Iraqi Journal of Veterinary Sciences, Vol. 37, Supplement I, 2023 (29-35)
Proceedings of the 1st International Conference of Pharmaceutical and Applied Medical Sciences, Knowledge University

Iraqi Journal of Veterinary Sciences

www.vetmedmosul.com

Phenotypic and genotypic study of sarcocystosis in Iraqi domestic goats (*Capra hircus*)

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Article information

Article history:
Received 03 April, 2023
Accepted 30 November, 2023
Available online 06 December, 2023

Keywords:
Sarcocystis
Trichinoscopy
Squeezing
PCR
goats

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Abstract

The study was designed to estimate the prevalence of Sarcocystosis in Iraqi domestic goats (*Capra hircus*) in Wasit Governorate, Iraq. 100 muscle (esophagus and diaphragm) samples were collected from slaughtered goats between October 2019 and March 2020. All muscle samples were examined by traditional microscopic examination (trichoscopy, squeezing, and staining using Giemsa stain) and Molecular detection by conventional PCR, where the total infection rate was 89%. Ten positive samples from PCR positive results were selected randomly for DNA analysis to obtain the partial nucleotide sequence of the small subunit ribosomal RNA gene (ITS1). The PCR product was processed as a wave-like shape at (608bp). After that, sequences were recorded in NCBI with ID No. (MW052225, MW052226, MW052231, and MW052232) for *S. hircicanis*, (MW052224, MW052227, MW052230) for *S. capracanis*, and (MW052223, MW052228, MW052229) was close related to NCBI-BLAST *S. gigantea*); then this gene sequences data were compared with another publication world in NCBI using phylogenetic tree analysis which showed NCBI-BLAST homology sequence identity between them, and these results were confirmed 99.83% identity with China isolates. In conclusion, the molecular diagnosis of the current study also revealed that goats were infected with *S. gigantea* for the first time in the world and in Iraqi goats.

DOI: 10.33899/ijvs.2023.139270.2947,

Introduction

Sarcocystis is one of the most common parasites affecting animals and humans (1,2). *Sarcocystis* is a ubiquitous parasite of many different animals; some species are specific to the intermediate host, while others have a more comprehensive range of hosts (3,4). Currently, 225 species of *Sarcocystis* have been identified and recognized. Goats are exposed to many viral, bacterial, and parasitic diseases, including genus *Sarcocystis*, which lead to significant losses (5,6). Within the species, Apicomplexa, protozoan parasites of the genus *Sarcocystis* have an obligatory two-host life cycle that requires two separate hosts in a prey-predator relationship: a definitive host (in which the sexual stage develops by producing oocysts/sporocysts in the intestine mucosa of carnivores predator when, after eating mature sarcocysts in animal muscles (meat), they become infected with bradyzoites that develop in the gastrointestinal tract, and an intermediate host, they reproduce asexually forming sarcocysts in the vascular endothelial and striated muscle cells of herbivores prey and omnivores when, after ingesting sporocysts in food or water contaminated with animal feces; humans can serve as both intermediate and definitive hosts with several *Sarcocystis* species (7). Three tissue cyst-forming *Sarcocystis* species were described in domestic goats; these include *S. capracanis*, *S. hircicanis*, and *S. moulie (S. caprafelis)* (8). Since the appearance of *Sarcocystis*, many changes depending on the locations and stages of development of sarcocysts and other conditions of the parasitized cell and molecular studies have been suggested to confirm species identification (9,10). The evidence of new

molecular techniques has provided new diagnostic means for parasitic infections; PCR reaction has been developed recently to differentiate Sarcocystis organisms genetically. Sequencing was performed on DNA product obtained from PCR reaction for extra species differentiation of *Sarcocystis*, while the importance of worldwide goat production, which is little known about the prevalence of *Sarcocystis* spp. in domestic goats (*Capra hircus*) (11-13). This study aims to detect and make a molecular diagnosis of *Sarcocustis spp*. in goats in Wasit province, Iraq.

Materials and methods

Samples collection

The samples of 100 from (the esophagus and diaphragm) were collected from different ages of 100 slaughtered goats (Capra hircus) examined in Wasit Province from October 2019 to the end of March 2020. Then, the samples were transported in refrigerator bags to the parasitology laboratory in the College of Veterinary Medicine-University of Baghdad.

