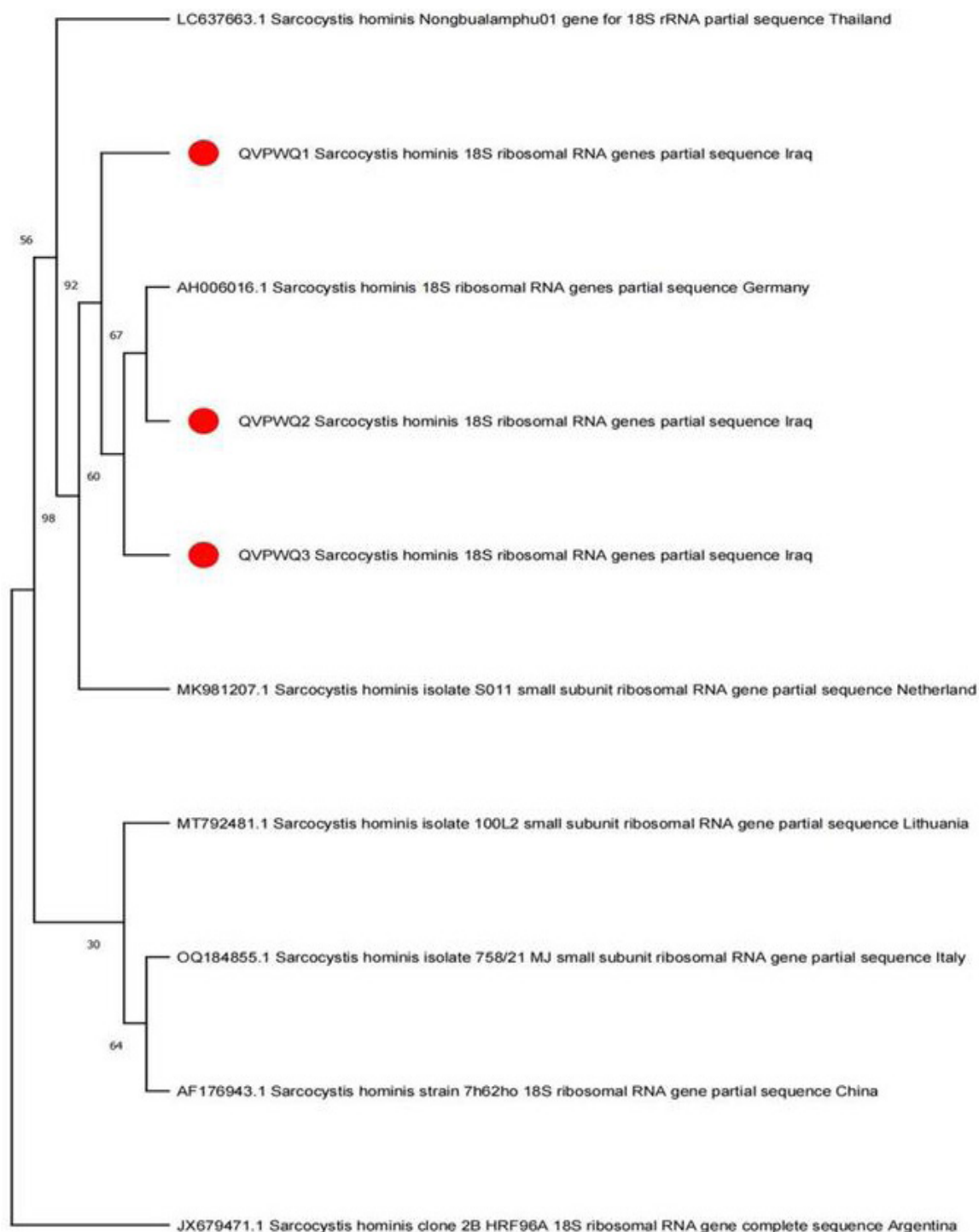


Figure 3: The complete phylogenetic tree of the genetic variations of the *Sarcocystis* isolate's 18S rRNA gene fragment. The sequenced S1, S2, and 23 variations are indicated by the red hue, and the corresponding NCBI *Sarcocystis hominis* deposited species are indicated by the black.

