Morphological and molecular characterization of Sarcocystis wenzeli in chickens (Gallus gallus) in China

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Research

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Abstract

Background: There has been considerable confusion concerning the number and classification of Sarcocystis spp. in chickens. Scarce nucleotide data of Sarcocystis spp. from chickens are provided in GenBank. The study aimed to investigate the morphological and molecular characteristics of Sarcocystis spp. found in chickens in China.

Methods: Tissues from 33 chickens were collected in 2019. Sarcocysts were observed using light (LM) and transmission electron microscopy (TEM). Individual sarcocysts from different chickens were selected for DNA extraction, and five loci, 18S rDNA, 28S rDNA, ITS1 region, the mitochondrial cox1 gene and the apicoplast rpoB gene, were amplified from each sarcocyst, sequenced and analyzed.

Results: Only S. wenzeli was found in 14 of 33 (42.4%) chickens. Under LM, the sarcocysts were microscopic and exhibited palisade-like villar protrusions measuring 1.5–2.8 μm. Ultrastructurally, the sarcocyst wall contained numerous stubby hill-like villar protrusions. The protrusions included scattered microtubules, which extended from the tips of the protrusions into the ground substance. The five loci were successfully sequenced and the sequences deposited in GenBank. At 18S rDNA, ITS1 and cox1, the most similar sequences in GenBank were those of Sarcocystis sp. obtained from the brains of chickens, i.e. 99.9–100%, 98.1–98.5% and 99.3% identity, respectively. The five loci (18S rDNA, 28S rDNA, ITS1, cox1 and rpoB) showed different levels of interspecific sequence similarity with other closely related species of Sarcocystis (e.g. 99.8%, 99.0–99.2%, 89.3-89.7%, 98.5%, and 97.5%, respectively, with S. anasi). Phylogenetic analysis based on four of the loci (18S rDNA, cox1, rpoB and ITS1) revealed that S. wenzeli formed an independent clade with Sarcocystis spp. that utilize geese or ducks as intermediate hosts and canines as the known or presumed definitive host.

Conclusions: To our knowledge, the sequences of 28S rDNA and rpoB reported here constitute the first records of genetic markers of Sarcocystis spp. in chickens. Based on molecular analysis, S. wenzeli might be responsible for the neurological disease in chickens, and ITS1 and rpoB are more suitable for discriminating it from closely related Sarcocystis spp. Phylogenetic analysis revealed that S. wenzeli presents a close relationship with Sarcocystis spp. in geese or ducks.

Background

Species of the genus Sarcocystis exhibit an obligate two-host life-cycle, with sexual development in the small intestine of the definitive host and asexual development in different tissues of the intermediate host, which are usually herbivores. To date, three species of Sarcocystis with chickens (Gallus gallus) as the intermediate host have been named: S. horvathi Rátz, 1908 [1], S. gallinarum Krause & Goranoff, 1933 [2] and S. wenzeli (Wenzel, Erber, Boch & Schellner, 1982) Odening, 1997 [3, 4]. However, there has been considerable confusion concerning the number and classification of species of Sarcocystis in chickens owing to the imperfection of the original description [5].
The correct identification of *Sarcocystis* species that might infect chickens is crucial for sarcocystosis control and prevention. The ultrastructure of sarcocysts is traditionally a reliable characteristic for identifying different *Sarcocystis* species in a given host. Currently, PCR assays and sequencing procedures are considered much more practical, accurate, and reliable methods for the delineation and identification of *Sarcocystis* species than traditional methods based on morphological characteristics [6, 7]. However, there are only one 18S rDNA sequence (783 bp), one ITS1 region (ITS1) sequence (923 bp) and one mitochondrial cox1 gene sequence (547 bp) of *Sarcocystis* sp. in chickens currently deposited in GenBank. All these nucleotide sequences were obtained from *Sarcocystis* in chickens associated with neurological lesions in Brazil, and the parasite was closely related to *S. anasi* and *S. albifrons* [8]. Nevertheless, *Sarcocystis* sp, found by these authors was not identified to the species level.