Detection of macroscopic cysts

The naked eyes detected macroscopic sarcocysts in the samples (esophagus and diaphragm). These samples were divided into two parts: the first was kept for the laboratory tests (trichinoscopy, squeezing, and acid pepsin digestion), and the second was held in deep freeze under -20 ºC for DNA extraction.

Trichinoscopy

A small piece of meat specimens (esophagus and diaphragm) in the size of a pinhead approximately 3-5 mm thick were by use of these small pieces of meat specimens was crushed firmly between two glass slides and examined under the microscope at (X10) magnification for diagnosis of the microscopic cyst of the parasite (14).

Squeezing

By using the garlic press (3-5) grams from each sample were put; then, these samples were ripped by a sterile scissor. After that, the pieces were placed in the presser, and a slight drop of meat juice was transferred to the slide, then stained with Giemsa stain and examined under a microscope (X 40) to observe the presence of bradyzoites (15).

Measurement of *Sarcocystis* cyst by ocular micrometer

The ocular eyepiece was replaced with one containing an ocular micrometer, then examined under X10 magnification to determine and measure cysts in both the esophagus and diaphragm (3).

Tissue DNA extraction

Genomic DNA from the esophagus and diaphragm muscle samples were extracted using gSYAN DNA mini kit extraction kit (tissue protocol) Geneaid, United States, and done according to company instructions.

Genomic DNA concentration and purity

The extracted genomic DNA was checked by using a Nanodrop spectrophotometer (THERMO. United States), which measured DNA concentration (ng/µL) and checked the DNA purity by reading the absorbance at (260 /280 nm).

Polymerase chain reaction (PCR)

PCR technique was performed for the detection of *Sarcocystis* spp. based on amplifying internal transcribed spacer one ribosomal RNA gene (ITS1) from the esophagus and diaphragm muscles goat samples.

Primers

The PCR primers were used to amplify a fragment of the internal transcribed spacer one ribosomal RNA (ITS1) gene to detect *Sarcocystis* spp. parasite designed in this study by using NCBI Genbank and primer three plus and create an online program; these primers were provided by (Scientific Researcher. Co. Ltd. Iraq) (Table 1).

Table 1: Primers design for this study

Primer	Sequence (5'-3')	Size
ITS1	F ACCGGCTGAACTTAAGCACA R GGGACCTACCTTTTGCACCA	608 bp

PCR master mix preparation

PCR master mix was prepared using (the AccuPower PCR PreMix Kit), and this master mix was done according to the company prescript (Table 2).

Table 2: PCR master mix preparation

PCR Master mix	Volume
DNA template	5µl
ITS1-rRNA gene forward primer (10pmol)	1µl
ITS1-rRNA gene reveres primer (10pmol)	1µl
Molecular grade water	13µl
Total volume	20µl

After that, these PCR master mix components mentioned above were placed in a standard PCR mix AccuPower PCR PreMix kit that contained all other elements needed for PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$, stabilizer, and tracking dye). Then, all the PCR tubes were transferred into an Exispin vortex centrifuge at 3000rpm for 3 minutes and then placed in a PCR Thermocycler (T100 Thermal cycler BioRad. the United States).

PCR thermocycler conditions

PCR thermocycler conditions were done by using a conventional PCR thermocycler system (Table 3).

Table 3: PCR thermocycler conditions

PCR steps	Temp.	Time	Cycle
Initial Denaturation	95◦C	5 min.	1
Denaturation	95◦C	30 sec.	
Annealing	58◦C	30 sec.	32
Extension	72◦C	1 min.	
Final extension	72◦C	5 min.	1
Hold	4◦C	Forever	-

DNA sequencing method

Ten positive samples from goats by PCR technique were subjected to sequencing to detect *Sarcocystis* spp. These PCR ITS-1 gene-positive products were sent by ice bag by DHL to Macrogen Company in Korea to perform the DNA sequencing by AB DNA sequencing system. The genetic analysis was done by phylogenetic tree analysis between local *Sarcocystis* species isolates and NCBI-Blast submission *Sarcocystis* species. Then, the identification species isolates were submitted to NCBI-GenBank.The DNA sequencing analysis by utilizing Molecular Evolutionary Genetics Analysis version 6.0. (Mega 6.0) and Multiple sequence alignment analysis of the partial small subunit ribosomal rRNA gene depends on an analysis of ClustalW alignment analysis. The development distances were computed by the Maximum Composite Likelihood method by phylogenetic tree UPGMA method.

