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Histomorphological and molecular survey of *Sarcocystis* spp. infecting sheep, cattle, and dogs in Jordan

H.Y. Abu Mhanna[1], R.M. Mukbel[1], W.M. Hananeh[2], H.B. Hammad[1] and M.N. Al-Sabi[1]

[1]Department of Basic Medical Veterinary Sciences, [2]Department of Veterinary Pathology and Public Health, Faculty of Veterinary Medicine, Jordan University of Science and Technology, Irbid, Jordan

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Correspondence:
R.M. Mukbel
rmmukbel@just.edu.jo

Abstract

Sarcocystosis in farm ruminants poses significant threats to animal health and causes economic losses. This study is the first to investigate the molecular diversity of *Sarcocystis* species in sheep, cattle, and dogs in Jordan. Samples from the diaphragm and esophagus of sheep and cattle in northern Jordan were examined by histomorphological and PCR. In contrast, dog fecal samples from central and northern Jordan were screened by PCR. PCR targeted the Cox1 and 18S rRNA genes for species identification. Histomorphology showed infection rates of 79.59% in sheep and 42.86% in cattle. PCR sequencing revealed higher infection rates: 97.41% in sheep and 91.84% in cattle. Among 266 dog samples, 48.12% tested positive. Cox1 gene sequencing identified multiple Sarcocystis species: *S. tenella*, *S. capracanis*, and *S. arieticanis* in sheep; *S. cruzi*, *S. hirsuta*, *S. hominis*, and *S. capracanis* in cattle; and *S. tenella*, *S. cruzi*, and *S. arieticanis* in dogs. The 18S rRNA gene sequencing was less effective at discriminating species. Infection rates were higher in stray dogs (54.25% and pet dogs 54.55% compared to breeding dogs 28.86%. The study reveals widespread and diverse *Sarcocystis* infections in Jordan's ruminants and dogs, with grazing sheep exhibiting particularly high infection rates. PCR sequencing of the Cox1 gene is the preferred molecular method for identifying *Sarcocystis* species. Controlling sarcocystosis in Jordan is complicated by the prevalence of stray dogs, underscoring the need for effective preventive strategies.

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Introduction

Sarcocystis is a unicellular protozoan parasite of the phylum Apicomplexa. More than 200 species have been reported to infect domestic animals, wild mammals, reptiles, and birds (1). *Sarcocystis* spp. have two-host life cycles in which the definitive host (usually a carnivore) harbors the parasite inside the intestine. In contrast, the parasite develops in the muscles of intermediate hosts (commonly herbivores) (2). *Sarcocystis* is distributed worldwide, with infection rates in sheep ranging from 5% to 100% (3). In Turkey, the dominant species detected in sheep is *S. tenella*, with a prevalence rate of 91% (4), while its reported prevalence rate in Saudi Arabia is 39.6% (5). In cattle, the global prevalence rate of *Sarcocystis* ranges between 36.2% and 100% (6-10). The most prevalent *Sarcocystis* species infecting cattle is *S. cruzi* (76.4%; 95% CI: 64.8–85%), followed by *S. hominis* (30.2%; 95% CI: 19.3–44%) (11). A local study in Serbia reported a prevalence rate of 4.5% in 134 dogs (12), whereas none of the 355 fecal samples from dogs in Calgary, Canada, revealed the presence of *Sarcocystis* infections (12,13). Similarly, the prevalence rates reported in dogs are relatively low in a number of countries; for example, they are 4.81% in Brazil (14), 4.5% in Canada (15), and 1.7% in the USA (16). In Jordan, only two studies previously investigated the presence of sarcocystosis in animals via microscopy and serology. The first was performed on sheep and goats in northern and central Jordan via the indirect

hemagglutination (IHA) test and trichinoscopy on esophageal and diaphragm samples. In that study, *Sarcocystis* spp. were identified in 50.1% of the 2693 sampled sheep and 56.4% of the 1261 sampled goats (17). Additionally, in this study, *S. moulei* was identified morphologically in both sheep and goat samples; however, *S. moulei* usually has goats as intermediate hosts (18). In addition, the identified species in sheep were *S. gigantea* (= *S. tenella*), *S. arieticanis*, and *S. medusiformis*, and those in goats were *S. caprcacanis* and *S. hircicanis* (17). No cattle samples were examined in that study, and the source of infections of these species was not identified. The second study screened the feces of dogs and reported an infection rate of *Sarcocystis* spp. in 8% of the 756 samples examined (19).

At that time, no molecular data were available to identify the species of *Sarcocystis* in dogs. Therefore, this study aimed to screen sheep and cattle samples collected from slaughterhouses in northern Jordan via histomorphology and PCR sequencing to identify the specific species of *Sarcocystis*. At the same time, dog fecal samples from central and north Jordan were screened for possible *Sarcocystis* infections via PCR sequencing.

Materials and methods

Ethics statement

Ethical approval was obtained from the institutional Animal Care Unit Committee (ACUC) at Jordan University of Science and Technology (Approval no. 20220096 & 202100215).

