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First molecular characterisation of *Sarcocystis miescheriana* in a pig carcass condemned during routine meat inspection in Poland

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Abstract

Introduction: This article presents the fourth detection of macroscopic cystic lesions due to sarcocystosis in domestic pigs during routine meat inspection worldwide, and the first molecular detection of *Sarcocystis miescheriana* in a domestic pig in Poland. Pigs can become intermediate hosts for *S. miescheriana* by accidental ingestion of oocysts or sporocysts present in food or water contaminated by the faeces of canids (definitive hosts). **Material and Methods:** The affected swine showed no clinical symptoms such as weight loss, dermatitis or dyspnoea suggesting sarcocystosis. The presence of grossly visible cyst-like lesions was noticed by veterinary inspectors during post-mortem meat inspection of pig carcasses at a slaughterhouse located in central Poland. Ten rice-grain-shaped white lesions were isolated from the muscle tissue for molecular analysis, and four other macroscopic cyst-like lesions were also isolated for histopathological and microscopy analysis. The molecular characterisation included amplification and sequencing of the cytochrome C oxidase subunit 1 mitochondrial gene. **Results:** The cyst-like structures were whitish, calcified, 1 cm long and 3 mm wide. The presence of S. *miescheriana* DNA was confirmed in all ten grossly visible cyst-like lesions. **Conclusion:** This study shows that *Sarcocystis* spp. may be present in swine muscle tissue and cause lesions leading to carcass discard. Further analyses are needed to fully recognise the prevalence and impact of *Sarcocystis* spp. on animal and human health, especially taking into account the possible presence of the zoonotic *S. suihominis*.

Keywords: *Sarcocystis miescheriana*, pig, cyst-like lesions, *cox1* mtDNA gene, meat inspection.

Introduction

Sarcocystis spp. (Apicomplexa: Sarcocystidae) are intracellular protozoa parasites with an obligatory prey–predator life cycle (10). Domestic and wild pigs may act as intermediate hosts for two species of *Sarcocystis*. These are *S. miescheriana* (syn. *S. suicanis*) and *S. suihominis* (22). Canids such as dogs, foxes, wolves, jackals and raccoon dogs, and probably also raccoons (Procyonidae family), can act as definitive hosts for *S. miescheriana*, whereas humans are definitive hosts for the zoonotic *S. suihominis* (15, 20, 27). The existence of a third species named *S. porcifelis* has been hypothesised, and it is assumed that this species is transmitted *via* cats; however, its existence has not been confirmed (1, 15). The intermediate hosts of *S. miescheriana* and *S. suihominis* become infected by ingesting sporulated oocysts or sporocysts present in food or water, while the definitive hosts can be infected by eating meat containing mature sarcocysts (10).

Generally, *Sarcocystis* spp. are more often detected in wild boars than in domestic pigs (15). However, the greater frequency of detection may simply be because studies on the prevalence of *Sarcocystis* spp. have been undertaken more often in wild boars than in domestic swine. Most of the data on sarcocystosis in domestic pigs

are outdated and do not include information about *Sarcocystis* species differentiation. According to some literature, the prevalence of *Sarcocystis* spp. in domestic pigs ranges from 3% to 43% (11, 15, 30), while recent data from India indicate the presence of *Sarcocystis* spp. in 82% of examined samples from this species. This might be explained by the low-input nature of the production systems raising pigs in India, where they are highly exposed to contact with different types of parasites including *Sarcocystis* spp. (6).

The prevalence of sarcocystosis in wild boars has been studied in recent years in Romania, Spain, Portugal, Latvia and Italy, and the results indicated prevalences of *Sarcocystis* spp. of 60.4%, 72.7%, 73.8%, 87.1% and 97%, respectively (4, 10, 24, 29, 31).

