Molecular Identification and Prevalence of Sarcocystis Suihominis in Pork Meat Intended for Public Consumption

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Abstract
Protozoa of the genus Sarcocystis are among the most prevalent parasitic forms found in pork. In order to rapidly assess the risk that these protozoa produce in the human population, a molecular method to detect and identify Sarcocystis suisominis needs to be implemented. The study aimed at characterizing the prevalence of Sarcocystis suisominis in pork obtained in the traditional households through PCR – RFLP method. The material was represented by seventy nine samples of diaphragmatic pillars collected between the period October 2014 – December 2014 from two local sanitary veterinary units from Alba and Cluj. The samples were processed first by trichinelloscopic compression method. All the positive fragments for Sarcocystis spp. were examined through PCR-RFLP method. The method used in the present study utilized the AluI restriction enzyme and had successfully differentiated the Sarcocystis suisominis from other Sarcocystis spp. prevalent in pork meat. The inspection of compressed muscle tissue has detected the presence of Sarcocystis suisominis in a high percentage of investigated samples. The prevalence of Sarcocystis suisominis in the examined samples was 26.58%. According to this result we can affirm that the risk of contamination in humans with Sarcocystis suisominis is high through consumption of contaminated pork meat, raised in the traditional system. In the present scientific research, we demonstrated that this method used for molecular identification of Sarcocystis suisominis has high accuracy and can be successfully applied for obtaining a certain diagnostic.

Keywords: meat, PCR, risk, Sarcocystis, traditional

INTRODUCTION
Sarcocystosis is one of the most prevalent parasitic diseases among wild and domestic animals in the world (Prakas and Butkauas, 2012). Sarcocystosis is widespread globally and evolve in all animal species, being conditioned by the action of multiple factors: geo-climatic, socioeconomic, growing conditions, diet, medication, species and age (Constantin, 2014).
Sarcocystis parasites belong to the phylum Apicomplexa, class Sporozoasida, order Eimerio-rina, family Sarcocystidae and genus Sarcocystis (Dubey and Odening, 2001).
Sarcocystis species are intracellular protozoan parasites with a requisite two-host life cycle based on a prey-predator (intermediate-definitive) host relationship (Fayer, 2004).
Muscular cysts of various Sarcocystis spp. are found in a broad spectrum of intermediate hosts, such as mammals (74%), birds (14%) and reptiles (10%). Only 0.5% of the intermediate hosts are fish (Prakas and Butkauas, 2012).
Sarcocystosis is a zoonotic and parasitic disease commonly seen in domestic animals such as pigs. Among these, *Sarcocystis suihominis* is important in terms of public health, as meat and meat products are the main source of infection in human beings. Therefore, the infection in human is produced through the ingestion of well-developed tissue cysts containing bradyzoites (Juyal and Bhatia, 1989). The early signs and symptoms of the infection with *S. suihominis* acquired through the consumption of raw or undercooked infected pork meat include nausea, diarrhea and vomiting (Tenter, 1995).

The WHO reported (1981) that, about 50% of parasitic cysts found in muscles of cattle and pigs belong to the species *S. hominis* and *S. suihominis*, respectively (Acha and Szyfres, 2003). High prevalence of muscles sarcocystosis in pigs was confirmed by data from India, where investigators found that *S.suihominis* and *S. meischeriana* have been determined in 47% and 43% of pigs, respectively (Saleque and Bhatia, 1991).

In pigs, the muscular cysts are found most commonly in cardiac, diaphragmatic and esophageal muscles. Cysts are white and cylindrical, ranging in size from a few micrometers to a few centimeters and may or may not be visible to the naked eye, being similar with a grain of rice (Cătoi, 2003).

A variety of molecular methods have been developed and used to detect and identify *Sarcocystis* spp. in order to assess the genetic diversity among this protozoan from different hosts. Ribosomal DNA sequences are the most common molecular markers used in *Sarcocystis* spp. differentiation (Dahlgren and Gjerde, 2008; Rosenthal et al., 2008; Xiang et al., 2009).