Study area and sample collection

From July 2021 until April 2022, tissue samples from the diaphragm and esophagus were collected by veterinarians during postmortem inspections of slaughtered sheep and cattle at slaughterhouses located in northern Jordan. The samples collected from 196 sheep originated from Irbid (n=104), Jordan Valley (n=41), and Mafraq (n=51), and from 50 cattle, the samples were collected from Irbid (n=27) and Jordan Valley (n=22). The age and area of sampling were noted for each sample. The samples were stored individually in sealed, sterilized plastic containers and transported in ice boxes to the Veterinary Parasitology Research Laboratory, Faculty of Veterinary Medicine, Jordan University of Science and Technology. A portion of each tissue sample was preserved in 10% formalin for histomorphological examination, and the other portion was stored at -20 °C for molecular analysis. Dog fecal samples (266) were collected from stray (n=153), pets (n=55), and breeding dogs (n=58) from central Jordan: Amman (n=123) and Zarqa (n=56), and northern Jordan: Irbid (n=87). The living conditions, age, sex, food type, and presence of diarrhea were noted for each sample. The fecal sample was kept in a sterile 1.5 mL microcentrifuge tube at –20 ℃ for molecular analysis.

Histomorphological analysis

The tissues were fixed in 10% formaldehyde for at least 24 hours. The tissues were then dehydrated in ascending grades of alcohol, cleared with xylene, infiltrated with paraffin wax, and then embedded in pure paraffin wax. The tissues were then embedded in paraffin blocks, and 4–5 µm thick sections were cut and stained with hematoxylin and eosin (20). Screening for the presence of microcysts of *Sarcocystis* was performed by a certified anatomic veterinary pathologist, and the results were recorded.

Molecular analysis

Approximately 5 grams from several parts of each tissue sample (esophagus/diaphragm) were homogenized individually via a mechanical tissue grinder (Karl Kolb, Germany). From the homogenate, genomic DNA was extracted via a QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The isolated genomic material was stored at –20 °C until further analysis. The quality of the isolated DNA was tested via a NanoDrop instrument (BioTek Gen5 microplate reader, USA). Genomic DNA from dog fecal samples was extracted via a soil DNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions, except that the extracted DNA was eluted in a 50 µL total volume and then stored at –20 ℃ until further analysis.

All samples were tested via conventional PCRs targeting two genes in *Sarcocystis* spp. In the first PCR, a 1050 bp fragment of the cytochrome C oxidase I gene (Cox1) was amplified with specific primers: forward primer SF1 and reverse primer SR9 (21). In the second PCR, a nearly 640 bp fragment of the 18S rRNA gene was amplified via the Sar-F1 and Sar-R1 primers (22) (Table 1). The PCR products were visualized via 1.5% agarose gel electrophoresis with a 100 bp DNA ladder (Promega, USA). The samples were then observed under UV light (150–200 nm) to visualize positive PCR products.

The PCR products were sequenced via conventional/2nd nested PCRs via Sanger sequencing (Macrogen™, Seoul, South Korea). PCR sequencing of the Cox1 gene was performed on all sheep and cattle tissue samples (esophagus and diaphragm), as well as all fecal samples from the dogs. PCR sequencing of the 18S rRNA gene was conducted on samples that did not produce a PCR product in the Cox1 reaction. Only molecular results of acceptable quality were included; samples with poor results were excluded.

The chromatograms of the obtained sequences were aligned with sequences available in GenBank via the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) via the U-gene® program (v52.0). Phylogenetic trees were constructed via the neighbor-joining algorithm, which is based on evolutionary distances with the maximum likelihood (ML) and maximum parsimony (MP) methods, and all sites were included via the Hirschberg (KAlign) algorithm via the Unipro UGENE®

program. The phylogeny was tested via the bootstrap method with 1,000 bootstrap replications. Finally, infections with *S. capracanis* in sheep samples were confirmed via species-specific PCR followed by sequencing. The PCR consisted of two primer sets targeting the Cox1 gene. In the 1st set, the VocaF1 and VocaR1 primers were used to amplify the 531 bp product, and in the 2nd set, the V2ca3 and V2ca4 primers were used to amplify 330 bp of the target gene (23).

Table 1: PCR sequences of primers used to amplify three target genes of *Sarcocystis* spp. from different sample sources. D: diaphragm. E: Esophagus. MM: Master mix, Tm: annealing temperature

Parasite	Gene	Primer	Primer sequence (5' to 3')	Sample source	Reference
Sarcocystis spp.	*cox1*	SF1 SR9	ATGGCGTACAACAATCATAAAGAA ATATCCATACCRCCATTGCCCAT	Sheep (D & E) Dog	(21)
Sarcocystis spp.	*18 s*	Sar-F1 Sar-R1	GCACTTGATGAATTCTGGCA CACCACCCATAGAATCAAG	Sheep, cattle, and dogs	(22)
S. capracanis	*cox1*	VocaF1 VocaR1 V2ca3 V2ca4	GTAAACTTCCTGGGTACTGTGCTGT CCAGTAATCCGCTGTCAAGATAC ATACCGATCTTTACGGGAGCAGTA GGTCACCGCAGAGAAGTACGAT	Sheep	(23)