Therefore, the aims of the present study were: (i) to obtain data on the prevalence of *Sarcocystis* in chickens using morphological characteristics; (ii) sequence and analyze the near-complete 18S rDNA, 28S rDNA, ITS1, cox1 and apicoplast rpoB gene (*rpoB*) of *Sarcocystis* species found in chickens in order to augment the species descriptions; and (iii) to investigate phylogenetic relationships of *Sarcocystis* species in chickens with known species of fowl-infecting *Sarcocystis* spp. using 18S rDNA, cox1, rpoB and ITS1 sequences.

### Methods

#### Morphological examination of sarcocysts from chickens

In total, tissues from 33 chickens were collected from Jiaojiaqing village, Shizong County, Yunnan Province, located in southwestern China, in July and December 2019. These chickens were free ranging and were raised by the local peasants. From each chicken, fresh tissue samples of the skeletal muscles and heart were examined for sarcocysts. In the laboratory, 20 pieces of 3 mm muscle from each collected sample were pressed and squeezed between two glass slides and inspected using a stereomicroscope. Individual sarcocysts were extracted and isolated from muscle fibers using dissection needles and processed for light (LM) and transmission electron microscopy (TEM) and DNA analysis. For TEM, four sarcocysts (two from chicken no. 4 and two from chicken no. 10) were fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4) at 4 °C and post-fixed in 1% osmium tetroxide in the same buffer, then dehydrated in a graded alcohol series and embedded in an epon-alaldite mixture. Ultrathin sections were stained with uranyl acetate and lead citrate and then examined using a JEM100-CX transmission electron microscope (JEOL Ltd., Tokyo, Japan) at 80 kV. For DNA isolation, individual cysts were stored in sterile water at -20 °C prior to processing.

#### DNA isolation, PCR amplification, cloning and sequence analysis

For DNA analysis, 5 individual sarcocysts isolated from different chickens were subjected to genomic DNA extraction using the TIANamp Genomic DNA Kit (Tiangen Biotech Ltd., Beijing, China) according to
the manufacturer's instructions. The Sarcocystis species were characterized at 5 loci within the 18S rDNA, 28S rDNA, ITS1, cox1 and rpoB genes. The near-complete 18S rDNA gene was amplified with the primer pair S1/SarDR [9, 10]; the near-full-length 28S rDNA gene was amplified with the primer sets KL1/KL3, KL4/KL5b, and KL6/KL2 [11]; the complete ITS1 region was amplified with the primer pair P-ITSF/P-ITSR [10]; the partial cox1 gene was amplified with the primer pair SF1/COIRm [6]; and the partial rpoB fragment was amplified with the primer pair rpoBF2 (5'-ATT TTT GTG GAT ATG ATT TTG AAG ATG C-3') and rpoBR2 (5'-AGT TTA GAT CCA GTT CTA CCG-3'), designed using OLIGO 7.60 (Molecular Biology Insights, Inc., West Cascade, USA) based on highly conserved regions of the rpoB sequences of Toxoplasma gondii, Neospora caninum, and Sarcocystis spp. deposited on GenBank. The PCR products were purified, cloned, sequenced, and assembled using the methods described in a previous report [12].

Phylogenetic analyses were conducted separately for the 18S rDNA, cox1, rpoB and ITS1 sequences using MEGA X software [13]. The selected sequences of the four loci of Sarcocystis spp. from various hosts were downloaded from GenBank, respectively, and aligned with the ClustalW program integrated in MEGA X applying a gap opening penalty of 10/10 and a gap extension penalty of 0.1/0.2 as pairwise and multiple alignment parameters, respectively. The alignment was subsequently checked visually; some sequences were truncated at both ends, so all sequences started and ended at the same nucleotide positions. The maximum parsimony (MP) trees were generated with a tree-bisection-regrafting (TBR) algorithm. The reliability of the MP phylograms was tested with the bootstrap method using 1000 replications.

