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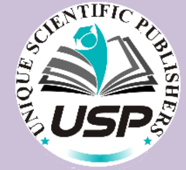
P-ISSN: 2304-3075; E-ISSN: 2305-4360

International Journal of Veterinary Science

www.ijvets.com; editor@ijvets.com



Research Article https://doi.org/10.47278/journal.ijvs/2024.206

Identification of Sarcocyst Species in Cattle Muscles: Experience of Kazakhstan

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Article History: 24-511 Received: 28-May-28 Revised: 03-Jul-24 Accepted: 06-Jul-24 Online First: 14-Jul-24

ABSTRACT

The paper presents the results indicating the spread of bovine sarcocystosis in the Kostanay region, Kazakhstan. Muscle samples from 358 cattle carcasses were examined for sarcocysts. The neck muscles in bulls from the eastern zone and the skeleton and diaphragm muscles in cows from the western zone were severely infected. The least infected were the neck muscles in bulls from the northern zone and the skeletal muscles in the southern zone. The extent of the infection equaled 77.4% of the studied livestock. Based on the molecular genetic analysis and comparison of cytochrome-c-oxidase (cox1) sequences, three types of bovine sarcocysts were identified for the first time in the Kostanay region: *S. cruzi, S. bovifelis*, and *S. dehongensis*. This study highlights the significant prevalence of sarcocystosis in the Kostanay region, demonstrating the utility of cox1 gene sequencing in identifying different *Sarcocystis* species. These findings underline the need for improved control and prevention strategies to mitigate the impact on cattle health and productivity.

Key words: Cattle, Sarcocystosis, Muscle samples, Prevalence, Molecular genetic analysis

INTRODUCTION

Sarcocystosis is a protozoan disease common among cattle, pigs, and horses. It is characterized by damage to muscle tissue, has an acute or subclinical course, and causes a decrease in animal productivity. In acute cases, the body temperature rises to 40.5°C, the feed intake decreases, diarrhea appears, depression is noticeable, and milk yield decreases. With a chronic course, sick animals lose weight, their mucous membranes are anemic, and the submandibular space swells. They develop shortness of breath, muscle weakness and subsequently paresis and paralysis of the extremities, and pregnant animals abort. With severe damage in ruminants, hemorrhages are found in the stomach, intestines, and parenchymal organs; besides that, nephritis, myocarditis, ulceration of mucous membranes, edema, myositis, and foci of necrosis (Yatusevich et al. 2020) are observed.

Sarcocystosis is a poorly studied disease caused by parasitizing heteroxenous coccidia belonging to the *Sarcocystis* genus. Representatives of the Sarcocystidae family, sarcocysts develop with the participation and successive change of two hosts. The first (definitive) host is carnivorous animals in whose intestines sarcocysts go through the sexual stage and form invasive oocysts and sporocysts. The second (intermediate) host is herbivorous and omnivorous animals, in which the development of protozoa occurs asexually (Morre et al. 2011; Dubey 2015; Novak and Engashev 2018; Rosenthal 2021).

Bovine sarcocystosis is caused by six *Sarcocystis* species *(S. cruzi, S. hominis, S. heydorni, S. rommeli, S. hirsuta,* and *S. bovifelis)*. Each of the six species has its definitive host. According to the results of molecular studies based on the analysis of the cytochrome-c-oxidase (cox1) gene sequence, as an intermediate host, cattle can contain tissue cysts of five of the *Sarcocystis* species (*S. cruzi, S. hominis, S. hirsuta, S. rommeli,* and *S. heydorni)* (Salimov et al. 2014; Gjerde et al. 2016; Imre et al. 2019; Tamura et al. 2021).

Cite This Article as: Bermukhametov Z, Suleimanova K, Prakas P, Tomaruk O, Shevtsov A, Abdygulov B, Mustafin B, Baimenov B, Sokharev Y and Rychshanova R, 2024. Identification of sarcocyst species in cattle muscles: experience of Kazakhstan. International Journal of Veterinary Science x(x): xxxx. https://doi.org/10.47278/journal.ijvs/2024.206

For *S. hominis* and *S. heydorni*, the common definitive host is humans. The disease in humans most often does not manifest itself, although occasionally symptoms appear in the form of fever, chills, and digestive disorders. *S. cruzi*, transmitted through the Canine family, is recognized as the most pathogenic species. It reduces the meat and dairy productivity of cattle and causes abortions and deaths. *S. hirsuta* infects cats and forms macroscopic cysts (Gjerde 2016). *S. rommeli* is also carried by cattle as an intermediate host, while its definitive host has not been established (Dubey et al. 2016).