Results

Macroscopic examination

The results of the macroscopic examination in the diaphragm showed oval or cylindrical macrocysts similar to milky white rice grain size (Figure 1).

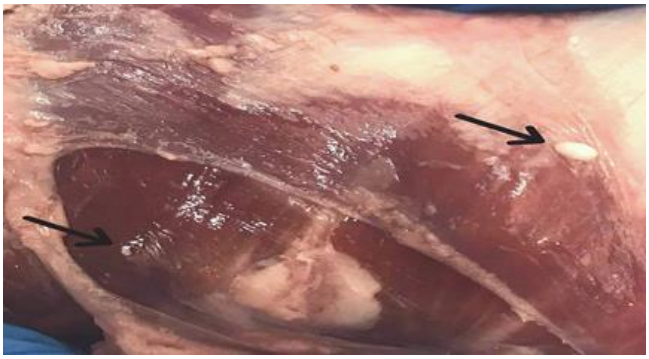

Figure 1: Goats diaphragm muscle, with white oval thin and thick cyst (sarcocysts).

Microscopic examination

Using the microscopic trichoscopy technique, different shapes of sarcocysts were revealed, such as elliptical and ovoid forms in the esophagus (Figure 2) which are divided into compartments with dissimilar measurements. The dimensions were recorded in the esophagus 121.2-28.1×17.2-3.6 µm with different measurement ranges 71.6*9.5 µm and in the diaphragm 111.8-34.76*15.42-4.9 at rang 65.5*9.2 µm.

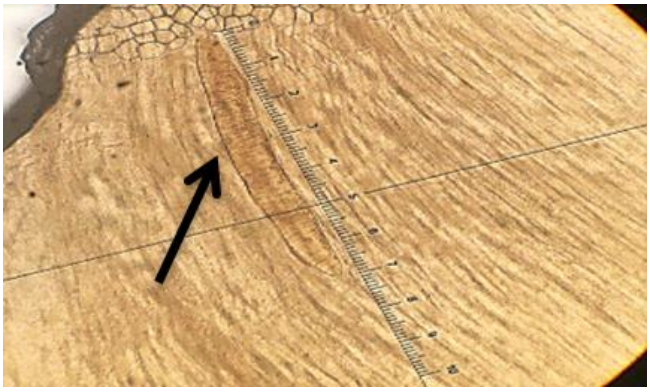

Figure 2: The microcyst of *Sarcocystis* spp. by trichinoscopy showed esophagus cyst septa dividing the internal compartments as dark structures (X 10).

Characterization of bradyzoite

Morphologically, unstained bradyzoites appeared brownish and clear under a light microscope, whereas bradyzoites stained dark blue with Giemsa stain. Bradyzoites (Cystizoites) appeared in different morphological characteristics and sizes; they have a banana shape with a little pointed anterior end and rounded posterior end with no apparent nucleus, usually near the last third part close to the posterior (Figure 3).

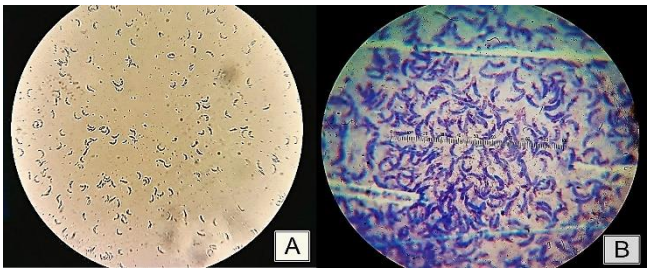

Figure 3: The bradyzoites (cystizoite) of goats *Sarcocystis* spp. A: After exploding the microcyst, 40x. B: stained with Giemsa stain, 100x.

Molecular detection of *Sarcocystis* spp. by using conventional polymerase chain reaction technique

The PCR technique was detected using an internal transcribed spacer with one ribosomal RNA gene (ITS1)

amplified to diagnose *Sarcocystis* spp. After PCR, it was analyzed by an agarose gel electrophoresis 1.5% stained by ethidium bromide stain using the voltage at 100 volts and 80 AM for 1 hour. The positive DNA bands were 608 bp (Figures 4 and 5).

Figure 4: Agarose gel electrophoresis image of the PCR product analysis of ITS1 gene in Sarcocystis spp. from esophagus tissue of goat samples. Where M: marker (100-2000bp); lane 1= negative control and lanes = (2, 19) shows negative Sarcocystis spp. and lanes = (3-18, 20, 21) shows positive Sarcocystis spp. at 608bp PCR product.