In the case of 18S rDNA, the final alignment comprised a total of 29 nucleotide sequences from 26 taxa and 2100 aligned positions, and Besnoitia besnoiti (GenBank: DQ227418), N. caninum (GenBank: U16159) and T. gondii (GenBank: U03070) were chosen as outgroups to root the tree. At cox1, the final alignment comprised 28 cox1 nucleotide sequences from 28 species and 1020 aligned positions with no gaps, and T. gondii (GenBank: JX473253), Hammondia trittiae (GenBank: JX473247) and B. besnoiti (GenBank: XM029362743) were used as outgroup species. At rpoB, the final alignment comprised 20 rpoB nucleotide sequences from 19 species and 694 aligned positions with no gaps, and T. gondii (GenBank: AF095904) and N. aninum (GenBank: AF138960) were used as outgroup species to root the tree. At ITS1, the final alignment comprised 25 ITS1 nucleotide sequences from 21 species and 1206 aligned positions, and B. tarandi (GenBank: MH217579) and N. caninum (GenBank: U16159) were chosen as outgroups.

Results

LM and TEM examination of S. wenzeli sarcocysts

Only sarcocysts resembling those of S. wenzeli were found in 14 of 33 (42.4%) chickens. Sarcocysts were found in skeletal muscles but not in the heart. Using LM, the sarcocysts of the parasite were observed to be microscopic, measuring 381–3585 × 48–154 μm (n = 30). The sarcocyst wall exhibited numerous short palisade-like villar protrusions measuring 1.5–2.8 μm (n = 40) in length (Fig. 1a). The cysts were
septate, and their interior compartments were filled with lancet-shaped bradyzoites measuring 9.2–12.6 × 1.5–3.5 μm (n = 40) (Fig. 1b).

Four sarcocysts from both chickens were examined using TEM, all of which appeared to have walls that were ultrastructurally similar and closely resembled the “type 9k” cyst wall. The sarcocyst wall contained numerous stubby hill-like villar protrusions that were up to 1.2 μm long and 1.0 μm wide and were lined with an electron-dense layer that appeared thicker at the tips of the protrusions (Fig. 1c). Within the protrusions, there were numerous scattered fine, electron-dense granules and scattered microtubules. The microtubules extended from the tips of the protrusions into the ground substance, where they crossed microtubules originating from neighboring protrusions (Fig. 1d). The protrusions were spaced at intervals of 0.3–1.1 μm from each other. Small invaginations of the primary wall were present on the lateral aspect of the protrusions and in the spaces between protrusions. The layer of ground substance beneath the protrusions was 0.3–0.4 μm in thickness; septa were evident within the cysts (Fig. 1c).

**Molecular analysis**

Genomic DNA was extracted from the 5 individual sarcocysts of *S. wenzeli* isolated from different chickens, and 18SrDNA, 28SrDNA, ITS1, cox1 and rpoB were successfully amplified using their DNA as templates. The five 18SrDNA sequences of *S. wenzeli* were 1747 bp in length and shared an identity of 99.8–100% (average 99.8% identity). Therefore, only 4 sequences (GenBank: MT756990-MT756993) were submitted to GenBank. The most similar sequence in GenBank was that of *Sarcocystis* sp. isolate Chicken-2016-DF-BR (MN845627) from chicken (99.9–100% identity, average 99.9% identity), followed by *S. anasi* (EU553477) from mallard duck (*Anas platyrhynchos*) (99.8% identity), *S. albifrons* (EU502868) from white-fronted goose (*Anser albifrons*) (99.7% identity) and *S. rileyi* (KJ396583) from common eider (*Somateria mollissima*) (99.5% identity).

The five 28SrDNA sequences of *S. wenzeli* were 3279 bp in length and shared 99.7–100% identity (average 99.9% identity). Therefore, only 4 sequences (GenBank: MT756986-MT756989) were deposited in GenBank. The most similar sequence in GenBank was that of *S. albifrons* (EF079885) (99.3–99.1% identity, average 99.2% identity), followed by *Sarcocystis* sp. (MH898978) from Temminck’s stint (*Calidris temminckii*) (98.9–99.2% identity, average 99.1% identity), *S. anasi* (EF079887) (99.0–99.2% identity, average 99.1% identity), *S. comixi* (EU553480) from hooded crow (*Corvus cornix*) (98.6–98.9% identity, average 98.8% identity) and *S. rileyi* (GU188426) (98.3–98.5% identity, average 98.4% identity).