Statistical analysis

Correlations between potential risk factors (including age, area of collection, sample origin, living conditions, sex, dog breed, food type, and the presence of diarrhea in the dogs) and *Sarcocystis* infection were evaluated via the chi-square test implemented in SPSS® version 25.0. Statistical significance was considered at $p<0.05$. Agreements between histopathology and PCR were calculated via the kappa test (24). The percentage of infection was presented with a 95% confidence interval (CI) based on the Clopper–Pearson (exact) method (25).

Results

Histomorphological examination in sheep and cattle

In sheep, 156 out of 196 samples were found to be infected with *Sarcocystis* (microcysts) (Figure 1), resulting in an overall infection rate of 79.59% (95% CI: 73.95–85.23; Table 2). Infections in sheep were detected in both the diaphragm and the esophagus in 92 samples (46.94%; 95% CI: 39.79–54.18), 33 samples in the diaphragm alone (16.83%; 95% CI: 11.88–22.82), and 31 samples in the esophagus alone (15.82%; 95% CI: 11.01–21.69). No differences in the infection rates were evident in terms of the age of the sampled sheep or their geographic distribution.

In cattle, *Sarcocystis* spp. were detected in 21 out of 49 cattle samples (42.8%; 95% CI: 28.82–57.79; Table 2). The infections were found in microcysts in both the diaphragm and the esophagus in nine samples (18.37%; 95% CI: 8.76–32.02), four samples in the diaphragm alone (8.16%; 95% CI: 2.27–19.6), and eight samples in the esophagus alone (16.33%; 95% CI: 7.32–29.65).

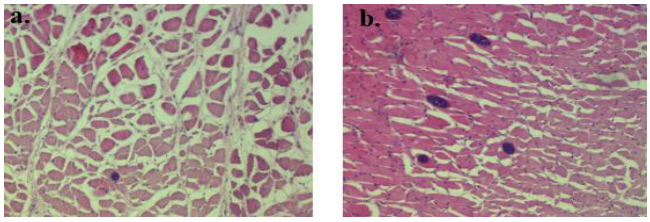

Figure 1: Histological section from the diaphragm of an infected sheep with *Sarcocystis* spp. in northern Jordan. (a) Microscopic cysts of *Sarcocystis* spp. with no inflammatory reaction (b) Heavy microscopic cysts of *Sarcocystis* spp. Infection. H&E. 100X,

Table 2. *Sarcocystis* spp. were detected via histomorphological examination of the diaphragm and/or esophagus of sheep and cattle sampled in northern Jordan from July 2021 until April 2022, according to the area of collection and the age of the host

		Sheep		Cattle	
		N	n (%; CI)	N	n (%; CI)
Area	Irbid	10	81 (77.88; 68.69 – 85.43)	27	10 (37.04; 19.4 – 57.63)
	Jordan Valley	41	30 (73.17%, 59.61 – 86.73)	22	11 (50%; 28.22 – 71.78)
	Mafraq	51	45 (88.24%, 79.39 – 97.08)	0	-
Age	=< 1 year (sheep) =< 2 years (cattle)	147	110 (74.83%, 67.81 – 81.85)	22	9 (40.91%, 20.36 – 61.45)
	> 1 year (sheep) > 2 years (cattle)	49	46 (93.88%, 87.61 – 100)	27	12 (44.44%, 25.70 – 63.19)
Total		196	156	49	21 (42.86; 28.81 – 57.79)

The percentage of infection (%) is presented with a 95% confidence interval (CI). N: Number of animals examined; n: number of infected.

Molecular screening of *Sarcocystis* spp. in sheep and cattle

PCR revealed that *Sarcocystis* spp. were detected in 188 out of the 193 DNA-extracted sheep samples with at least one gene (97.41%; 95% CI: 94.06–99.15). PCR sequencing of the CoxI gene (1050 bp) resulted in the detection of *S. tenella* in 69 sheep samples, the majority of which had 100% matching to sequences deposited in GenBank (for example, accession number MK419987.1, an isolate from a sheep, Spain). In contrast, some isolates had a lower percentage of matching to sheep isolates deposited in GenBank, for example, a matching of 97.17% (accession number: MW768895.1; sheep, Austria). Additionally, PCR sequencing of the CoxI gene resulted in the detection of *S. capracanis* in 12 sheep samples, with matching percentages ranging from 98.94% (KU820977.1; goat, China) to 99.79% (PP668138.1; goat, Egypt) (Table 3)

PCR sequencing of the 18S rRNA gene (609 bp) resulted in the detection of *S. capracanis* in six sheep samples, *S. tenella* in one sheep sample, and *S. arieticanis* in two sheep samples (99%; MF039330.1, sheep, China, and 100%; PQ538540.1, & MK420017.1, sheep, Spain). However, the 18S rRNA gene was unable to discriminate between different *Sarcocystis* species, such as *S. tenella*, *S. capracanis*, and *S. cruzi*, in many samples.