Figure 5: Agarose gel electrophoresis image of the PCR product analysis of ITS-1 gene in *Sarcocystis* spp. from diaphragm tissue of goat samples. Where M: marker (100-2000bp); lane 1= negative control and lanes = (3, 19) shows negative *Sarcocystis* spp. and lane = (2, 4-18) shows positive *Sarcocystis* spp. at (608bp) PCR product.

Total infection rate of *Sarcocystis* spp. in goats according to PCR

According to conventional PCR analysis, the total infection rates of goats' DNA samples showed 89 (89%) positive that of 100 examined tissue samples collected from goats in Wasit province (Table 4).

Table 4: Total infection rate of *Sarcocystis* spp. in goats according to polymerase chain reaction

Host	Examined No.	Infected No.	Percentage %
Goats	100	89	89

Sequencing analysis

Ten samples of PCR products out of 89 positive PCR samples were collected and sequenced using forward and reverse primers. The sequences were manipulated in gene bank database NCBI accession numbers: sample No.1 (MW052223), No.2 (MW052224), No.3 (MW052225), No.4 (MW052226), No.5 (MW052227), No.6 (MW052228), No.7 (MW052229), No.8 (MW052230), No.9 (MW052231), No.10 (MW052232) (as in Appendix). These sequences were blasted at the NCBI-BLAST program to present highly similar sequences found in NCBI. Sequences accentuation using references of ITS-1 gene of *Sarcocystis* parasite confirmed that four out of ten Iraqi *Sarcocystis* species goats isolates (MW052225, MW052226, MW052231, and MW052232) were closely related to *S. hircicanis* (KU820984.1) isolates, with identity score as (99.83 - 99.65 and 99.48%), while another three samples of *Sarcocystis* goats isolates (MW052224, MW052227, MW052230) were closely related to *S. capracanis* (MT772238.1) isolates, with identity score as (99.32 - 99.66%), furthermore, three sample of *Sarcocystis* goats isolates (MW052223, MW052228, MW052229) was closely related to NCBI-BLAST *S. gigantea*, (L24384.1) with identity score (94.62 - 99.66 %) (Table 5).

Table 5: The NCBI-BLAST homology sequence identity between Iraqi *Sarcocystis* spp. goats isolates and NCBI-BLAST isolates

Sarcocystis spp. local goats isolate No.	Accession number	NCBI-BLAST Homology Sequence identity (%)			
		Identical *Sarcocystis* spp.	Accession number	Country	Identity (%)
No.1	MW052223	*S. gigantea*	L24384.1	Australian	94.62
No.2	MW052224	*S. capracanis*	MT772238.1	Iran	99.32
No.3	MW052225	*S. hircicanis*	KU820984.1	China	99.83
No.4	MW052226	*S. hircicanis*	KU820984.1	China	99.65
No.5	MW052227	*S. capracanis*	MT772238.1	Iran	99.66
No.6	MW052228	*S. gigantea*	L24384.1	Australian	99.66
No.7	MW052229	*S. gigantea*	L24384.1	Australian	99.66
No.8	MW052230	*S. capracanis*	MT772238.1	Iran	99.66
No.9	MW052231	*S. hircicanis*	KU820984.1	China	99.48
No.10	MW052232	*S. hircicanis*	KU820984.1	China	99.48

NCBI-GenBank submission

The local *Sarcocystis* spp. were submitted in the NCBI-Genbank database to get the Gene bank accession number for Iraqi *Sarcocystis* isolates in Iraq based on sequence analysis of small subunit ribosomal RNA gene (ITS1) region with the accession no. (MW052223, MW052224, MW052225, MW052226, MW052227, MW052228, MW052229, MW052230, MW052231, MW052232). Samples were recorded and waiting for the release date on the NCBI-Gen bank website.

Multiple sequence alignment analysis

Multiple sequence alignment analysis of small subunit ribosomal RNA gene in local *Sarcocystis* spp. goats isolate, and NCBI-Genbank *Sarcocystis* species isolate. The multiple alignment analysis was constructed using the Clustal (W) alignment tool (MEGA 6.0 version). That showed the nucleotide alignment similarity as (*) and substitution mutations in small subunit ribosomal RNA genes between different *Sarcocystis* species isolates (Figure 6).