Although there is a considerable amount of information about sarcocystosis, it remains incomplete and requires further research. Rutaganira and Glamazdin (2022) note that the spread of bovine sarcocystosis represents a financial burden for food producers due to the disease's ability to reduce livestock productivity. Subsequently, the problems of malnutrition and food insecurity take root, and countering them requires increased control in the field of veterinary parasitology and food biotechnology.

Dubey and Rosenthal (2023) indicate a connection between sarcocystosis and inflammatory processes of striated muscles called bovine eosinophilic myositis. Although myositis does not have clinical manifestations, it is detected after cutting the carcass. The sale of meat from an infected animal is impossible, which causes economic losses in agriculture.

Sivkova (2018) considers the sarcocyst invasion in moose, which is widespread in the Perm Territory, Russia. With a high invasion intensity, infected animals develop pathologies that lead to their death.

Thus, the spread of sarcocysts is dangerous for various animal species and humans. Moreover, sarcocystosis is a serious problem for agriculture due to the loss of cattle productivity and health. Studying the disease and its pathogens will make it possible to more accurately assess the scale, determine the conditions and places of its spread, and develop further recommendations for its localization and prevention. Identifying sarcocyst species specific to certain territories will provide an opportunity to effectively control the disease caused by them, leveling potential economic and biological damage.

In Kazakhstan, information on sarcocystosis in animals has not been updated since the 80s (Levchenko 1962; Eshtokina and Pak 1982; Khan 1985; Popov 1988), and only in 2008, sarcocystosis in sheep was described in West Kazakhstan (Kereev et al. 2008).

In general, there is currently no information about this invasion in Kazakhstan. Our study aimed to evaluate the prevalence of bovine sarcocystosis in the Kostanay region, Kazakhstan.

MATERIALS AND METHODS

The research was conducted according to the requirements and ethical principles for animal experimentation in European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes presented by Council of Europe in 1987.

The study material consisted of samples of bovine muscle tissue taken at slaughterhouses in four districts of the Kostanay region (Karasu, Denisov, Uzunkol, and Nauyrzym) from October 2022 to April 2023. The Kostanay region is located in the north of Kazakhstan and borders five regions of the country (Aktobe, Ulytau, Karaganda, Akmola, and North Kazakhstan) and three regions of the Russian Federation (Orenburg, Chelyabinsk, and Kurgan) (Fig. 1).

An initial visual inspection of the samples was carried out, followed by histological and microscopic studies using compressor microscopy. The sarcocysts found in the samples were subjected to molecular genetic analysis.

Fig. 1: Map of Kazakhstan showing Kostanay region – Study area (Source: Courtesy of Ontheworldmao.com).

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Animal carcasses were examined, and three samples of muscle tissue from different parts of each carcass were selected: the neck, diaphragm peduncles, and skeletal muscles, weighing no more than 50g. A total of 987 muscle tissue sections were collected (338 from the neck, 329 from the diaphragm, and 320 from the skeleton) from 358 cattle carcasses. 172 belonged to bulls, 186 to cows.

Thin sections were prepared from selected pieces of muscle tissue for compressor microscopy (Federal Service for Supervision in the Sphere of Consumer Rights Protection and Human Welfare, 2011). From the muscle tissue samples, 14 sections (28 sections from the sample) the size of an oat grain were cut along the muscle fibers with curved eye scissors. Next, the muscle parts were put on a synthetic mesh and soaked in a 0.2% aqueous solution of methylene blue for 20-30 minutes. After staining, the pieces of muscle with the synthetic mesh were placed on filter paper to drain and remove excess staining agent. The samples were kept on filter paper for only a few seconds. Then the stained muscle pieces were immersed in a 1.5% acetic acid solution for 15-20 minutes to lighten the samples. To avoid gluing, light mixing was carried out. Next, the samples were placed again on filter paper for drainage and then transferred to a glass compressor consisting of two glass plates tightened with screws.

Muscle tissue samples stained with methylene blue were scanned using a light microscope at magnifications x40, x100, and x400 and photographed using a Vision camera. According to the criteria for assessing the intensity, the invasion was conditionally classified as significant (over 200 sarcocysts in 28 sections), medium (51-200 sarcocysts), or slight (up to 50 sarcocysts) (Nikonorova 2020).