Molecular techniques such as PCR and its variants are widely used to genetically determine diversity among many organisms and species and have been applied to many phylogenetic and taxonomic works (Güçlü et al., 2004).

Another important technique used in the diagnosis of *Sarcocystis* spp. is the RFLP technique (restriction fragment length polymorphism analysis). This method is based on the fragmentation of the nucleic acids obtained from the PCR reaction. In the RFLP analysis, nucleic acids are digested by the restriction enzyme. After digestion, it realizes a specific pattern of fragments of nucleic acids. Comparison between the fragments obtained define the variation of sequences (Stojecki, 2012).

Taking into account the fact that in Romania this disease is still a major concern of public health, further studies are needed to better identify and characterize new methods that could help at the identification of *Sarcocystissuihominis*. Therefore the aim of our study was to determine the prevalence of *Sarcocystissuihominis* in pork obtained in the traditional households through PCR – RFLP method.

MATERIALS AND METHODS

Sample collection

The material subjected to research was represented by seventy nine samples of diaphragmatic pillars isolated from pigs raised in the traditional system. The samples were collected between October 2014 and December 2014 from two local sanitary veterinary units from Alba and Cluj, located in the center of Transylvania. Meat samples had different sizes and were wrapped individually in plastic bags, separately for each animal. The samples were preserved by congelation, being transported for examination at the laboratory of «Inspection and food control of feed and animal origin products» in the Veterinary Medicine Faculty from Cluj – Napoca.

The collected sample were processed first by trichinelloscopic compression method, from each area mentioned above there were 28 tissue fragments displayed on the compressors’ blade, treated with clarifying solution (KOH 3%, NaOH 3%) for a better high lightening of the microsarcocysts. The samples were examined with the screen trichinelloscope, in order to establish the presence or the absence of the *Sarcocystis* cysts. All the positive fragments for *Sarcocystis* spp. were examined through PCR-RFLP method.

DNA extraction

DNA extraction was performed by using a specific extraction kit Genomic ISOLATE II (Bioline), utilized for extraction from animal and human tissue and cell culture.

The first stage consisted of weighing out 25 mg of muscle tissue from each sample and suspended in 180 μl of Lysis Buffer GL and 25 μl Proteaseine K solution in individual 1.5 ml microcentrifuge tubes and then vortexed. The samples were incubated at 56°C in a thermobloc for 3 hours and vortex every
30 minutes, to complete lysis of the samples. After incubation, the samples were vortexed again. In each sample was added 200 μl lysis solution (Lysis Buffer G3) and then vortexed. After this step, the samples were incubated at a temperature of 70°C for 10 minutes and after the time were vortexed again. 210 μl ethanol were added to each sample and then processed through a genomic DNA mini spin column containing a silica membrane to which the genomic DNA binds.

After washing the DNA, the „ISOLATE II Genomic DNA Spin Column – green“ were introduced in 2 ml tubes, then the sample was added and then centrifuged at 11,000 rpm for 60 seconds. The resulting supernatant was removed and the tubes were reutilized.

Contaminants and impurities such as salts, metabolites and cellular components are effectively removed by simple washing steps with two different buffers, adding first 500 μl Wash Buffer GW1 and then centrifuged at 11,000 rpm for 60 seconds and second adding 600 μl Wash Buffer GW2 while the same protocol for centrifugation was applied and the supernatant removed. After removing the liquid, the samples were again centrifuged for 1 minute at 11,000 rpm in order to remove any ethanol left inside.

For each sample, the „ISOLATE II Genomic DNA Spin Column“ was transferred in 1.5 ml Eppendorf tubes and added 100μl preheated Elution Buffer G, (70°C) was added directly onto the silica membrane. The sample was incubated at room temperature for 1 minute, then centrifuged for 1 minute at 11,000 rpm.

DNA samples resulting from the whole process were collected and stored in Eppendorf tubes.