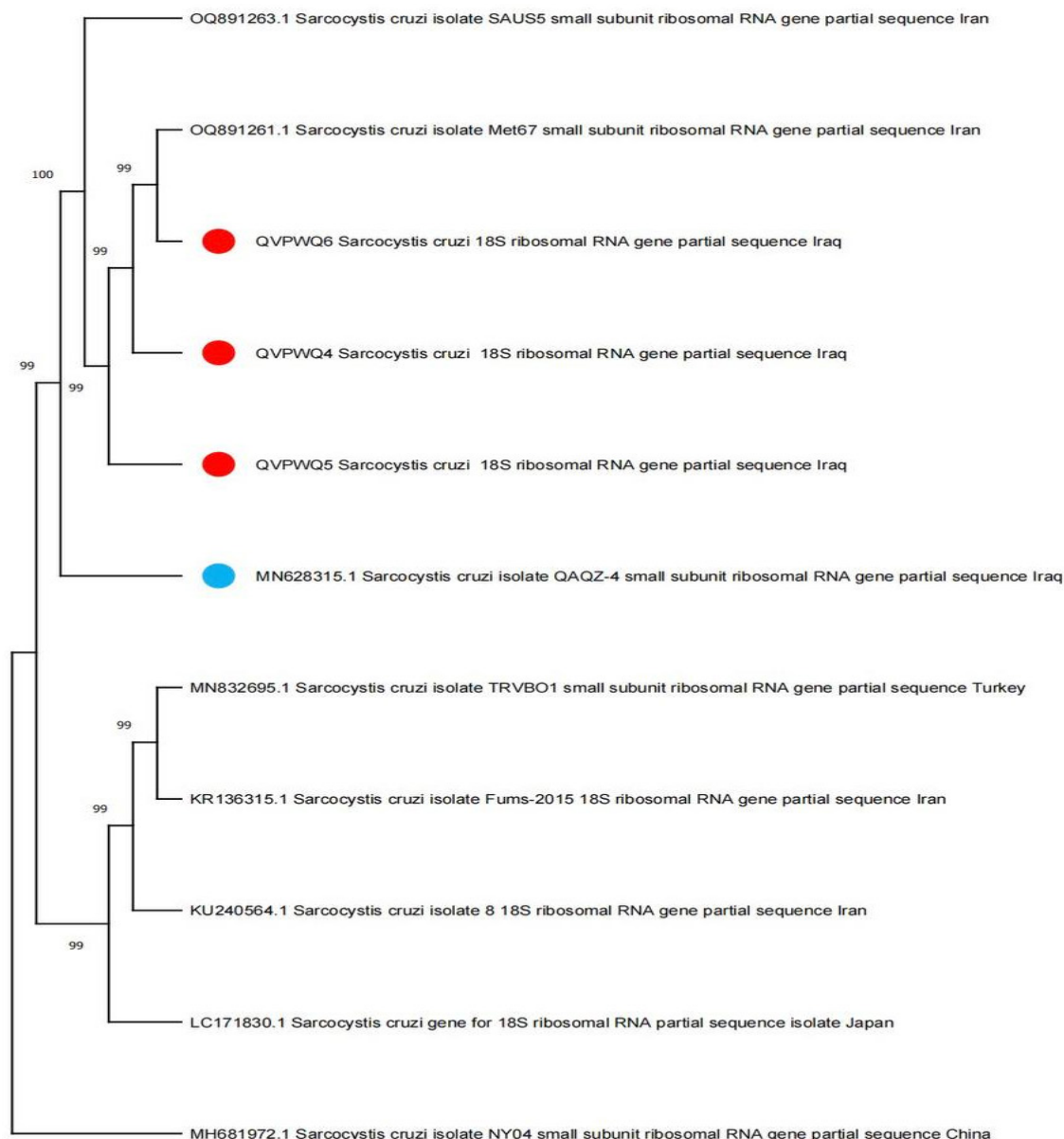


Figure 4: Four isolates of *Sarcocystis cruzi* are included in the full phylogenetic tree of genetic variations of the 18S rRNA gene fragment. The sequenced S4, S5, and S6 variations are shown by the red color, while the corresponding NCBI *Sarcocystis cruzi* deposited species are indicated by the black line color.