For further molecular genetic identification of the detected sarcocysts, 57 parasites were taken from the neck muscles. The samples were selected randomly, considering the extent of invasion in the sample collection areas.

Using dissecting needles under a microscope, native sarcocyst isolates free of muscle fibers were obtained. The DNA of single sarcocysts was isolated using the PureLink Genomic DNA Kits (Thermo Fisher Scientific, USA) following the manufacturer's protocol.

For species identification, a fragment of the cox1 gene was sequenced using SF1 ATGGCGTACAACAATCATAAAGAA and SR9 ATATCCATACCRCCATTGCCCAT primers (Gjerde 2013, 2014; Metwally et al. 2019). The polymerase chain reaction (PCR) was carried out in a total volume of 30µl, including 10mM tris HCl pH 8.5, 50mM KCl, 200 nmol of each primer, 2.5mM $MgCl_2$, 200µm of deoxyribonucleotides, 2 units of DNA polymerase HotStrart Taq (Sintol, Russia), and 7µL of DNA. Amplifications were carried out in the Eppendorf Master Cycler gradient thermal cycler (Germany). The PCR program included the initial stage of denaturation at 95°C for 5min, followed by 35 amplification cycles at 95°C for 30s, 57°C for 40s, and 72°C for 1min, as well as the final stage of extension at 72°C for 5min. The PCR products were visualized after separation on 2% agarose gel and staining with ethidium bromide.

The purification of the PCR products was carried out with Agencourt AMPure XP (Beckman Coulter) magnetic particles. Sequencing was performed using the BigDye Terminator v3.1 kit (Thermo Fisher Scientific, USA), following the manufacturer's instructions. The samples were separated using an Applied Biosystems 3500 genetic analyzer (Thermo Fisher Scientific, USA). The resulting gene sequences were analyzed using Unipro UGENE and compared with the sequences of closely related organisms deposited in GenBank using the BLAST tool.

The history of evolution was reconstructed using the neighbor-joining method (Saitou and Nei 1987). The optimal tree graph was shown. The percentage of reproduction of tree graphs in which the corresponding taxa were grouped in the bootstrap test (1,000 replicas) was shown next to the branches (Felsenstein 1985). The tree graph was drawn at scale, with branch lengths in the same units as the evolutionary distances used to reconstruct the phylogenetic tree. The evolutionary distances were calculated using the Tamura method with three parameters (Tamura 1992) and expressed in units of the number of base replacements per zone. The velocity variation between the zones was modeled using a gamma distribution (shape parameter = 5). This analysis included 130 nucleotide sequences. Codon positions included 1st+2nd+3rd+non-coding. All positions with less than 5% coverage were excluded, that is, less than 95% of missing data, ambiguous bases, and alignment gaps in any position were resolved (the partial deletion option). The final data set after sequencing and removal of the end poorly read fragments consisted of 769 nucleotide positions. Evolutionary analyses were carried out in the MEGA X software (Kumar et al. 2018).

To assess the differences in Sarcocystis infection rates between regions and between types of animals, Chi-Square test was performed.

RESULTS AND DISCUSSION

During the visual examination, we did not detect pathological changes (exhaustion, hydremia, discoloration, calcification of muscle tissue, degenerative changes) and macrocysts formed by parasites in the muscle samples. However, microscopic studies showed that most neck, diaphragm, and skeletal muscle samples contained *Sarcocystis* tissue cysts. This finding aligns with Rubiola et al. (2024), who reported a high prevalence of Sarcocystis species in cattle carcasses affected by bovine eosinophilic myositis in Northern Italy. Their study identified several Sarcocystis species, including S. hominis, S. bovifelis, and S. cruzi, in both intralesional and extralesional tissues, indicating the widespread nature of these parasites in bovine muscle tissues. The association of S. hominis and S. bovifelis with eosinophilic myositis suggests a significant pathogenic role of these species, which may also contribute to the high infection rates observed in our study.

Against it, Jauregui et al. (2024) investigated the prevalence and distribution of Sarcocystis spp. in cattle from the Peruvian tropical Andes and found an exceptionally high prevalence of 96.2% using direct microscopy and 100% using histopathology. Their findings highlighted the esophagus as having the highest prevalence of Sarcocystis, emphasizing the need for comprehensive screening across various muscle types. This complements our study's results, where extensive infection was noted in neck, diaphragm, and skeletal muscles, underscoring the necessity for thorough monitoring and control measures in diverse anatomical regions.