**Detection and identification of Sarcocystis suisominis**

Detection and identification of Sarcocystis suisominis was performed using PCR-RFLP. The amplification of the target gene, 18srRNA gene was done using specific primers of sarFS‘-GGATAAC GCT GAT ATC AGT-3‘and sarR 5‘-GGC AAA TGC TTT CGC AGT AG-3’ (Rosenthal, 2009). Based on databases, the amplicon size of S. Suishominis is 900 pb.

In order to apply the PCR protocol, samples were processed as following: in 200 μl Eppendorf tubes were added 12.5 μl MasterMix, 1 μl Forward primer, 1 μl Reverse primer, 4 μl DNA and 6.5 μl pure water, with a final volume of 25 μl.

Amplification of samples was carried out by using a Thermocycler (G-STORM). This stage involves subjecting samples to different temperatures in order to reconstitute the amplified DNA fragment. The amplification program was done with an initial denaturation of 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 45 sec, annealing at 56°C for 45 sec and extension at 72°C for 45 sec with an elongation step of 5 min at 72 °C at the last cycle. The protocol ends by reaching a temperature of 4°C.

RFLP analysis was performed using the Alul restriction enzyme, specific for Sarcocystis suisominis. The reaction was carried out by adding 2.5 μl buffer solution, 5 μl amplified DNA, 16 μl pure water and 1.5 μl Alul restriction enzyme, with a total reaction volume of 25 μl. Before adding the enzyme, the reaction components were vortexed, the enzyme being the last one added. After addition of enzyme, the sample is homogenized by mild pipetting.

After preparing the samples according to protocol, they were introduced in thermocycler at a temperature of 37°C for 5 minutes. To stop the reaction of DNA fragments restriction, the samples were maintained for 20 minutes at a temperature of 65°C.

The electrophoretic profiles were carried out by the migration of the DNA samples in an agarose gel of 1.5% concentration. Reading and interpreting reaction was performed using the imaging system TransUV BioRad.

**Statistical interpretation**

For the interpretation of our results we used the OriginPro (Software version 8.5) and applied the ANOVA one-way and the least significant difference test. The Origin program is intended for plotting data and statistical analysis of biological experimentation with large or small data, integrating one or more variables.

**RESULTS AND DISCUSSION**

A total of 79 samples of pork meat were examined for the presence of microscopic sarcocysts.

After applying the compression method we have identified the presence of cysts of Sarcocystis spp. in the examined samples of diaphragm muscle
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In this study, of the total samples examined (n = 79), Sarcocystis spp. was molecular identified by PCR in 51.9% of cases.

In order to differentiate *Sarcocystis suihominis* from the other *Sarcocystis* subspecies found in pigs we applied the RFLP method. *Sarcocystis suihominis* was found in a proportion of 51.2% in the samples molecular identified, the remaining 48.8% representing other subspecies of *Sarcocystis* with specificity and pathogenicity for pigs, but non-pathogenic to humans.

After applying the entire protocol, the results have revealed the presence of *S. suihominis* in a high percentage of samples (21.26%).

Based on the EC Regulation 854/2004 on official controls on meat intended for human consumption, the presence of *Sarcocystis* in the carcass of slaughtered animals is not covered by routine meat inspection. However, the high prevalence rate of infection, the presence of *Sarcocystis* species considered potentially zoonotic and the growing habit, in European countries, to eat raw or undercooked meat, brought some authors to consider the genus *Sarcocystis* as a potential foodborne risk.
possible zoonotic risk for consumers (Bucca et al., 2010; Chiesa et al., 2013).

Intestinal sarcocystosis in humans is fairly widespread in Europe, with a prevalence ranging from 1.6% to 10.4% (Dubey et al., 1989). These results are based on microscopic examination of fecal samples, a technique that cannot distinguish between *S. suihominis* and *S. bovihominis*, both of them being pathogenic to humans.