Table 3: Summary of *Sarcocystis* sp. sequencing results obtained from three different animals sampled in central and northern Jordan from July 2021 until April 2022. The accession numbers represent the best-matching isolates and references in the GenBank database. The identity percentages represent the match between this study's isolate sequences and the reference sequences.

Animal	n	Species	GenBank accession number	Identity %
Cox1				
Sheep	69	*S. tenella*	MW768895.1 & MK419987.1	97.17 - 100
	12	*S. capracanis*	PP668139.1, MW848335.1, & KU820977.1	98.94 - 99.79
Cattle	13	*S. cruzi*	MT796939.1, KC209599.1, & LC171861.1	97.45 - 99.31
Dog	2	*S. cruzi*	KT901079.1	98.56 & 99.9
	6	*S. tenella*	PP668137.1, MK419984.1, & MW768896.1	97.07 - 99.9
18 s rRNA				
Sheep	1	*S. tenella*	MG515218.1 & KP263752.1	100
	6	*S. capracanis*	MW832493.1 & KR155191.1	99.40 - 100
	2	*S. arieticanis*	MF039330.1, PQ538540.1 & MK420017.1	99 & 100
	1	*S. tenella/S. capracanis/S. cruzi*	MW832474.1, MW832480.1, & LC214880.1	100
	49	*S. tenella/S. cruzi*	MW832474.1, MT445218.1, & LC214880.1	98.84 - 100
Cattle	1	*S. capracanis*	MW832493.1 & KR155191.1	99.4
	8	*S. cruzi*	MN197849.1, KR155206.1, & OL305830.1	98.59 - 99.60
	8	*S. pilosa/S. leivinei/S. cruzi/S. hjroti*	LC481027.1, KU247921.1 & JN256124.1	99.38 - 99.79
	3	*S. hirsute*	KT901163.1 & LC171839.1	99.6
	2	*S. hominis*	JX679470.1 & MT792481.1	98.20 & 98.61
Dog	19	*S. tenella*	MH236177.1, MT569891.1, & LC364049.1	99.82 - 100
	2	*S. arieticanis*	MF039330.1, PQ538540.1, & MK420017.1	99.62 & 100
	2	*S. cruzi*	MH129611.1, MN197849.1 & OL305830.1	97.75 - 99.63
	2	*S. tenella/S. capracanis/S. cruzi*	MW832474.1, MW832480.1, & LC214880.1	99.63 & 99.81
	2	*S. levinei/S. cruzi*	KU247921.1, KT306827.1, & AF176935.1	98.35 & 98.74
	3	*S. tenella/S. cruzi*	MW832474.1, MT445218.1, & LC214880.1	97.24 - 100

PCR sequencing revealed that the prevalence rate of *Sarcocystis* spp. in cattle was 91.84% (45/49; 95% CI: 80.40–97.73). PCR sequencing of the Cox-1 gene resulted in the detection of *S. cruzi* in eight samples, with matching percentages ranging from 97.45% to 99.31% (MT796939.1; cattle, Lithuania, KC209599.1; cattle, Argentina, and LC171861.1; cattle, USA). PCR sequencing of the 18S rRNA gene resulted in the detection of *S. cruzi* in eight samples, with percentages ranging from 98.59 to 99.60 (MN197849.1, KR155206.1, & OL305830.1; cattle, Iraq, Malaysia, and Egypt), *S. hominis* in two samples, with matching percentages: 98.20% (MT792481.1, cattle, Lithuania) and 98.61% (JX679470.1; cattle, Germany), *S. hirsuta* in three samples (99.60% matching to KT901163.1 & LC171839.1, cattle, New Zealand), and *S. capracanis* in one sample (99.40%: MW832493.1; Barbary sheep, Spain & KR155191.1; Australian feral goat, Malaysia). Low discrimination-sequencing results were obtained for the eight cattle samples via the sequence of the 18S rRNA. The alignment showed equal matching percentages to the GenBank isolates of *S. pilosa*, *S. leivinei*, *S. cruzi*, and *S. hjroti*.

Molecular examination of dogs' fecal samples

Dog fecal samples were screened by PCR of the Cox1 and 18S rRNA genes, which revealed the presence of *Sarcocystis* infections in 128 out of the 266 tested samples (48.12%; 95% CI: 41.98–54.30). The prevalence rate was significantly higher among stray dogs from the Zarqa area, among dogs that did not have diarrhea, and among dogs living near other animals (Table 4).