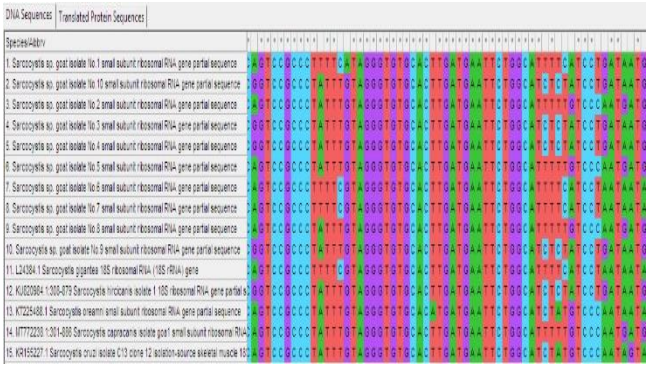

Figure 6: Multiple sequence alignment analysis of small subunit ribosomal RNA gene in local *Sarcocystis* spp

Phylogenetic analysis

Phylogenetic tree analysis based on ITS-1 gene partial sequence in local *Sarcocystis* spp. goats isolates from Wasit province that were used for genetic *Sarcocystis* species identification. The phylogenetic tree was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). The local *Sarcocystis* isolates No.1, No. 6, and No.7 were closely related to NCBI-BLAST *S. gigantea* isolates Australian (L24384.1). The local *Sarcocystis* isolates No.2, No.5, and No.8 were closed related to NCBI-BLAST *S. capracanis* isolate Iran (MT772238.1). In contrast, the local *Sarcocystis* isolates No.3 and No.4, No.9, and No.10 showed closed related to NCBI-BLAST *S. hircicanis* isolates China (KU820984.1) at total genetic changes (0.04-0.01%) (Figure 7).

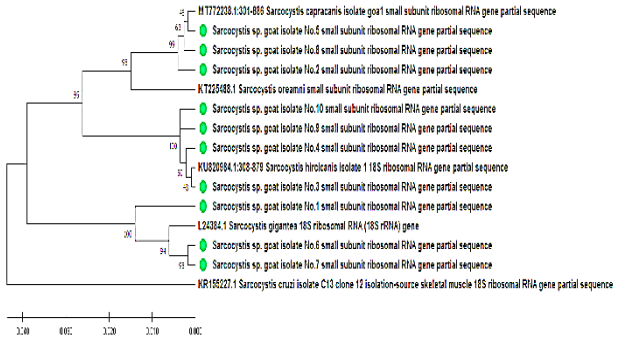

Figure 7: Phylogenetic tree analysis based on ITS-1 gene sequence in local goats *Sarcocystis* spp.

Discussion

Sarcocystosis is a common zoonotic protozoal parasitic disease affecting a wide range of domestic ruminants, and some of them can generate significant economic losses when causing clinical and subclinical disease (16,17). *Sarcocystis* spp. can be distinguished into two forms: macroscopic and microscopic cysts can invade different organs of their intermediate host (18). The results showed a macroscopic cyst similar to the grain of rice milky-white in color, round to ovoid, and buried in the muscle fibers (19). The study showed the presence of several forms of microscopic cyst, including spindle, circular, elliptical, cylindrical, and twisted shapes, in the diaphragm 111.8-34.76*15.42-4.9 µm (20). The difference in shape may be due to the age of the cyst and the duration of its stay in the intermediate host. Bradyzoite (Cystizoite) characterization appeared in different forms and sizes, showing as banana form with a little pointed anterior end and rounded posterior end containing a nucleus. The results agree with what was recorded by Nasr *et al.* (20). In this study, the conventional PCR technique used specific forward and reverse primer designs to detect *Sarcocystis* spp. positive band of 608bp. The total infection rates of goats using molecular technique showed 89(89%) positive samples out of 100 that examined DNA samples in Wasit province. In Iraq, few studies reported Sarcocystosis by molecular methods; Al-Saadi *et al.* (15) recorded Sarcocystosis in sheep in the Karbala governorate 90.78%, and Dakhil *et al.* (21) recorded overall rates of *S. fusiformis* and *S. Moulei* infection in Iraqi water buffaloes were 2.8% and 0.1% respectively. The prevalence reported in 95 (90.48 %) in Selangor, Malaysia farm goats and recorded 50.4 % (61/121) in goats (22) and in Tunisia (23), while in Brazil (24) examined 270 goat's samples and found prevalence in goats 50.7%. The differences between this and other molecular studies results may depend on different methods used to extract genomic DNA, various factors affecting the amplicon production, the absence of the parasite in the organ, or its loss due to frequent freezing and analysis. The