The estimated prevalence of *Sarcocystis* in pigs in central Europe is approximately 35% for breeding animals and approximately 10% for fattening pigs (Daugschies, 2006). The results of a cross-sectional study on the seroprevalence in breeding sows, carried in the German federal state of Hesse indicated a percentage of 25% of the tested animals positive (Damriyasa et al., 2004). Species specification was not possible in this study because of cross-reactivity of the antigen used in the test, though it was suspected that *S. suihominis* is probably more common. Older studies, carried out between 1963 and 1974, reviewed in Heydorn (1977) and 1978 to 1994 (cited in Damriyasa et al., 2004) report infections of 2.8% - 60% in Germany, 1.1% - 95% in Poland, 7.4% - 32% in Austria, 25% in Hungary, 10.5% in Bulgaria, 1.8% - 3.5% in Denmark, 18% in Iowa (USA), and 16% in Japan. Where results were differentiated, the reported prevalence in older animals (sows and boars) was higher compared to fattening pigs. However, the differences and wide percentage ranges were not only considered the result of using various detection methods but also significantly influenced by differences in husbandry and hygiene factors (Taylor et al., 2010).

A study of literature before 2000 revealed a single report using PCR-RFLP technique to investigate the diversity of the genus *Sarcocystis*; this study involved isolating *S. gigantean* from two slaughterhouses (Jeffries et al., 1996), without there being a report subsequently published about the interspecific variation of the parasites of this kind. The prevalence of this parasite in meat has been declared in many countries (Ginawi and Shommein, 1977), but unfortunately a proper investigation in Romania has not yet been published. Clinical signs of human intestinal sarcocystosis are particularly digestive system disorders such as nausea, vomiting, diarrhea (Dubey et al., 1989), which appeared especially in immunocompromised patients (Velásquez et al., 2008). According to data released by Saleque et al. (1990), intestinal sarcocystosis can be prevented by thoroughly cooking or freezing meat to disable the action of bradyzoites from the sarcocysts. Sarcocysts in pig muscles became noninfectious for puppies after cooking meat at 60, 70, and 100°C for 20, 15, and 5 min, respectively. Freezing at -4°C and -20°C, for 48 and 24 h, respectively, also rendered bradyzoites in pork noninfectious (Fayer et al., 2004).

Visual inspection at the slaughterhouse will identify macroscopic lesions, not necessarily caused by the zoonotic species, but does not allow species differentiation. Species differentiation can be carried out using various methods (Taylor et al., 2010).

In the last decade, nearly all molecular approaches to the detection and diagnosis of species of *Sarcocystis* were based on the amplification and characterization of ribosomal
DNA. Such methods have differentiated among parasites localized in the tissues of various livestock (and wildlife) hosts, including those of swine and cattle. For *S. hominis* and *S. suihominis*, such molecular methods have been applied to the encysted forms found in beef and swine muscle.

Thus, *Sarcocystis hominis* and *S. suihominis* represent just two out of hundreds of species mentioned in the Sarcocystidae, which also includes parasites variously assigned to the genera *Toxoplasma*, *Besnoitia*, *Frenkelia*, *Hammondia*, *Neospora*, and *Sarcocystis*. For every study of *S. hominis* or *S. suihominis*, more than 100 have been published for *Toxoplasma magnagondii* owing to its especially clinical significance as a major threat to human reproductive health and a serious opportunistic pathogen of AIDS. This justifiably disproportionate research effort has resulted in a suite of experimental methods and assays, including tools of modern molecular genetics, immunological reagents, in vitro culture protocols, *in vivo* phenotyping, genome sequences, population genetics, expression analysis, and more.

Molecular methods can and should be employed to aid diagnosis of human infection with *S. hominis* and *S. suihominis*. Such methods, moreover, offer great potential in exploring the evolutionary origins and exposure routes of other human parasites whose identity and provenance remains enigmatic (Rosenthal, 2009).

Molecular techniques should become a routine, because they can be applied directly to identify certain species of *Sarcocystis*.

**CONCLUSIONS**

The results of this study demonstrate how the PCR-RFLP method, developed in this study, represents an effective and rapid method for the identification of *Sarcocystis suihominis* in pork meat. It is recommended that the consumption of pork meat and meat products to occur after an appropriate heat treatment to avoid the risk of contamination by eating pork produced in the traditional system, because from the survey conducted the *Sarcocystis suihominis* was detected in the analyzed samples in a percentage of 26.58%.

**REFERENCES**


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