Table 4: Risk factors associated with infections with *Sarcocystis* spp. in dogs in central and northern Jordan from July 2021 until April 2022. The infections were diagnosed on the basis of PCR sequencing of the CoxI and/or 18S rRNA genes via fecal samples. N: Number of animals examined; n: number of infected. The prevalence rates (%) are presented with 95% confidence intervals (CIs). Superscribed letters ([a], [b], and [c]) beside the brackets indicate the infection ratio of each group within the variable. N.S.: not significant

Variable	Level	N	*Sarcocystis* infection (n) (%; 95% CI)	*p* value
Region	Amman	123	60 (48.78, 39.67 – 57.95) [a]	0.037285 [b, c]
	Irbid	87	30 (34.48, 24.61 – 45.44) [b]	
	Zarqa	56	36 (64.29, 50.36 – 76.64) [c]	
Dog Type	Stray	153	83 (54.25, 46.01 – 62.32) [a]	0.018974 [a, c] 0.040605 [b, c]
	Pet	55	30 (54.55, 40.55 – 68.03) [b]	
	Breeding	58	15 (25.86, 15.25 – 39.04) [c]	
Age	≤ 1 year	78	41 (52.56, 40.93 – 63.99)	N.S.
	1 < Age ≤ 3	160	73 (45.62, 37.74 – 53.67)	
	3 < Age ≤ 9	17	8 (47.06, 22.98 – 72.19)	
	Unknown	11	6 (54.55, 23.38 – 83.25)	
Sex	Female	149	84 (56.38, 48.02 – 64.47)	N.S.
	Male	96	36 (37.50, 27.82 – 47.97)	
	Unknown	21	8 (38.10, 18.11 – 61.56)	
Habitat Indoor/Outdoor	Indoor	94	46 (48.94, 38.48 – 59.46)	N.S.
	Outdoor	172	82 (47.67, 40.02 – 55.41)	
Food Type	Cooked	45	24 (53.33, 37.87 – 68.34)	N.S.
	Raw	207	97 (46.86, 39.91 – 53.90)	
	Unknown	14	7 (50.00, 23.04 – 76.96)	
Diarrhea	No	177	102 (57.63, 49.99 – 65.01)	N.S.
	Yes	63	23 (36.51, 24.73 – 49.60)	
	Unknown	26	3 (11.54, 2.45 – 30.15)	
Other animals nearby	No	53	23 (43.40, 29.84 – 57.72)	N.S.
	Yes	205	100 (48.78, 41.76 – 55.84)	
	Unknown	8	5 (62.50, 24.49 – 91.48)	

PCR sequencing of the Cox1 gene resulted in the detection of *S. tenella* in six dog samples, with matching percentages ranging from 97.07% to 99.90% (for example, MK419984.1; sheep, Poland), and *S. cruzi* in two dog samples, with percentages ranging from 98.56% to 98.99% (KT901079.1; cattle, Argentina). Furthermore, PCR sequencing of the 18S rRNA gene resulted in the detection of *S. tenella* in 19 dog samples, *S. cruzi* in two dog samples, and *S. arieticanis* in two dog samples. Low-accuracy sequence results were obtained for seven dog isolates via sequences of the 18S rRNA gene. Three dog samples had percentages equal to those of the GenBank isolates of *S. tenella* and *S. cruzi*; two samples had percentages equal to those of many GenBank isolates of *S. tenella*, *S. capracanis*, and *S. cruzi*; and two samples had percentages equal to those of the *S. levinei* and *S. cruzi* isolates. The results of the PCR sequencing were verified by phylogenetic trees (Figs. 2 and 3), which clearly clustered the isolates obtained here with other isolates deposited in GenBank.

The identified isolates from this study's PCR-sequencing data were submitted to GenBank with the following accession numbers: PV171112 to PV171117 as Cox1 sequences representing *S. tenella*, *S. cruzi*, and *S. capracanis*. PV195265 to PV195280 are 18S rRNA sequences representing *S. hirsuta*, *S. hominis*, *S. arieticanis*, *S. tenella*, *S. cruzi*, and *S. capracanis*.

Discussion

The results of the present study confirmed previous findings concerning the persistence of *Sarcocystis* infections in sheep, dogs, and cattle in Jordan. PCR-sequencing revealed that the currently reported prevalence rate of *Sarcocystis* spp. in sheep (97.41%) was higher than that

reported in Iraq (90%) (26), the USA (82.1% sheep) (27), India (37.3%) (28), and China (52.51%) (29). Sheep farming in Jordan is based on grazing in the countryside alongside herding dogs, in areas where wild canines such as stray dogs, foxes, and wolves also live and are frequently encountered (30). The currently reported high prevalence of *Sarcocystis* spp. in sheep clearly indicates the high exposure of grazing sheep in pastures to sporocysts shed by freely roaming dogs and other definitive hosts (31).