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Table 1: Infection with *Sarcocystis* in the cattle muscles by region

Districts	Type of animal	Place of sample collection					
		Neck muscles (n=338)		Diaphragm muscles (n=329)		Skeletal muscles (n=320)	
		No. of animals	No. infected (%)	No. of animals	No. infected (%)	No. of animals	No. Infected (%)
Uzunkol	bulls	53	28 (52.8)	38	24 (63.2)	37	24 (64.7)
Nauyrzym	bulls	62	37 (59.7)	59	36 (61.0)	52	30 (57.7)
Karasu	bulls	57	41 (71.9)	46	28 (60.9)	45	28 (62.2)
Denisov	cows	186	166 (89.3)	186	171 (91.9)	186	176 (94.6)
Chi-Square Value		18.21		7.25		7.91	

For neck muscles: P<0.0004 indicated a significant difference in infection rates between regions; For diaphragm muscles: P<0.064 suggested no significant difference in infection rates between regions; For skeletal muscles: P<0.048 indicated a significant difference in infection rates between regions.

Table 2: The extent of cattle invasion.

Type of animal	Age (years)	Number of examined animals	Number of infected animals	Percentage of infection (%)
Bulls	2-3	172	106	61.6
Cows	7-9	186	171	91.9
	Total	358	277	77.4

Chi-Square value=6.0; df=1; P<0.0001.

According to Table 1, the neck muscles in bulls from the Karasu district and the skeleton and diaphragm muscles in cows from the Denisov district were severely infected. The lowest infection rate was noted in the neck muscles in bulls from the Uzunkol district and the skeletal muscles in bulls from the Nauyrzym district.

The lowest infection rate was noted in the neck muscles in bulls from the Uzunkol district and the skeletal muscles in bulls from the Nauyrzym district. This observation aligns with the findings of Marandykina-Prakienė et al. (2023), who also reported variability in infection rates across different muscle types and regions. Their study on Sarcocystis species richness in sheep and goats from Lithuania found significant differences in the prevalence of Sarcocystis spp. in various muscle tissues, highlighting the complexity of infection patterns.

Marandykina-Prakienė et al. (2023) noted that the presence of non-canonical Sarcocystis species in sheep and goats suggests a broader host range than previously understood, which could contribute to varying infection rates in different anatomical regions.

The findings emphasize the importance of comprehensive molecular techniques for detecting Sarcocystis species. Marandykina-Prakienė et al. (2023) used cox1 sequence analysis to uncover unexpected host-specificity patterns, identifying species such as S. capracanis in sheep and S. arieticanis in goats. These insights into host-parasite relationships can inform our understanding of the lower infection rates observed in the Uzunkol and Nauyrzym districts, where specific environmental or genetic factors might limit the proliferation of Sarcocystis cysts in particular muscle groups.

The data in Table 2 indicate that of the 358 examined carcasses of bulls and cows, sarcocysts were found in 277 carcasses. The prevalence of invasion in bulls and cows equaled 77.4%. Of the studied 172 bull carcasses, 106 (61.6%) were infected. Of the studied 186 cow carcasses, 171 (91.9%) were infected.

In the neck muscles, skeletal muscles, and diaphragm peduncles, the bulk of *Sarcocystis* cysts had elongated-oblong, fusiform, and oval shapes with sharp and rounded ends. This align to the study by Portella et al. (2021), which examined the macroscopic, histological, and molecular aspects of Sarcocystis spp. infection in tissues of cattle and sheep. In their study, sheep tissues exhibited grossly visible white round to oval tissue cysts, whereas cattle tissues did not present grossly visible cysts but had white-yellow foci with irregular contours. These differences in macroscopic presentation align with our observation of variable infection rates across different muscle groups and regions. Histologically, they found circular to elongated, encapsulated, basophilic structures in the skeletal muscle fibers of sheep and inside cardiomyocytes in cattle. Our study's finding of lower infection rates in specific muscle groups, such as the neck muscles in bulls from Uzunkol and the skeletal muscles in bulls from Nauyrzym, might suggest a differential susceptibility or exposure to Sarcocystis infection in these muscle tissues. The cysts found in the fibers of the diaphragm muscles mostly had long sharp ends. Individual specimens of cysts in the neck muscles, diaphragm peduncles, and skeletal muscles had a fusiform shape and did not differ significantly in size. The length of the detected sarcocysts in cattle ranged from 0.5 to 0.7mm, and the width from 0.2 to 0.3mm.