Both *S. miescheriana* and *S. suihominis* can be pathogenic for pigs. Nevertheless, data on natural sarcocystosis in pigs are limited. A case of fatal sarcocystosis in a domestic pig was described by Caspari *et al.* (5), who proved the occurrence of this disease in a large white boar from a breeding stock of 88 animals on an indoor farm in Switzerland. The swine had symptoms of anorexia and reduced general condition and fever (40°C), and, despite an initial clinical improvement after pharmacological treatment, it eventually died of myocarditis. Histopathological and molecular investigations revealed the presence of *S. miescheriana* tachyzoites within lesions. Apart from the case mentioned above, the presence of *S. miescheriana* in domestic pigs has only been molecularly confirmed in China, India and Italy (25, 32, 35); cases of sarcocystosis leading to the development of grossly visible cyst-like lesions in domestic pigs resulting in carcass condemnation have recently been described in Italy, Nigeria and the West Indies (32, 28, 9), while a single case of gross lesions associated with *S. miecheriana* leading to carcass discard has been documented in a wild boar in Portugal (34). Natural *Sarcocystis* spp. infections in pigs are usually subclinical, possibly because of natural immunisation by low doses of *Sarcocystis* spp. oocysts and sporocysts, which might be found in the environment of free-ranging pigs (5). Experimental infections of domestic pigs have also shown that ingestion of sporocysts of either *S. miescheriana* or *S. suihominis* in low amounts results in subclinical infections (2, 5). In cases of the intake of large amount of sporocysts, symptoms such as weight loss, dermatitis, dyspnoea, skin purpura, muscle tremors, miscarriages and even death may occur (4, 15). Mild infections are usually asymptomatic and associated with low weight and poor body condition (12, 16). Nevertheless, sarcocystosis in domestic pigs may result in economic losses for farmers because of reduced carcass value and their potential condemnation at slaughterhouses.

Sarcocystosis is not notifiable within the EU; therefore, there are no specific regulations for the control or prevention of this disease (17, 18). European Union legislation has established specific requirements for the inspection and control of meat at various stages of the production process, including visual inspection of carcasses, inspection of organs and removal of parts affected by parasitic infestations or other diseases. Cysts of *Sarcocystis* spp. are not usually identified during meat inspection because most often they are microscopic in size. In the absence of regulations on the handling of meat containing *Sarcocystis* spp. cysts, the general provisions of European Commission Regulation (EC) 627/2019, stating that meat intended for human consumption must not contain parasites, should be applied. Therefore, if cysts or lesions suggesting the presence of *Sarcocystis* spp. are observed, the meat should not be passed for human consumption (17).

This study reports the first molecular detection of *S. miescheriana* in a pig carcass condemned during routine veterinary inspection in an abattoir in Poland.

Material and Methods

In September 2019, the veterinary inspectors responsible for post-mortem meat inspection of pig carcasses at a slaughterhouse in Opoczno (located in the central part of Poland) observed the presence of whitish rice-grain-shaped cyst-like lesions in a swine carcass. The lesions were located in the diaphragm, heart, shoulders, back and intercostal muscles. The affected animal showed no clinical symptoms of parasitic disease. The carcass was condemned, and samples of the infected muscle tissues were excised, stored at −20°C and sent to the Department of Parasitology and Invasive Diseases of the National Veterinary Research Institute (Puławy, Poland).

Gross examination. Following the procedure described by Rubiola *et al.* (32), a gross examination of the affected tissue was performed. Next, ten white rice-grain-shaped lesions were isolated from the muscle tissue and frozen at −20°C for further molecular analysis, and four other macroscopic cyst-like lesions were processed by histopathology and microscopy as follows.