The five *cox1* sequences of *S. wenzeli* were 1142 bp in length and were completely identical; therefore, only 1 sequence (MT761700) was submitted to GenBank. The most similar sequence in GenBank was that of *Sarcocystis* sp. isolate Chicken-2016-DF-BR (MN848337) from chicken (99.3% identity), followed by *S. albifrons* (MH138310) (98.6% identity), *S. anasi* (MH138311) (98.5% identity) and *S. rileyi* (KJ396582) (96.4% identity).
The five ITS1 sequences (MT756994-MT756998) of *S. wenzeli* were 1186–1187 bp in length and shared 99.0–99.9% identity, with an average identity of 99.4%. The most similar sequence was that of *Sarcocystis* sp. isolate Chicken-2016-DF-BR (MN846302) from chicken (98.1–98.5% identity, average 98.3% identity), followed by *S. anasi* (JF520779) (89.3–89.7% identity, average 89.5% identity) and *S. rileyi* (KJ396584) (78.8–79.0% identity, average 78.9% identity).

The five *rpoB* sequences of *S. wenzeli* were 844 bp in length and shared 98.9–100% identity, with an average identity of 99.3%, so only 2 sequences (MT761694 and MT761695) were submitted to GenBank. The most similar sequence in GenBank was that of *S. anasi* (MH138320) (97.5% identity), followed by those of *S. albifronsi* (MH138319) (97.4% identity) and *S. rileyi* (MF596308) (95.9% identity).

Although the newly obtained 18S rDNA, *cox1* and ITS1 sequences had the highest similarity with homologous sequences of *Sarcocystis* sp. isolate Chicken-2016-DF-BR, only the ITS1 sequence of this isolate was included in the phylogenetic analysis because sequences of the other two loci of the isolate were shorter than the rest and it would lower the phylogenetic signal of the analysis. In the phylogenetic tree constructed based on 18S rDNA (Fig. 2), *cox1* (Fig. 3), or *rpoB* (Fig. 4) sequences, *S. wenzeli* formed an individual clade with *S. anasi*, *S. albifronsi* and *S. rileyi* basal to a group comprising *Sarcocystis* spp. obtained from birds or terrestrial carnivores. Phylogenetic analysis based on ITS1 sequences (Fig. 5) revealed that *S. wenzeli* formed an individual clade with *Sarcocystis* sp. isolate Chicken-2016-DF-BR and *S. rileyi*, and this clade was within a group comprising *Sarcocystis* spp. obtained from birds or terrestrial carnivores.

**Discussion**

*Sarcocystis* spp. in chickens may cause severe myositis [14] and occasionally neurological disease [8, 15]. *Sarcocystis* infection in chickens has been reported in Hungary [1], Bulgaria [2], Russia [16], Papua New Guinea [14], Australia [14], Germany [3], the Czech Republic [17], Azerbaijan [18], China [19], Iran [20] and Brazil [8]. Three species of *Sarcocystis*, *S. horvathi*, *S. wenzeli* and *S. gallinarum*, have been proposed to be responsible for the sarcocysts observed in muscle tissues of chickens. The sarcocysts found in chickens have been divided into two types based on the shape of the bradyzoites. Banana-shaped sarcocysts are considered to be produced by *S. horvathi*, described in 1908, which is synonymous with *S. gallinarum* whereas lancet-shaped sarcocysts are attributed to *S. wenzeli*, described in 1982 [5]. The ultrastructure of the sarcocysts of *S. wenzeli* has been described in detail previously [19, 21] and is similar to the type 9k sarcocyst wall classified by Dubey et al. [5]. It is worth noting that morphologically similar sarcocysts have been observed in the lesser snow geese (*Anser caerulescens*) in Saskatchewan, although the species has not been named [22]. The fine structure of the sarcocysts of *S. horvathi* and *S. gallinarum* is still unclear. In our materials, only the sarcocysts of *S. wenzeli* were found and identified, based on the observation of lancet-shaped bradyzoites and the TEM analysis of sarcocysts. The 42.4% (14/33) prevalence rate of *Sarcocystis* identified in chickens was lower than the 94.78% (37/39) prevalence recently surveyed in Iran using the digestive method [20], but was higher than the 8.9% (17/191) prevalence based on microscopic detection reported in China in 2012 [19]. It needs to be stressed that...
only squash preparation was used to search for mature sarcocysts in tissues of chickens in the present study. Therefore, the prevalence rate of *Sarcocystis* surveyed in the village should be underestimated because of the low sensitivity of the method.