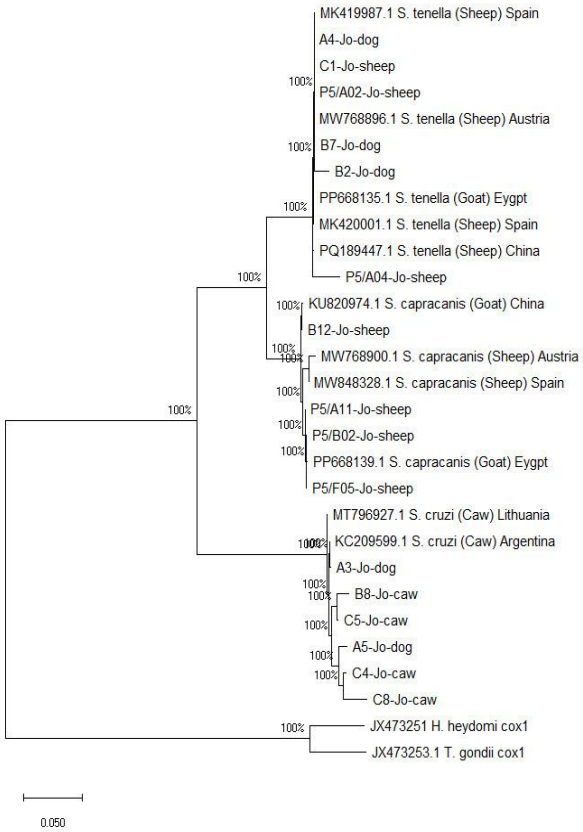

Figure 2: Phylogenetic tree of the CoxI gene. Neighbor-joining phylogenetic tree for members of the *Sarcocystis* based on *cox1* sequences of 113 *Sarcocystis* samples isolated in this study from sheep, cattle, and dogs (written as Spp.-jo-sample# sample type location-animal) and three GenBank entries identified as *Sarcocystis tenella*, *Sarcocystis capracanis*, and *Sarcocystis cruzi* (Accession Nos. MK419984.1, KU820977.1, and KC209599.1, respectively). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Bootstrap values < 50 are not shown. Sub-trees formed by two or more sequences of the same or closely related taxa have been collapsed.

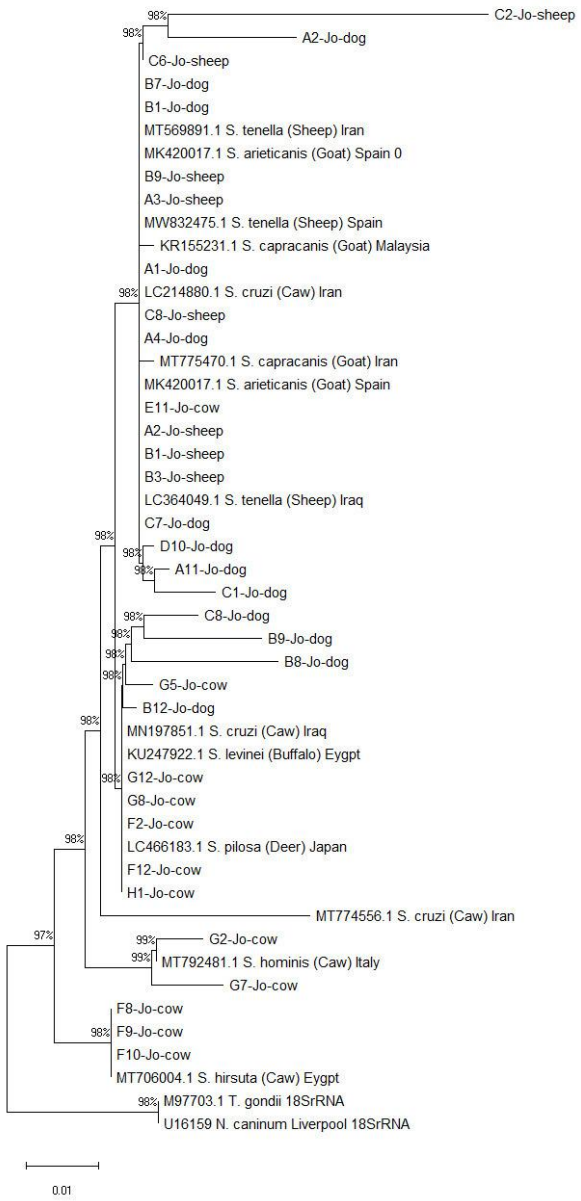

Figure 3: Phylogenetic tree of the 18S rRNA gene. Neighbor-joining phylogenetic tree for members of the *Sarcocystidae* based on *18S rRNA* sequences of 91 *Sarcocystis* samples isolated in this study from sheep, cattle, and dogs (written as Spp.-jo-sample# sample type location-animal) and seven GenBank entries identified as *Sarcocystis tenella*, *Sarcocystis capracanis*, *Sarcocystis hominis*, *Sarcocystis arieticanis*, and *Sarcocystis hirsuta*. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Bootstrap values < 50 are not shown. Sub-trees formed by two or more sequences of the same or closely related taxa have been collapsed.

The current PCR-sequencing results revealed the dominance of the canonical species *S. tenella* in Awassi sheep, followed by the noncanonical species *S. capracanis*, both of which can be transmitted by canids (3). This might explain the current molecular linkage between sheep and dog isolates of *S. tenella* and *S. arieticanis*. These two species are pathogenic to sheep and might cause abortion, fever, anemia, and anorexia (32). The herein reported presence of both *S. tenella* and *S. arieticanis* in sheep is in general agreement with previous reports from Italy (33), Brazil (34), China (29), and Iraq (26).