Histological examination. The muscle tissue samples containing macroscopic cysts were isolated, transferred into plastic containers and frozen at −80°C. Afterwards, samples were fixed in 10% neutral buffer formalin (Alpinus Chemia, Solec Kujawski, Poland). The fixed tissues were then rinsed in tap water and processed with a series of reagents: anhydrous ethyl alcohol (Avantor Performance Materials, Gliwice, Poland) at concentrations increasing from 70% to 99,8%, xylene (Avantor Performance Materials) for clearing and paraffin as an infiltrant. They were then embedded in paraffin blocks (Elektro Med, Niepołomice, Poland) and subsequently cut using a rotary microtome into 3.5- and 4.0-µm-thick sections. The paraffin sections were automatically stained with GILL 2 haematoxylin (Thermo Scientific, Loughborough, UK) and eosin 1% solution (Chempur, Piekary Śląskie, Poland) and were mounted with Shandon Consul-Mount

medium (Thermo Scientific) for evaluating the lesions in basic histopathology. The histological samples were observed using Axio Lab.A1 and Axio Imager.M2 microscopes (Carl Zeiss, Jena, Germany). Photographs were recorded and analysed using ZEN 2.3 software (Carl Zeiss).

Molecular characterisation. Following the procedure described by Rubiola *et al.* (32), the ten cystic lesions previously isolated from the muscle tissue were subjected to DNA extraction. The DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). SF1 (21) and SR11 (20) primers targeting the cytochrome C subunit 1 mitochondrial gene (*cox1* mtDNA) were used to amplify an expected fragment of approximately 1,100 base pairs (bp) of *Sarcocystis* spp. as previously described by Rubiola *et al.* (32); concurrently, the collected samples were tested for the presence of *Taenia* spp. DNA applying the PCR protocol targeting the *cox1* mtDNA gene described by Bowles *et al.* (3). Each PCR run included negative controls (reagent blanks and DNA extracted from negative wild boar samples) and a positive control (*S. miescheriana* DNA isolated from positive wild boar muscles and *Taenia saginata* DNA isolated from cysts of *Cysticercus bovis* in the Department of Veterinary Sciences of Turin University, Italy).

The amplified PCR products were enzymatically purified with ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced on a SeqStudio Genetic Analyzer (Thermo Fisher Scientific Applied Biosystems, Foster City, CA, USA). The obtained sequences were manually assembled into consensus sequences using MEGA X and compared with other sequences deposited in GenBank using the NCBI basic local alignment search tool BLASTN in a sequence similarity search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (26).

The phylogenetic analysis was performed using the Neighbor-Joining method within MEGA X: 70 partial sequences from 57 taxa of the *cox1* mtDNA gene were aligned using the ClustalW algorithm, including the 10 sequences generated herein and 59 *Sarcocystis* spp. sequences recovered from GenBank. *Neospora caninum* was used as the outgroup species to root the tree. Tree reliability was assessed by the bootstrap method (1,000 replications).

Results

Morphological characterisation. The pathological changes found in the muscle tissue of the infected pig were grossly described as whitish, rice-grain-shaped, cyst-like structures. The length of ten of them was from 0.5 to 1.2 cm, and their diameters ranged from 2.7 to 3.1 mm (Fig. 1).

The two rice-shaped cystic structures subjected to histological examination were characterised by a necrotic central core surrounded by a dense lymphocytic inflammatory infiltrate, connective tissue fibres and oedema (Fig. 2).

Molecular characterisation. The amplification of the *cox1* mtDNA gene fragment revealed the presence of *Sarcocystis* spp. DNA in all ten randomly sampled cystic lesions, while all tested samples tested negative for the presence of *Taenia* spp. DNA. The 999–1074 bp sequences generated for the PCR products showed 94.49–99.61% identity with *Sarcocystis miescheriana* sequences submitted to GenBank (accession Nos OR859843–OR859862, OQ472068–OQ472077, MT070614–MT070635, MH404185–MH404227 and LC349977–LC349980) and 80.2% or lower identity with any other sequence retrieved from GenBank (the 80.2% identity was with *S. suihominis*). The phylogenic analysis based on *cox1* mtDNA sequences revealed the close relationship of the *S. miescheriana* sequences obtained in the present study with the *S. miescheriana* sequences generated from an Italian domestic pig affected by macroscopic sarcocystosis (accession Nos OQ472068–472077) and with *S. miescheriana* sequences obtained from Italian (accession No. MH404227.1) and Latvian (accession No. MT070629.1) wild boars (Fig. 3). Eight out of the ten *S. miescheriana* sequences obtained in this investigation showed 100% identity with each other, while two sequences had 1–10 nucleotide differences. The sequences generated in the present study were submitted in GenBank under accession Nos PP136039–PP136048.