Nucleotide sequence analysis has proven to be a useful tool for delineating or identifying species of *Sarcocystis* from the same or different hosts, and different genetic markers have revealed different levels of intra- or interspecific sequence diversity [6, 7, 12]. There are only one 18SrDNA sequence, one ITS1 sequence and one *cox1* sequence of *Sarcocystis* sp. obtained from brains of two chickens in Brazil currently available in GenBank. In the present study, five loci (18SrDNA, 28SrDNA, ITS1, *cox1* and *rpoB*) from *S. wenzeli* were sequenced and analyzed, to the best of our knowledge, for the first time. Among them, 28SrDNA and *rpoB* constitutes the first records of *Sarcocystis* species in chickens. In our analysis, the sequences of the five loci (18SrDNA, 28SrDNA, ITS1, *cox1* and *rpoB*) of this parasite presented high intraspecific similarities of 99.8–100%, 99.7–100%, 99.0–99.9%, 100%, and 98.9–100%, respectively. When comparing these sequences with those deposited on GenBank, sequences of 18SrDNA, ITS1 and *cox1* of *S. wenzeli* shared high similarities with those of *Sarcocystis* sp. isolate Chicken-2016-DF-BR obtained from brains of two chickens, i.e. 99.9–100%, 98.1–98.5%, and 99.3% identity, respectively. Therefore, the unrecognized species of *Sarcocystis* associated with meningoencephalitis in chickens from Brazil in 2020 [8] could be inferred as *S. wenzeli* owing to the high similarities of the three loci. The first case of *Sarcocystis*-associated encephalitis in chickens was diagnosed in the USA in 1995 [15], and the species of *Sarcocystis* was not identified because of no sarcocysts observed in brain samples of chickens, similar to the case occurred in Brazil in 2020 [8]. The sequences of the five loci (18SrDNA, 28SrDNA, ITS1, *cox1* and *rpoB*) of *S. wenzeli* exhibited different levels of similarity compared with closely related *Sarcocystis* species, sharing 99.8%, 99.0–99.2%, 89.3–89.7%, 98.5% and 97.5% identity with the corresponding sequences of *S. anasi*, and 99.5%, 98.3–98.5%, 78.8–79.0%, 96.4%, and 95.9% identity with those of *S. rileyi*. Therefore, ITS1 and *rpoB* appeared to be more suitable to for distinguishing *S. wenzeli* from other *Sarcocystis* spp., especially closely related species of *Sarcocystis*, than the 18SrDNA, 28SrDNA and *cox1* loci.

This study also established the phylogenetic relationships between *S. wenzeli* and *Sarcocystis* spp. in different hosts based on 18SrDNA, *cox1*, *rpoB* and ITS1 sequences. The topologies of the trees inferred from these sequences were highly similar and revealed that *S. wenzeli* presents a close relationship with *Sarcocystis* sp. isolate Chicken-2016-DF-BR, *S. rileyi*, *S. albifronsi* and *S. anasi*. The later three species utilize geese or ducks as intermediate hosts, and the definitive hosts of *S. rileyi* and *S. albifronsi* are canines, but that of *S. anasi* is still unknown [23, 24]. Based on experimental infection, the definitive hosts of *S. wenzeli* were confirmed to be both cats and dogs [3, 25], which is peculiar and differs from the situation for all known *Sarcocystis* spp. found in domestic animals, which use only either cats or dogs as their definitive host. However, *Sarcocystis* sporocysts were not found in the feces of cats fed breast muscle sample from over 2000 chickens from grocery stores in the USA, although the muscle was not examined microscopically for sarcocysts or bradyzoites [26].
Conclusions

