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 suis hominis* needs to be implemented. The study aimed at characterizing the prevalence of *Sarcocystis suis hominis* 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 suis hominis* from other *Sarcocystis* spp. prevalent in pork meat. The inspection of compressed muscle tissue has detected the presence of *Sarcocystis suis hominis* in a high percentage of investigated samples. The prevalence of *Sarcocystis suis hominis* in the examined samples was 26.58%. According to this result we can affirm that the risk of contamination in humans with *Sarcocystis suis hominis* 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 suis hominis* 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 Butkausas, 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 Butkausas, 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 sarcocystiosis 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 *Sarcocystis suihominis*. Therefore the aim of our study was to determine the prevalence of *Sarcocystis suihominis* 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 absense 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 Proteinase 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 100 µl 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 Eppendorff tubes.

Detection and identification of *Sarcocystis suis hominis*

Detection and identification of *Sarcocystis suis hominis* was performed using PCR-RFLP. The amplification of the target gene, *18srRNA* gene was done using specific primers of sarF5'-GGA TAA CCT GGT AAT TCT ATG-3' and sarR 5'-GGC AAA TGC TTT CGC AGT AG-3' (Rosenthal, 2009). Based on databases, the amplicon size of *S. Sui hominis* is 900 pb.

In order to apply the PCR protocol, samples were processed as following: in 200 µl Eppendorff 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 *AluI* restriction enzyme, specific for *Sarcocystis suis hominis*. The reaction was carried out by adding 2.5 µl buffer solution, 5 µl amplified DNA, 16 µl pure water and 1.5 µl *AluI* 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

Fig. 1. *Sarcocystis* spp. cyst - the compression method on trichinoscope screen

Fig. 2. The electrophoretic profile of 900 bp fragment corresponding to *Sarcocystis* spp. ; M - standard scale of 100 base pairs; Samples 2, 5, 6, 10, 11, 14, 16, 17 – the evidence of the presence of specific DNA for *Sarcocystis* spp.

tissue in a percent of 72,15 %. The parasitic forms are shown in Figure 1.

After examining and identifying cysts in muscle tissue by compression exam we passed to the molecular identification of *Sarcocystis* spp. by PCR method. The simplex PCR method with specific primers revealed the fragment corresponding to common gene (18S) for all species of *Sarcocystis*, shown in Figure 2.

Using the *AluI* restriction enzyme we are allowed to distinguish between *S. sui hominis* and the rest of the species of *Sarcocystis*. This enzyme has the ability to cut the fragment corresponding to this sub-species in two restriction sites and obtaining in the final three fragments at: 450, 300 and 150 base pairs. This can be seen in Figure 3 where a significant part of the samples were restrictive and thus identified as positive for *S. sui hominis*.

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 sui hominis* from the other *Sarcocystis* subspecies found in pigs we applied the RFLP method. *Sarcocystis sui hominis* 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. sui hominis* 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 *Sarcocysts* in the carcass of slaughtered animals is not covered by routine meat inspection. However, the high prevalence rate of infection, the presence of *Sarcocysts* 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

Fig. 3. The electrophoretic profile of restriction fragment of 400, 300, 150 bp corresponding to *Sarcocystis suihominis*; M - standard scale of 100 base pairs; Samples 1, 3, 4, 5, 6, 7, 9, 10, 11, 12, 14, 15, 17 – The evidence of specific DNA restriction fragments for *Sarcocystis suihominis*

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. bovis*, 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 (Dauguschies, 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. gigantea* 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. sui hominis*, more than 100 have been published for *Toxoplasma gondii* 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 sui hominis* 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 sui hominis* was detected in the analyzed samples in a percentage of 26.58%.

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