Discussion

This study presents the fourth documented detection of macroscopic cystic lesions due to sarcocystosis in a domestic pig worldwide, and the first molecular detection of *S. miescheriana* in a domestic pig in Poland. Recently, the first report describing the presence of gross cyst-like lesions associated with the presence of *S. miescheriana* was published by Rubiola *et al.* (32), while a second report of macrocysts of *S. suihominis* was reported a few months ago in Nigeria (28). Pigs affected by sarcocystosis do not usually show clinical symptoms of the disease, and *S. miescheriana* cysts are usually microscopic; therefore, they are rarely noticed during routine meat inspection in slaughterhouses. Although reports of swine carcass condemnations due to sarcocystosis can be retrieved from outdated publications, the first molecularly confirmed case of sarcocystosis in swine resulting in grossly visible cystic lesions was only described in 2023 in Italy (15, 32). In the present study and in the previous first report, the macroscopic cystic lesions detected had similar appearances and sizes and represented only occasional findings; further studies are needed to investigate abnormal responses such as these of host tissues to *S. miescheriana* infections.

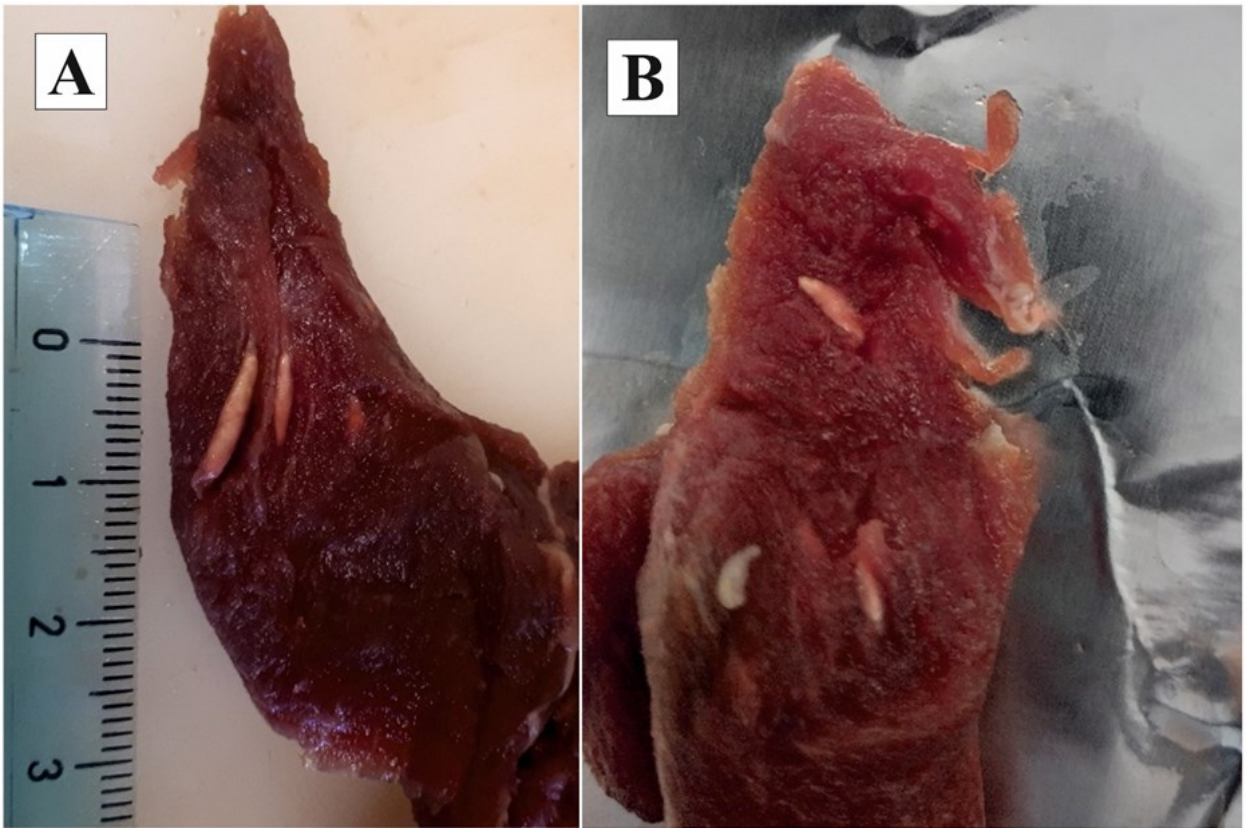

Fig. 1. Macroscopic cystic lesions observed in swine muscle tissue

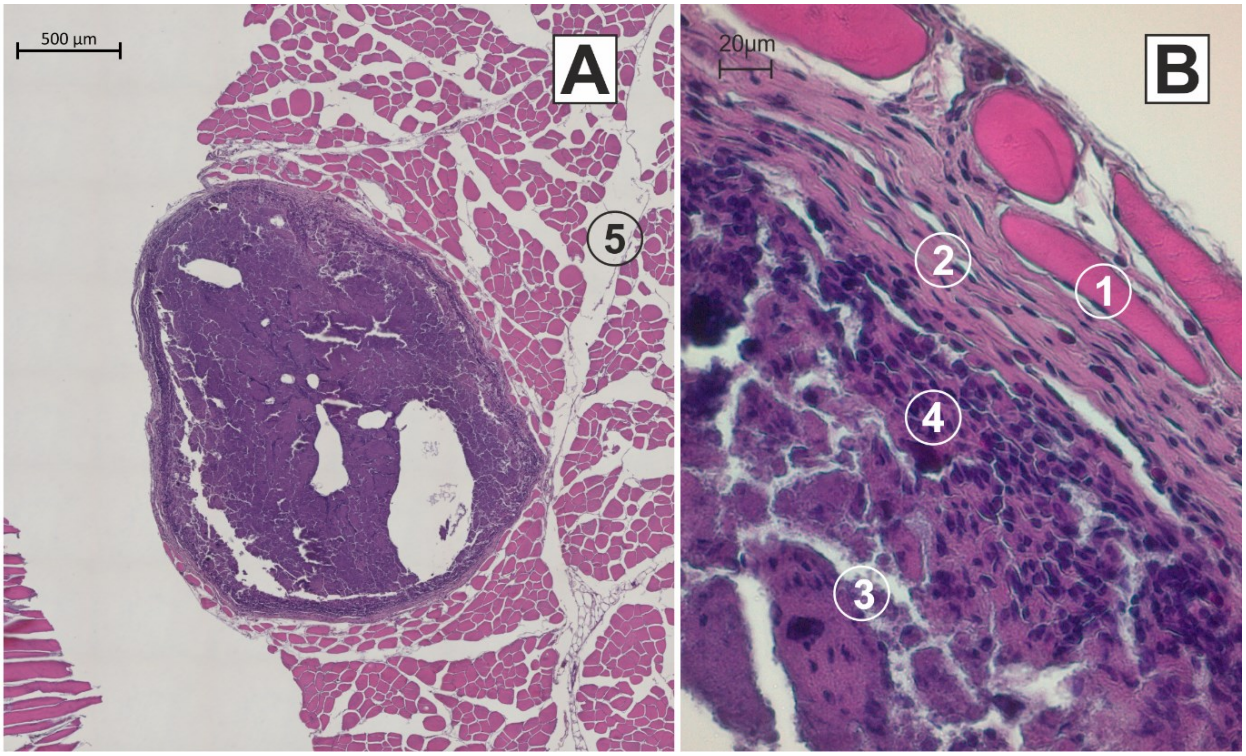