In summary, we found a high prevalence rate of *Sarcocystis* in free-range chickens in China, and only *S. wenzeli* was identified based on the cyst ultrastructure. Five loci (*18S* rDNA, ITS1, *28S* rDNA, *cox1* and *rpoB*) of the parasite were sequenced, analyzed and deposited in GenBank. Based on molecular analysis, *S. wenzeli* might be an agent caused neurological disease in chickens. Among these genetic markers, ITS1 and *rpoB* are more suitable for discrimination among closely related *Sarcocystis* species. Phylogenetic analysis revealed that *S. wenzeli* shows a close relationship with *Sarcocystis* spp. that use geese and/or ducks as intermediate hosts and canines as definitive hosts.

Abbreviations

LM: light microscopy; TEM: transmission electron microscopy; PCR: polymerase chain reaction; *18S* rDNA: 18S ribosomal DNA; *28S* rDNA: 28S ribosomal DNA; ITS1: intergenic transcribed spacer region 1; *cox1*: cytochrome oxidase subunit 1; *rpoB*: RNA polymerase beta subunit; MP: maximum parsimony; TBR: tree-bisection-regrafting.

Declarations

Acknowledgements

Not applicable.

Ethics approval and consent to participate

The present study was approved by the Animal Ethics Committee of Yunnan University (permission number AEC2015021), and all authors declare that they have participated in this work.

Consent for publication

Not applicable.

Availability of data and materials

The data collected for this study are available from the corresponding author upon request.

Competing interests
The authors declare that they have no competing interests.

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**Authors’ contributions**

JH conceived the study and prepared the manuscript. JP, CM and ZH collected the samples and performed the molecular experiments. YY and HZ preformed the investigation of prevalence and morphological observation. SD and JT analyzed and interpreted the results. All authors read and approved the final manuscript.

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**References**

1. Rátz I. Szakosztályunk ülései. Állattani Közlemények. 1908;7:177–8.
2. Krause C, Goranoff S. Ueber Sarkosporidiosis bei Huhn und Wildente. Z Infektionskr Haustier. 1933;43:261–78.
3. Wenzel R, Erber M, Boch J, Schellner HP. Sarkosporidien-Infektion bei Haushuhn, Fasan und Bleßhuhn. Berl Münch Tierärztl Wochenschr. 1982;95:188–93.
4. Odening K. 1997. Die *Sarcocystis*-Infektion: Wechselbeziehungen zwischen freilebenden Wildtieren, Haustieren und Zootieren. Zool Garten N F. 1997;67:317–40.
5. Dubey JP, Calero-Bernal R, Rosenthal BM, Speer CA, Fayer R. Sarcocystosis of animals and humans, 2nd ed. Boca Raton: CRC Press. 2016; p. 277–80.
6. Gjerde B. Phylogenetic relationships among *Sarcocystis* species in cervids, cattle and sheep inferred from the mitochondrial cytochrome c oxidase subunit I gene. Int J Parasitol. 2013;43:579–91.
7. Huang ZM, Ye YL, Zhang HZ, Deng SS, Tao JJ, Hu JJ, et al. Morphological and molecular characterizations of *Sarcocystis miescheriana* and *Sarcocystis suihominis* in domestic pigs (*Sus*...
8. Wilson TM, Sousa SKH, Paludo GR, de Melo CB, Llano HAB, Soares RM, et al. An undescribed species of *Sarcocystis* associated with necrotizing meningoencephalitis in naturally infected backyard chickens in the Midwest of Brazil. Parasitol Int. 2020;76:102098.

