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Short communication

Occurrence and first molecular characterization of *Sarcocystis* spp. in wild boars (*Sus scrofa*) and domestic pigs (*Sus scrofa domesticus*) in Romania: Public health significance of the isolates

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ABSTRACT

Domestic and wild pigs, as intermediate hosts, can harbor tissue cysts of three *Sarcocystis* species namely *S. miescheriana*, *S. suihominis* and *S. porcifelis*. Out of them, *S. suihominis* is zoonotic. Romania is a country with high consumption of raw and/or undercooked traditional pork products. This fact may greatly favor the acquiring of the zoonotic *Sarcocystis* infections by humans, as definitive host. Based on this consideration and in order to investigate the occurrence and public health significance of *Sarcocystis* spp. in two western counties (Caraş-Severin and Timiş) of Romania, a total of 165 heart samples from hunted wild boars (*Sus scrofa*, n = 101) and home slaughtered domestic pigs (*Sus scrofa domesticus*, n = 64) were screened using microscopic fresh examination and molecular methods. Microscopic examination revealed the presence of sarcocysts in 60.4% of wild boars, and 23.4% of domestic pigs. Genetic characterization of isolates through the PCR-RFLP procedure, targeting the 18S rRNA gene, was successfully achieved for all microscopically positive samples, indicating the presence of a single species, *S. miescheriana*, in both hosts. The identity of 13 selected *S. miescheriana* isolates was also confirmed through sequencing. The tested hosts older than 27 months were found to be significantly higher infected ($p < 0.05$) with *Sarcocystis* than the 6 to ≤27 months age group. Although the human infective *S. suihominis* has not been registered, for a more reliable epidemiological picture, further molecular studies enrolling a larger number of animals and diagnosis on human intestinal *Sarcocystis* infections are still necessary.

1. Introduction

The globally distributed species of the genus *Sarcocystis*, that cause sarcocystosis, are considered strict two-host life cycle intracellular coccidian (phylum Apicomplexa) parasites affecting a wide range of warm-blooded and poikilothermic animals, including humans. Within the life cycle of the parasite, definitive hosts (e.g. domestic and wild carnivores, humans, non-human primates) become infected by carnivorism, after ingestion of sarcocysts contained tissues of intermediate hosts (e.g. herbivores and omnivores) (reviewed by Dubey, 2015).

Wild boars and domestic pigs, as intermediate hosts, can acquire the parasite ingesting environmentally resistant mature oocysts shed by the definitive hosts, and can harbor tissue cysts of three species, namely *Sarcocystis miescheriana*, *S. suihominis* and *S. porcifelis*. Out of them, only *S. suihominis* is zoonotic and the taxonomic status of *S. porcifelis* is still unclear (Kia et al., 2011; Dubey, 2015).

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In naturally infected pigs with *Sarcocystis* fever, muscular disorders, and weight loss have been frequently recorded (Caspari et al., 2011; Kia et al., 2011). Human infections linked to the consumption of infected raw and/or undercooked meat and meat-derived products, during sporocyst excretion, are characterized most frequently by diarrhea and abdominal pain (Kaur et al., 2016). Usually, during the routine meat control by veterinary practitioners, which refers to the visual inspection and artificial digestion method in order to detect only *Trichinella* larvae, the presences of sarcocysts in the fresh meat go unobserved. Unlike the non-molecular methods used with research aim (e.g. cysts and/or merozoites isolation after pepsin digestion of muscle samples or histopatological and fresh examination of tissue samples), the current PCR assays, and sequencing procedures are considered reliable tools to differentiate the morphologically similar sarcocyst at the species level (Yan et al., 2013; Dubey, 2015).

The cultural habits of humans in Romania are strongly related to consumption of raw and/or undercooked traditional pork products (e.g. sausages and marinated loin) providing from home slaughtered backyard pigs or hunted wild boars in the winter season. In rural areas the meat obtained after slaughtering is mainly intended for familial consumption and controlled by designated veterinarians. Thus, there are numerous favorable conditions within parasite life cycle for possible linkage between intermediate and definitive host.

Considering the lack of any available scientific report, and in accordance with the EU Directive no. 2003/99/EC (European Commission, 2003; Taylor et al., 2010) requirement, which refers to the necessity of the monitoring of zoonotic agents in animals by each member states, such as those of the genus *Sarcocystis*, the present study aimed to investigate the occurrence and public health significance of *Sarcocystis* spp. in wild boar and home slaughtered backyard pigs from Western Romania.