Fig. 2. Cross-sections of a calcified gross cystic lesion and the surrounding skeletal muscle tissue from a condemned pig carcass observed after staining with haematoxylin and eosin solution. Histopathological changes in the muscle tissue are shown. 1 – muscle fibres; 2 – connective tissue fibres forming a capsule around the cyst; 3 – necrotic lesions in the centre of the cyst; 4 – dense lymphocytic inflammatory infiltrate; 5 – oedema in the connective tissue of the muscle

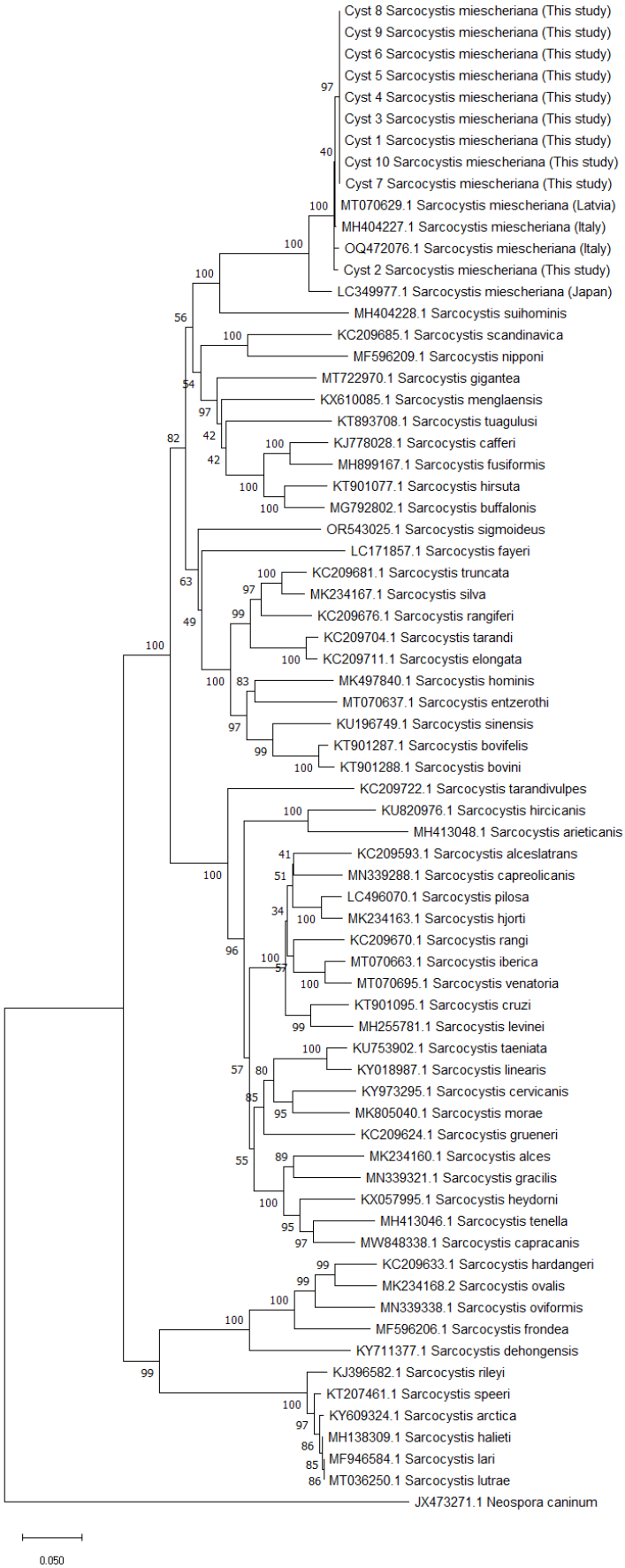

Fig. 3. Neighbor-Joining phylogenetic tree based on 70 partial sequences from 57 taxa of the *cox1* mtDNA gene, including 56 members of the Sarcocystidae family and a *Neospora caninum* sequence as outgroup. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches (1,000 replicates), together with the accession numbers of GenBank partial sequences