Japan *S. hirsuta* strain and Germany *S. hirsuta* strain also Egypt *S. hirsuta* strain.

Phylogenetic tree in the present study is comprehensive based on 18S rRNA 220bp amplicons. An aligned nucleic acid sequence, for total numbers irrespective of the protozoan variants, in this comprehensive tree was 9 sequences. Investigated S10, S11, and S12 *Sarcocystis* samples were distributed into main species of this genus all over the scanned 18S rRNA-based variants sequence-related organisms [Figure 5](#).

It was found that the studied variants were located in distinct positions within this tree. Three sample of *Sarcocystis*, namely S10, S11, S12 was found to be positioned within the *Sarcocystis heydorni*

phylogenetic portion in the vicinity to accession number (QVP Q10 QVC WQ11, QVC WQ12) which belonged to the Iran *Sarcocystis heydorni* strain and China *Sarcocystis heydorni* strain Japan *Sarcocystis heydorni* strain and Germany *Sarcocystis heydorni* strain also Egypt *Sarcocystis heydorni* strain.

In order to identify the species of *Sarcocystis* spp. infection and to compare it with other *Sarcocystis* spp. in GenBank, which provides information on the novel strain of *Sarcocystis* spp. in Iraq, DNA sequencing and phylogenetic analysis are thought to be the most essential methods. Babylon province in slaughtered cattle help in the control and of this parasite infection with prevent and control of the zoonosis type ([Dubey and Rosenthal, 2023](#)).