2. Materials and methods

Between October 2015 and February 2016, a total of 165 entire heart samples, as predilection sites for cysts of *Sarcocystis* spp. (Malakauskas and Grikienienė 2002; Hvizdosová and Goldová, 2009; Calero-Bernal et al., 2015), from wild boars (*Sus scrofa*) ($n = 101$ samples) and domestic pigs (*Sus scrofa domesticus*) ($n = 64$) were collected in two counties (Caraş-Severin and Timiş) of Western Romania. In rural areas of the screened counties the backyard and free-ranging pigs are considered the most important meat providing livestock. Data providing from Sanitary Veterinary and Food Safety Directorates from county level indicate that yearly, around of 28,000 specimens are sacrificed for familial consumption. Also, the wild boar is the most preferred game species and annually over 2000 from a total of ~6400 wild animals (official data) are hunted and intended for human consumption. The enrolled wild boars were shot by hunters in 22 hunting grounds, within an organized framework by veterinary diagnostic authorities, according to the current legal regulations and during the yearly survey of the swine fever. After shooting the boars were eviscerated and the obtained carcasses were subjected to routine veterinary postmortem inspection and *Trichinella* detection. The game meat, found fit for human consumption, was divided within the hunting group members. Samples of the free ranging and backyard pigs from rural areas, home-slaughtered during winter season around Christmas and New Year celebrations, were kindly provided by the owners. It is important to mention that the adequate management of the slaughtered meat in the surveyed area is frequently hampered by the existence of poor hygienic conditions, associated with the lack of the proper slaughter facilities both households as well as field conditions.

During sampling, information about dates of hunting, location of hunting grounds, gender and age class of the wild boars based on the tooth eruption criteria (Cotta et al., 2001) were recorded. For domestic pigs, epidemiological data were obtained from owners, which confirmed the presence of definitive hosts (dogs and/or cats) in their backyards in all situations. Heart samples were stored at −20 °C until further analysis.

In the day of examination (from 3 to 27 days elapsed between sampling and processing) the samples were defrozed at room temperature. Muscle tissues (10 g) of the heart apex region from each animal were processed for detection of the presence of sarcocysts using microscopic fresh examination method as previously described by Moré et al. (2011). Between 5 and 15 individual sarcocysts or cysts fragments, recognized under an optical microscope at 50–100× magnification, were collected from each positive samples, placed in 1.5 ml microcentrifuge tubes containing 50 μl of distilled water, and stored at −20 °C until further molecular analysis.

Genomic DNA of the collected sarcocysts was extracted using the Isolate Genomic DNA kit (Bioline Reagents Limited®, London, U.K.) according to the manufacturer's instructions. The genus-specific polymerase chain reaction (PCR) was performed using amplification conditions as previously described by Yang et al. (2001), targeting the 18S ribosomal RNA gene (915 bp long section) and using the 2L forward (5′-GGATAAACCGTGGTAATTCTATG-3′) and 3H reverse (5′-GGCAAATGCTTTCGCAGTAG-3′) specific primer set. Amplifications included positive and negative controls. Subsequently, in order to reveal the polymorphism between *S. miescheriana* and *S. suihominis*, the PCR products were subjected to restriction fragment length polymorphism (RFLP) analysis using the conventional *Ssp*I (New England BioLabs®, Beverly, MA, USA) enzyme as described by Yang et al. (2002). Amplification and digested products were visualized on 2.2% agarose gel stained with MidoriGreen™ (Nippon Genetics®, Europe Gmbh). Thirteen selected samples, derived from domestic pigs ($n = 5$) and wild boars ($n = 8$) providing from various hunting grounds, showing strongest specific PCR bands were purified and sequenced in both directions (Macrogen Europe®, Amsterdam, the Netherlands) using the same primers. The obtained sequences were trimmed and aligned using Clustal X software and their similarity analysis with other Gen Bank™ database retrieved isolates was carried out using BLAST analysis (available online: http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic relationships of *Sarcocystis* spp. were constructed using Neighbour – Joining (NJ) algorithm implemented in MEGA 6.0 (Tamura et al., 2013) under the Tamura 3-parameter +G model of evolution. The reliability and robustness of the phylogenetic tree were tested by bootstrap analysis with 1000 replicates. Two representative *S. miescheriana* sequences were deposited into GenBank™ under accession numbers KX008292-KX008293.

The possible significant differences between recorded epidemiological data were examined using the nonparametric Pearson's Chi – square test (Excel 2007, Microsoft, Redmond, WA), and a probability [p] value ≤ 0.05 was considered as statistically significant.

3. Results

The study results are summarized in Table 1. Altogether a total of 61 out of 101 (60.4%) wild boars, and 15 out of 64 (23.4%) domestic pigs were tested *Sarcocystis* positive by microscopically fresh examination, meaning a significant difference ($p = 0.0001$) between the two host species. Under light microscope the thick-walled and ovoid elongated cysts (up to 1500 μm long and 200 μm wide; Dubey, 2015) appeared with serrated surface, showing numerous internal septa dividing the organs into compartments. Molecular characterization of *Sarcocystis* isolates through the PCR-RFLP procedure, targeting the 18S ribosomal RNA gene, was successfully