Although *S. capracanis* is thought to be a goat-specific species, Formisano and Aldridge (35) reported for the first time *S. capracanis* infection in sheep and presumed that sheep were accidental intermediate hosts, however, a few recent works, such as that published by Marandykina-Prakienė, Butkauskas (23), confirmed the presence of *S. capracanis* infection in three different muscle tissues, with detection rates ranging from 10.6% to 42.6% when direct and nested PCRs were applied. In addition, *S. capracanis* infection was recorded in European mouflon (*Ovis gmelini musimon*) and Barbary sheep (*Ammotragus lervia*), with 98.1–98.3% and 97.9–99% match with domestic *S. capracanis* isolates from goats, respectively (36,37). The definitive hosts of *S. capracanis* were not identified in this study and might be farm-living dogs or canines other than dogs. Therefore, a larger sample size from dogs is needed to exclude their contribution to environmental contamination with other *Sarcocystis* spp.

In the present study, molecular detection revealed a higher prevalence rate of *Sarcocystis* spp. Histopathology revealed that the prevalence rates were 79.59% and 42.86% in sheep and cattle, respectively. Moreover, via PCR sequencing, the prevalence rates inferred from the same samples were 97.41% and 91.84% in sheep and cattle, respectively. Notably, few studies have compared the latter two detection methods. Most of the published works have depended on histopathology for detection and PCR sequencing to confirm the identity of the infecting species or have used each of the latter methods solely for screening and/or species discrimination (38). However, the molecular approach resulted in a higher infection rate (83.33%) than histopathology (77.33%) in the examined sheep muscle in Iran (31). However, this was not the case in the report by Sarafraz, Spotin (39), where 92% of the examined tissues were found to be infected via meat digestion followed by microscopy, and only 83% of the samples were found to be infected via PCR. Dalir Ghaffari, Dalimi (40), Salehi, Spotin (41), and Shakeri and Adhami (42), via microscopy, reported that almost 100% of *Sarcocystis* bradyzoites were detected in cattle and sheep in Iran. Following PCR and sequencing, all the cattle samples were *S. cruzi*-infected, while the sheep samples were *S. tenella*, *S. arieticanis*, and *S. gigantea*/*S. moulei*.

The results of this study also revealed the presence of *Sarcocystis* spp. in farmed cattle in Jordan, with a high prevalence rate (91.84%). This rate was comparable to that reported in cattle in some Asian countries, such as in Iraq (92.5%) (26) and India (91.33%) (43); African countries, such as Egypt (85.7%) (44); Tunisia (cattle; 58.6%) (45); and Europe, such as Italy (cattle; 96%) (46). Several factors, such as farm management practices and climatic factors, might contribute to the persistence of sarcocystosis in farm animals (43). Cattle farming in Jordan is solely based on closed farming systems where cattle are housed with no access to grazing areas in the countryside; hence, contamination with dog feces is expected to be limited. However, the high prevalence of sarcocystosis in farmed cattle clearly indicates the widespread contamination of cattle feedstuff with sporocysts shed by livestock guardian dogs, freely roaming dogs, and other definitive hosts (47). Here, the PCR-sequencing results linked cattle isolates of one species of *Sarcocystis*, i.e., *S. cruzi*, with dog isolates of the same species. In the literature, cattle infected with *S. hirsuta* had cats as the main definitive hosts, whereas cattle infected with *S. hominis* had humans as the main definitive hosts (3); neither definitive host was screened in this study.

In other countries, the prevalence of sarcocystosis in dogs ranges between 0% and 9% (12,13). The high prevalence rate of sarcocystosis in dogs, depending on the sampled region, might be explained by other linked risk factors, among which the presence of other nearby animals was clearly related to sarcocystosis. The Zarqa region sampled herein is a land where many farms are located, and it is known for its rich wildlife (48). On the other hand, the currently reported higher infection rate in stray dogs can be linked to their dependence on uncooked meat, which in turn increases the risk of contamination of the environment with oocysts of *Sarcocystis* (49).

Sarcocystis infections are commonly screened via molecular methods. For example, PCR sequencing of the Cox1 gene from sheep isolates revealed a prevalence rate of 88% in Iraq (26), 39.5% in Saudi Arabia (5), 77.33% in Iran (31), 13.20% in Egypt (50), and 52.2% in Italy (33). Barcoding via the Cox1 gene is recommended for discriminating between particular species, such as *S. pilosa* and *S. hjorti* in the Sika deer (51). On the other hand, *S. cruzi* was more frequently detected with the Cox1 primers SF1 and SR9, which were used in this study, but *S. hominis*, *S. hirsuta*, and *S. heydorni* might not be detected at all via the above primers (52), which was in agreement with our results.