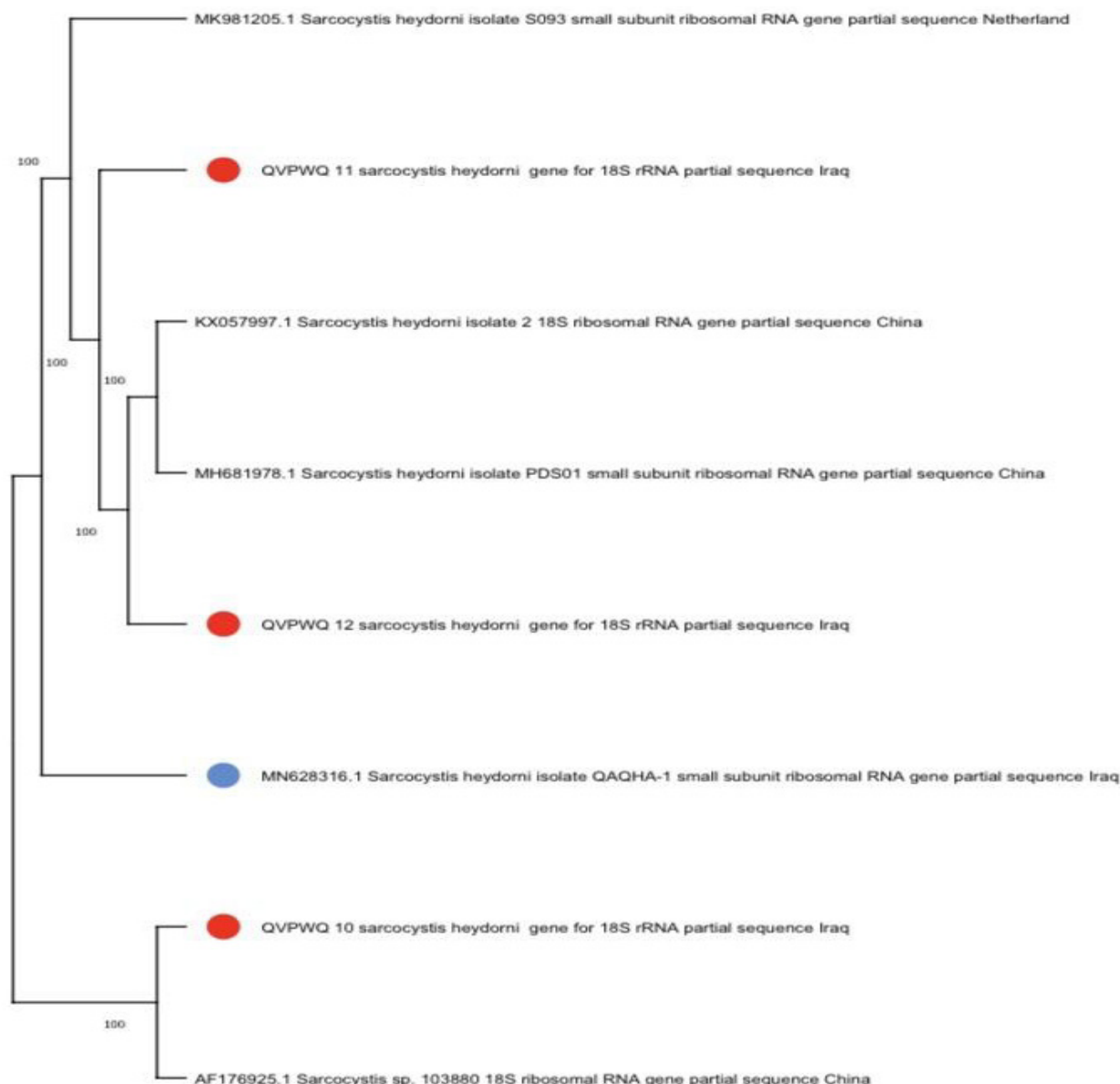


Figure 5: Isolates of *Sarcocystis heydorni* are included in the full phylogenetic tree of genetic variations of the 18S rRNA gene fragment. The sequenced S10, S11, and S12 variations are shown by the red color, while the corresponding NCBI *Sarcocystis heydorni* deposited species are indicated by the black line color.
Phylogenetic tree analysis of samples (S10, S11, S12)

The results of DNA sequencing of slaughtered cattle were collected from different regions of Babylon province which were examined using the 18S rRNA gene (174 bp) of the parasitic *Sarcocystis* species, which include *S. hydroni*, *S. cruzi*, *S. homini*, and *S. hirsuta*. The 18S rRNA sequences shared 100% identity with *S. cruzi*, *S. homini*, *S. hirsuta*, and *S. hydroni*, according to BLAST searches and the phylogenetic topology of 12 bootstraps of our samples, which showed strong sequence similarity to recognized species. The phylogenetic tree's data revealed the differences and similarities between the Iraqi strain and other strains from across the globe. It was discovered that the *S.*

cruzi phylogenetic region was closely positioned, which belongs to a Chinese strain and Iran and Japan and turkey strain *S. homini*. With regard to either close positioning was detected beside Thailand strains, Germany strain, Netherlands strain, Lithuania strains, Italy strain China strain and Argentina strain. Samples of the investigated *Sarcocystis* were found to belong to *S. hydroni* samples were localized relatively close together; they were clustered in the vicinity to a Netherlands strain and China strain and Iraq strain. *S. hirsuta* close positioning was detected beside China strain, Iran strain, Japan strain, Germany strain, Egypt strain and Norway strain.

Conclusions and Recommendations

Isolation and identification of *Sarcocystis hominis*. As one important zoonotic *Sarcocystis* species which are important for public health, and high prevalence rate infection by *Sarcocystis* in both slaughtered cow and imported beef in Babylon province using PCR technique. This means meat must be checked and inspected before use. According to the molecular study this first study revealed *S. hominis* in Iraq isolate in the slaughtered cattle and imported meat samples in Babylon province.

Novelty Statement

This study aimed to identify the *Sarcocystis* parasite because it is a common disease. The study aimed to know the rate of infection in animals as well as to know other types that have not been isolated and recorded in Iraq, such as *Sarcocystis hirsuta*, which was diagnosed using PCR technology and was registered in the Gene Bank in the United States of America. This study is also the first in Babylon Governorate using PCR.

Author's Contribution

Wisam Naser Kadhim: Helped with the idea, creation, and editing of the manuscript, approved the final version of the manuscript.

Qassim Gawad Ameer: Worked on article drafting and revision statistical analysis.

Conflict of interest

The authors have declared no conflict of interest.

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