Molecular Genetic Research

Of the obtained 769 nucleotides, 38 sequences (PP128380_Denis_1, PP128381_Denis_10, PP128383_Denis_12, PP128384_Denis_13, PP128385_Denis_14, PP128388_Denis_18, PP128389_Denis_19, PP128390_Denis_2, PP238391_Denis_20, PP128393_Denis_23, PP128394_Denis_24, PP128396_Denis_3, PP128398_Denis_5, PP128399_Denis_6, PP128402_Karasu_1, PP128404_Karasu_13, PP128405_Karasu_14, PP128406_Karasu_15, PP128407_Karasu_17, PP128409_Karasu_20, PP128410_Karasu_22, PP128411_Karasu_3, PP128412_Karasu_4, PP128413_Karasu_5, PP128415_Karasu_7, PP128416_Karasu_8, PP128417_Karasu_9, PP128419_Nauruz_12, PP128421_Nauruz_17, PP128422_Nauruz_18, PP128424_Nauruz_25, PP128429_Uzunk_12, PP128430_Uzunk_19, PP128431_Uzunk_20, PP128432_Uzunk_24, PP128433_Uzunk_25, PP128434_Uzunk_26, PP128436_Uzunk_9) clustered with the sequence KC209599 *S. cruzi.* These sequences formed

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33 genotypes (Fig. S1) with a divergence of up to 1.3% (10 nucleotides) (sample PP128431_Uzunk_20) from the sequence KC209599 *S. cruzi*. Most of the identified polymorphisms did not lead to amino acid substitution, only one missense mutation was found in the samples (PP128384_Denis_13, PP128388_Denis_18, PP128433_Uzunk_25, and PP128415_Karasu_7) and three missense mutations were detected in PP128431_Uzunk_20.

15 sequences (PP128382_Denis_11, PP128386_Denis_15, PP128387_Denis_17, PP128395_Denis_25, PP128397_Denis_4, PP128401_Denis_9, PP128408_Karasu_2, PP128418_Nauruz_11, PP128420_Nauruz_13, PP128423_Nauruz_24, PP128425_Nauruz_6, PP128426_Nauruz_7, PP128427_Nauruz_8, PP128428_Nauruz_9, and PP128435_Uzunk_6) clustered with KT900996 *S. bovifelis* and formed 12 genotypes (Fig. S1). The maximum divergence of the obtained sequences with the sequence KT900996 *S. bovifelis* (Table S1) equaled 0.9% (seven nucleotides). Samples PP128386_Denis_15, PP128387_Denis_17, PP128395_Denis_25, PP128428_Nauruz_9, PP128418_Nauruz_11, PP128423_Nauruz_24 and PP128435_Uzunk_6 each had one amino acid substitution.

Four sequences (PP128392_Denis_22, PP128400_Denis_8, PP128403_Karasu_12, PP128414_Karasu_6) clustered with KY711377 *S. dehongensis* and formed three genotypes (Fig. S1). The maximum divergence of the obtained sequences with the sequence KY711377 *S. dehongensis* (Table S2) equaled 0.3% (two nucleotides). One amino acid substitution was found in samples PP128392_Denis_22 and PP128400_Denis_8.

When analyzing the data, we found a high prevalence of sarcocyst invasion in all areas of the region. Our results are close to those of similar studies conducted in 2019 in Lithuania, where the extent of invasion ranged from 44.9 to 98.1%, and cysts were localized in six different types of cattle muscles. In Brazil, the extent of invasion ranged from 70 to 94%; in Italy, Sarcosporidia cysts were found in 78.1% of animals and were most often found in the esophagus; in the Netherlands, cysts were found in 82.7% of animals (Pena et al. 2001; Domenis et al. 2011; Hoeve-Bakker et al. 2019; Januškevičius et al. 2019).

Molecular genetic analysis showed the presence of three types of sarcocyst pathogens in the muscles of cattle belonging to farms in the Kostanay region: *S. cruzi, S. bovifelis,* and *S. dehongensis*. The latter infests the Asian buffalo, living mainly in China, India, and other countries (Pena et al. 2001; Domenis et al. 2011; Chen et al. 2017; Hoeve-Bakker et al. 2019). *S. cruzi* has a higher divergence; according to many data, it is the most ancient and widespread parasite and has the highest level of genetic polymorphism in a fragment of the cox1 gene, which is confirmed by our study and implies its long-term circulation in the Kostanay region. According to our results, the most common species was *S. cruzi* (62 samples, 69.7%). The number of samples with *S. bovifelis* was 20 (22.5%), and *S. dehongensis* was found in seven samples (7.9%). The obtained data correspond to conclusions by other researchers, with most studies noting the highest prevalence of *S. cruzi* in cattle (Prakas et al. 2020).