The identification of *Sarcocystis* spp. based on cyst morphological features is not recommended, because these features can vary through the different stages of cyst development and by their location, and may also be affected by the fixation methods used (10, 19, 33). The microscopic appearances of the cystic, granulomatous lesions containing *S. miescheriana* DNA were similar in this and in the Italian study. Light microscopy observations have revealed pathological, inflammatory changes around the cystic lesion, including a thick layer of host connective tissue; similar pathological changes have been previously observed in other studies (1, 14). While *S. miescheriana* DNA was detected in each cystic lesion, neither sarcocyst wall nor bradyzoites were observed in light microscopy. The most probable reason for the destruction of the wall and degradation of the bradyzoites was the long freezing of muscle tissue containing the gross lesions. According to Chen *et al.* (7), after prolonged freezing, the bradyzoite structure becomes indistinct and their arrangement becomes disordered.

Molecular methods have been increasingly applied in recent studies on sarcocystosis, allowing the rapid identification of *Sarcocystis* spp. In the present work, the phylogenic analysis based on the *cox1* mtDNA sequences revealed the close relationship of the *S. miescheriana* specimens detected in the present study and the *S. miescheriana* sequences obtained from pigs and wild boars in Italy and Latvia, while a lower similarity was recorded with *S. miescheriana* sequences obtained from wild boars originating from China (23, 31, 32). This observation supports the hypothesis that genetic diversity might be correlated with the geographical location (31).

In this case, as well as in the case described by Rubiola *et al.* (32), the source of the infection was unknown. However, taking into account the type of pig farming system typical in the region of Poland where the pig originated, it can be assumed that the pig was raised on a large farm with strict surveillance and hygiene measures. Therefore, one of the possible scenarios is that *S. miescheriana* could have been transferred to the barn with straw used as bedding material which was contaminated by dogs' or other definitive hosts' faeces (5). This is supported by the popularity of the deep bedding system in the area where the infected pig was raised.

S. miescheriana is not usually pathogenic for domestic pigs; nevertheless, infected animals may be underweight, have poor body condition (1, 5) and develop grossly visible cystic lesions (32). Therefore, this parasite can lead to a decrease in carcass value and even to condemnation of meat in extreme cases, such as the one reported here. As a result, *Sarcocystis* spp. infections can cause economic losses for farmers (13). Health effects in animals can also affect their welfare and quality of life. So far, there is only one report describing naturally acquired clinical sarcocystosis in pigs (5). However, the lack of such described cases is not a result of the absence of *Sarcocystis* spp. in pig breeding stock environments, because there are reports indicating the occurrence of this parasite in many countries, including European ones (11). The very low case count does not imply that the knowledge in this subject should not be updated and completed. Therefore, it is especially important to focus on the monitoring, control and prevention of *Sarcocystis* spp. infections (8).

Conclusion

This is the fourth worldwide report showing the presence of macroscopic cystic lesions associated with the presence of *Sarcocystis* spp. in domestic pigs. These reports were published recently, and hence indicate the growing interest in and awareness of the presence of *Sarcocystis* spp. in meat-producing animals among veterinary inspectors, which can notice the lesions and condemn the carcass. Nevertheless, usually the sarcocysts are microscopic in size and therefore are not identified during routine meat inspection. For this reason, further analyses are needed to expand our knowledge of the *Sarcocystis* spp. prevalence among meat-producing animals and to fully recognise their impact on animal and public health.

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Animal Rights Statement: No approval of research ethics committees was required because the samples used in this study were taken from a slaughtered pig.

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