Despite having relatively low copy numbers in eukaryotic cells, the 18S rRNA gene has also been used to barcode *Sarcocystis* (53,54). The 18S rRNA gene can better discriminate between more distant species, but less so for closely related species. In contrast, the Cox1 sequences can better distinguish between closely related species than the 18S rRNA gene can (21). Previous work revealed that Cox is more reliable for the differentiation of closely related

thick-walled *Sarcocystis* spp. The 18S rRNA gene is, since Cox1 has more SNPs, indicating much greater variation than those sequenced by 18S rRNA (52). However, a review of the molecular data obtained from the partial sequencing of the 18S rRNA gene from several studies questioned the host specificity of some *Sarcocystis* spp. and suggested assigning the cattle species of *S. levinei* as *S. cruzi* and *S. buffalonis* as *S. hirsuta* (53,54). These results indicate the need to explore the genome of *Sarcocystis* and select other genes for accurate barcoding of its species.

Conclusions

Diverse species of *Sarcocystis* have been identified in the meat of sheep and cattle in northern Jordan and in dogs in north and central Jordan. The source of *Sarcocystis* infections in grazed and farmed ruminants has yet to be identified. The presence of nonconclusive sequencing results for several isolates from all animals via the 18S rRNA gene and the nonspecific matching of *Sarcocystis* spp. The Cox1 gene suggests the need to identify other genes for accurate barcoding of infections with sarcocystosis. The presence of several species of *Sarcocystis* of medical and veterinary importance in stray, pet, and breeding dogs, as well as in the examined meat prepared for human consumption, poses a risk of contracting infections in human consumers. Controlling *Sarcocystis* infections is challenging under current conditions in Jordan and calls for collaborative efforts under One Health initiatives to plan effective preventive measures.

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication and/or funding of this manuscript.

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المسح النسيجي والجزيئي لأنواع طفيلي الحويصلات الصنوبريه التي تصيب الأغنام والأبقار والكلاب في الأردن

هديل ابو مهنا[١]، رامي محمود مقبل[١]، وائل محمود حننه[٢]، هيفاء بكر حماد[١] و محمد نافع السبع[١]

[١]قسم العلوم الطبية البيطرية الأساسية، [٢]قسم علم الأمراض البيطرية والصحة العامة، كلية الطب البيطرية، جامعة العلوم والتكنولوجيا الأردنية، إربد، الأردن

الخلاصة

يشكل داء الحويصلات الصنوبريه في المجترات الزراعية تهديدات كبيرة لصحة الحيوان ويسبب خسائر اقتصادية. هذه الدراسة هي الأولى التي تبحث في التنوع الجزيئي لأنواع طفيلي الحويصلات الصنوبريه في الأغنام والأبقار والكلاب في الأردن. تم فحص عينات من الحجاب الحاجز والمريء للأغنام والأبقار في شمال الأردن عن طريق علم الأمراض النسيجي وتفاعل البوليميراز المتسلسل، في حين تم فحص عينات براز الكلاب من وسط وشمال الأردن عن طريق تفاعل البوليميراز المتسلسل. استهدف تفاعل البوليميراز المتسلسل جينات *Cox1* و*18S rRNA* لتحديد الأنواع. أظهر علم الأمراض النسيجي معدلات إصابة بنسبة ٧٩,٥٩٪ في الأغنام و٤٢,٨٦٪ في الأبقار. كشف تسلسل تفاعل البوليميراز المتسلسل عن معدلات إصابة أعلى: ٩٧,٤١٪ في الأغنام و٩١,٨٤٪ في الأبقار. من بين ٢٦٦ عينة من الكلاب، كانت نتيجة اختبار ٤٨,١٢٪ إيجابية. حدد تسلسل جين *Cox1* أنواعًا متعددة من الحويصلات الصنوبريه: *S. tenella* و*S. capracanis* و*S. arieticanis* في الأغنام، *S. cruzi* و*S. hirsuta* و*S. hominis* و*S. capracanis* في الأبقار؛ و*S. tenella* و*S. cruzi* و*S. arieticanis* في الكلاب. كان تسلسل جين ١٨*S rRNA* أقل فعالية في التمييز بين الأنواع. كانت معدلات الإصابة أعلى في الكلاب الضالة (٥٤,٢٥%) والكلاب الأليفة (٥٤,٥٥%) مقارنةً بكلاب التكاثر (٢٨,٨٦%). تكشف الدراسة عن انتشار وتنوع إصابات طفيلي الحويصلات الصنوبريه في المجترات والكلاب في الأردن، مع ارتفاع معدلات الإصابة بشكل خاص في الأغنام الرعوية. يُعد تسلسل تفاعل البوليميراز المتسلسل (*PCR*) لجين *Cox1* الطريقة الجزيئية المفضلة لتحديد أنواع طفيلي الحويصلات الصنوبريه. تُعقّد مكافحة طفيلي الحويصلات الصنوبريه في الأردن انتشار الكلاب الضالة، مما يؤكد الحاجة إلى استراتيجيات وقائية فعالة.