9. Fischer S, Odening K. Characterization of bovine *Sarcocystis* species by analysis of their 18S ribosomal DNA sequences. J Parasitol. 1998;84:50–4.

10. Kutkiené L, Prakas P, Sruoga A, Butkauskas D. The mallard duck (*Anas platyrhynchos*) as intermediate host for *Sarcocystis wobeseri* nov. from the barnacle goose (*Branta leucopsis*). Parasitol Res. 2010;107:879–88.

11. Mugridge NB, Morrison DA, Heckeroth AR, Johnson AM, Tenter AM. Phylogenetic analysis based on full-length large subunit ribosomal RNA gene sequence comparison reveals that *Neospora caninum* is more closely related to *Hammondia heydorni* than to *Toxoplasma gondii*. Int J Parasitol. 1999;29:1545–56.

12. Hu JJ, Liu TT, Liu Q, Esch GW, Chen JQ, Huang S, et al. Prevalence, morphology, and molecular characteristics of *Sarcocystis* in domestic goats (*Capra hircus*) from Kunming, China. Parasitol Res. 2016;115:3973–81.

13. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Mol Biol Evol. 2018;35:1547–9.

14. Munday BL, Humphrey JD, Kila V. Pathology produced by, prevalence of, and probable life-cycle of a species of *Sarcocystis* in the domestic fowl. Avian Dis. 1977;21:697–703.

15. Mutilib A, Keirs R, Maslin W, Topper M, Dubey JP. *Sarcocystis*-associated encephalitis in chickens. Avian Dis. 1995;39:436–40.

16. Golubkov IA. Infection of dogs and kittens with *Sarcocystis* from chickens and ducks. Veterinaria (Moscow). 1979;77:55–6.

17. Pecka Z. Muscular sarcocystosis of fowls and pheasants in Czechoslovakia. Veterinářství. 1990;40:314–5.

18. Memmedov I. Prevalence of *Sarcocystis* species in some bird in Nakhchivan Autonomous Republic. Kafkas Univ Vet Fak Derg. 2010;16:857–60.

19. Chen X, He Y, Liu Y, Olias P, Rosenthal BM, Cui L, Zuo Y, Yang Z. Infections with *Sarcocystis wenzeli* are prevalent in the chickens of Yunnan Province, China, but not in the flocks of domesticated pigeons or ducks. Exp Parasitol. 2012;31:31–4.

20. Vahedi Noori N, Salehi A, Razavi M, Masoumi M. Poultry carcasses investigation of parasitic *Sarcocystis* infection in native in Mazandaran (North part of Iran). Anim Husb Dairy Vet Sci. 2019;3:1–3.

21. Mehlhorn H, Hartley WJ, Heydorn AO. A comparative ultrastructural study of the cyst wall of 13 *Sarcocystis* Protistologica. 1976;12:451–67.
22. Wobeser G, Leighton FA, Cawthorn RJ. Occurrence of *Sarcocystis* Lankester, 1882, in wild geese in Saskatchewan. Can J Zool. 1981;59:1621–4.

23. Kutkienė L, Prakas P, Sruoga A, Butkauskas D. Description of *Sarcocystis anasi* nov. and *Sarcocystis albifrons* sp. nov. in birds of the order Anseriformes. Parasitol Res. 2012;110:1043–6.

24. Prakas P, Liaugaudait S, Kutkienė, L, Sruoga A, Švažas S. Molecular identification of *Sarcocystis rileyi* sporocysts in red foxes (*Vulpes vulpes*) and raccoon dogs (*Nyctereutes procyonoides*) in Lithuania. Parasitol Res. 2015;14:1671–6.

25. Mao JB, Zuo YX. Studies on the prevalence and experimental transmission of *Sarcocystis* in chickens. Acta Vet Zootech Sinica. 1994;25:555–9.

26. Dubey JP, Hill DE, Jones, JL, Hightower AW, Kirkland E, Roberts JM, et al. Prevalence of viable *Toxoplasma gondii* in beef, chicken, and pork from retail meat stores in the United States: Risk assessment to consumers. J Parasitol. 2005;91:1082–93.