experience of the examiner and the number of samples have had a significant impact on the rate of infection. The DNA sequence and phylogenetic analysis are considered the most critical approaches to identifying the species of different pathogen infections and to comparing the strain of the pathogen, which may be a parasite, bacteria, virus, or another microorganism with the similar and different strain that spreads in the world (25-27). In the present study, five PCR products from 89 positive PCR samples were checked using NCBI-BLAST analysis of Sarcocystis species sequenced and compared with other *Sarcocystis* spp. Sequencing in Gene Bank gives an idea about the new strain of Iraqi Sarcocystis spp. in Wasit province to help control this disease. The results of DNA sequencing of goats were collected from different regions of Wasit province, which were checked using references of ITS1 gene of *Sarcocystis* parasite, including *S. capracanis*, *S. hircicanis*, and *S. gigantea*. The phylogenetic tree results showed similarities and differences between the Iraqi strain, neighboring countries, and the distant world; these indicated the highest homology with China and equal similarity with Australia and Iran. The results of a phylogenetic tree and sequence analysis of ITS1 coding gen of *Sarcocystis* spp. were *S. hircicanis* 40% (4/10) and *S. capracanis* 30% (3/10), *Sarcocystis capracanis* was found in goats' meat samples from Brazil, Malaysia, Egypt, Tunisia and Saudi Arabia (19,28,29). Another study identified *S. capracanis* and *S. hircicanis* in domestic goats from Kunming city (30). Our study found *S. gigantea* 30% of *S. gigantea* (3/10) that infect sheep have been diagnosed in goats. To our facts, there is no information yet on *S. gigantea* in goats. In this study, we report the natural infection of *S. gigantea* in Iraqi goats for the first time in the world; the results of the phylogenetic tree indicated the highest homology with the Australian strain of *S. gigantea* 99.62 to 99.66% identity; previously, a species of *Sarcocystis* that infects sheep in goats has been diagnosed for the first time by Hong *et al.* (30) who recorded 2.91% as positive for *S. tenella* by light, electron microscopic and molecular examination in Korean native goat. This convergence and divergence of findings among countries are due to the diversity in the resemblance between the breeds isolated in Iraq and the world breeds separated in groups far from Iraq.

Conclusion

According to the molecular study, *Sarcocystis hircicanis* and *Sarcocystis capracanis* are the main distribution species in goats in Wasit province, Iraq. The molecular research first recorded *Sarcocystis gigantea* in a goat in Iraq.

Acknowledgments

The authors thank the College of Veterinary Medicine, University of Baghdad, for their assistance and cooperation.

Conflict of interest

The authors declared that there is no conflict of interest.

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التشخيص التقليدي والجزيئي لداء الحويصلات الصنوبرية في الماعز في محافظة واسط، العراق

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الخلاصة

هدفت هذه الدراسة لمعرفة انتشار الحويصلات الصنوبرية في الماعز في محافظة واسط من خلال فحص ١٠٠عينة عضلية (لمرئ والحجاب الحاجز) جمعت للمدة كانون الثاني ولغاية أذار ٢٠٢٠. فحصت العينات بالطرئق التقليدية (منظار الشعريات، العصارة وصبغة كمزا لتلوين العينات)، وباستعمال تفاعل البلمرة التتابعي التقليدي في التقنية الجزيئية، وكان معدل الإصابة الكلي٨٩%. وتم اختيار عشر عينات موجبة لهذا الفحص لتحليل الحمض النووي وللحصول على مجموعات النيوكليوتيدات من المورث ITS-1 وتم تسجيل تسلسل المورث في المركز الوطني الدولي لمعلومات التقانة الحيوية بالأرقام والأنواع وكالاتي (MW052225, MW052226, MW052231 و *S. hircicanis*(MW052232 ورقم MW052224, MW052227, *S. capracanis*(MW052230) ورقم (MW052223, وبتحليل بيانات تسلسل *S. gigantea* (MW052228, MW052229 المورثات مع أنواع الصنوبريات المعزولة في البنك المركزي للمعلومات التقنية الحيوية ، فأظهرت النتائج تطابق ٩٩,٨٣% مع العزلات الصينية. لقد كشف التشخيص الجزيئي للدراسة الحالية كذلك عن إصابة الماعز بنوع *S. gigantea* لأول مرة في العالم وفي الماعز العراقي.