Table S1: The divergence of the obtained sequences with the sequence KT900996 *S. bovifelis*

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
KT900996_Sarcocystis_bovifelis																
PP128382_Denis_11	0.003															
PP128386_Denis_15	0.004	0.004														
PP128387_Denis_17	0.004	0.004	0.000													
PP128395_Denis_25	0.004	0.004	0.000	0.000												
PP128397_Denis_4	0.003	0.003	0.004	0.004	0.004											
PP128401_Denis_9	0.003	0	0.004	0.004	0.004	0.003										
PP128408_Karasu_2	0.001	0.001	0.003	0.003	0.003	0.001	0.001									
PP128418_Nauruz_11	0.009	0.009	0.008	0.008	0.008	0.009	0.009	0.008								
PP128420_Nauruz_13	0.001	0.001	0.003	0.003	0.003	0.001	0.001	0.000	0.008							
PP128423_Nauruz_24	0.003	0.003	0.004	0.004	0.004	0.003	0.003	0.001	0.009	0.001						
PP128425_Nauruz_6	0.004	0.004	0.005	0.005	0.005	0.004	0.004	0.003	0.009	0.003	0.004					
PP128426_Nauruz_7	0.004	0.004	0.005	0.005	0.005	0.004	0.004	0.003	0.011	0.003	0.004	0.005				
PP128427_Nauruz_8	0.001	0.001	0.003	0.003	0.003	0.001	0.001	0.000	0.008	0.000	0.001	0.003	0.003			
PP128428_Nauruz_9	0.009	0.009	0.011	0.011	0.011	0.009	0.009	0.008	0.005	0.008	0.009	0.009	0.011	0.008		
PP128435_Uzunk_6	0.003	0.003	0.004	0.004	0.004	0.003	0.003	0.001	0.009	0.001	0.003	0.004	0.004	0.001	0.009	

A = KT900996_Sarcocystis_bovifelis; B= PP128382_Denis_11; C = PP128386_Denis_15; D = PP128387_Denis_17; E = PP128395_Denis_25; F = PP128397_Denis_4; G = PP128401_Denis_9; H = PP128408_Karasu_2; I = PP128418_Nauruz_11; J = PP128420_Nauruz_13; K = PP128423_Nauruz_24; L = PP128425_Nauruz_6; M = PP128426_Nauruz_7; N = PP128427_Nauruz_8; O = PP128428_Nauruz_9 P = PP128435_Uzunk_6

Table S2: The divergence of the obtained sequences with the sequence KY711377 *S. dehongensis.*

	KY711377_Sarcocystis_dehongensis	PP128392_Denis_22	PP128400_Denis_8	PP128403_Karasu_12	PP128414_Karasu_6
KY711377_Sarcocystis_dehongensis					
PP128392_Denis_22	0.001				
PP128400_Denis_8	0.003	0.004			
PP128403_Karasu_12	0.001	0.003	0.001		
PP128414_Karasu_6	0.001	0.003	0.001	0	

Conclusion

Based on the conducted molecular diagnostic research, three types of sarcocysts were first identified in cattle muscles: *S. cruzi* with 62 samples (69.7%), *S. bovifelis* with 20 samples (22.5%), and *S. dehongensis* with seven samples (7.9%). The sequencing of the cox1 gene showed applicability for the identification of sarcocyst species. Phylogenetic analysis using the neighbor-joining method showed that the isolates obtained are related to Sarcocystis samples from New Zealand, Lithuania, Poland, Germany, Argentina, and China, which have conducted similar research in this area. Additionally, the sequencing results of the cox1 gene demonstrated its applicability for identifying different varieties of Sarcocystis.

Acknowledgment

This paper is part of a doctoral dissertation on veterinary parasitology within the framework of the grant funding with the individual registration number (IRN) AP14869992 "Monitoring the spread of sarcocystosis in domestic animals in the context of food safety". We would like to express our gratitude to the management and veterinarians of the slaughterhouses for their help and assistance in examining carcasses and collecting samples for scientific research.

Authors' Contribution

ZhB, KS, BB – coordination of material collection and work in the field; PP, OT, BM – coordination of manuscript preparation; ASh, BA, YS – DNA extraction and sequencing; KS, BB, RR – data processing and analysis and manuscript preparation; RR – project supervision. All authors have read and agreed to the published version of the manuscript.

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