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Identification of *Sarcocystis* spp. by polymerase chain reaction and microscopic examination in various beef products (minced meat, meatballs, fermented sausage)

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Abstract: *Sarcocystis* species are the most commonly encountered parasitic organisms in animals. *Sarcocystis hominis* (*Sarcocystis bovihominis*), the species found in beef cattle, is not visible macroscopically in muscle tissue. In recent years it has been established that, in addition to being a zoonotic parasite transmitted via the consumption of beef, the parasite produces toxins and causes food poisoning. Therefore, this study was undertaken in order to determine the extent to which *Sarcocystis* species are present in minced meat, meatballs, and fermented sausages produced from beef and marketed in Kars. A total of 150 samples, 50 of each product, were collected in the city center from butcher's shops, from businesses that produce such items, and from general stores. In order to detect sarcocysts in the samples, the cysts revealed by an enzymatic digestion test were examined microscopically. Genomic DNA extracted from the samples was stored at –20 °C for analysis by polymerase chain reaction (PCR). The microscopic analysis revealed that the parasite was present in 2 samples of minced meat (4%) and 1 sample of meatballs (2%). Analysis by PCR revealed that the parasite was present in 14 samples of minced meat (28%), 34 samples of meatballs (68%), and 1 sample of sausage (2%). These results indicate that the use of PCR in the routine analysis of meat preparations for the presence of *Sarcocystis* species should be increased, since the application of this technique would provide analysts with more conclusive results than examination by microscope.

Key words: *Sarcocystis* spp., microscopic examination, beef products, PCR

1. Introduction

Sarcocystosis is one of the most commonly encountered parasitic diseases, both in Turkey and worldwide. Since the definitive hosts of this parasite are carnivores, including humans, the intermediate hosts are generally those animals whose meat is consumed by the definitive hosts, in particular ruminants such as cattle, sheep, and goats, as well as poultry and pigs. *Sarcocystis hominis* and *S. suihominis*, of which cattle and pigs are the intermediate hosts, are of zoonotic importance, since humans are the definitive host. These two species are known to cause intestinal sarcocystosis in people (1,2).

Apicomplexan parasite *Sarcocystis* species produce sarcocysts, which are found predominantly in the striated muscles of the intermediate hosts. The size and shape of sarcocysts may differ by species and according to their degree of maturity. While some remain microscopic, others become macroscopic. Sarcocysts are most commonly found in the esophagus and diaphragm of the intermediate host, as well as in the tongue, pharynx, and larynx and to a lesser extent in the skeletal muscles. Whereas in sheep, sarcocysts may be seen both macroscopically and microscopically, depending upon the particular species, in cattle there are only microscopic cysts (3,4). The fact that only microscopic cysts are found in cattle makes the inspection of beef for the zoonotic species *S. hominis* more difficult, which raises public health concerns (2,5). Moreover, the importance of this issue has been increased in recent years by evidence that sarcocysts secrete toxins that contaminate food (6).

The intake of mature cysts via the consumption of raw or undercooked beef and pork causes infection in humans. The onset of clinical infection with *S. hominis* usually starts 3 to 6 h after consumption of the meat; its symptoms are nausea, stomachache, and diarrhea. In the case of infection with *S. suihominis*, clinical symptoms appear in 6 to 48 h and its clinical progression is more serious (1). In Malaysia, observation over a period of 26 days of

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89 patients diagnosed with infection by *S. nesbitti* revealed that 94% presented with fever, 91% with muscle pain, 87% with headache, and 40% with coughing (7).

The conventional method for the identification of sarcocystosis is to use microscopic and histological techniques (8,9). However, in recent years, a number of molecular diagnostic techniques have been applied in the identification of the parasite in meat products (10–12).

Numerous studies, in Turkey (13–16) and throughout the world (8–10,17–19), have revealed a high level of prevalence of sarcocystosis in animals slaughtered for meat. It is of considerable importance to identify the extent to which the zoonotic species of *S. hominis* is present in beef products in the Kars region. The aim of this study was to determine the prevalence of *Sarcocystis* in the meat products of the region using microscopic examination and molecular techniques, and to compare the effectiveness of the methods used.

2. Materials and methods

2.1. Collection of samples

Beef products (50 minced meat, 50 meatballs, and 50 fermented sausage samples), which were collected from different restaurants, markets, and butchers in Kars Province, were taken to the laboratory and examined.

2.2. Microscopic examination

In this study, a modified trypsin digestion method was used, essentially as described elsewhere (20), to reveal sarcocysts. Ten grams of each sample was placed into a separate Falcon tube with a capacity of 50 mL, and the tubes were filled with 0.2% trypsin solution. After adding some glass beads, the tubes were vortexed for 30 s and incubated at room temperature for 20 min. The resulting homogenate was then sieved through a tea strainer to remove large particles and centrifuged at 2000 × g for 5 min. The supernatant fluid was discarded and the sediment was resuspended (1). Half of the sediment was removed and stored for DNA extraction; the remaining half was examined under a light microscope with magnification of 4×, 10×, and 40× for the presence of the parasitic stages (sarcocysts and bradyzoites). Species identification was attempted according to the tissue cyst and the cyst wall morphology (21).

2.3. Polymerase chain reaction (PCR) analysis

2.3.1. DNA extraction

Genomic DNA was extracted from digested samples using the QIAmp DNA Mini Kit (QIAGEN, Germany) according to the manufacturer's protocol. The resulting DNA samples were kept frozen at –20 °C until used as templates for PCR amplification of the mitochondrial cytochrome c oxidase subunit-I gene (*cox-I*), complete *18S rRNA* gene, and/or the partial *cox-1*. The concentration and the suitability of DNA were measured and evaluated using a NanoDrop ACTGene-UVS99 model spectrophotometer.

2.3.2. PCR primers and protocols

It has recently been argued that a ~1100-bp-long portion of *cox-I* is a powerful molecular marker for differentiating *Sarcocystis* species in cattle (22). The species-specific primers for *Sarcocystis* species SF1 (5'-ATG GCT TAC AAC AAT CAT AAA GAA-3') as forward primer and SR9 (5'-ATA ATCC ATA CCR CCA TTG CCC CAT-3') as reverse primer (Integrated DNA Technology) were used for the PCR (22). For the PCR amplification of the *cox-1* gene, the reaction mixture contained 2.5 µL of PCR buffer-S, 0.5 µL of dNTPs, 0.75 µL of Taq DNA polymerase, 2 µL of $MgCl_2$, 20 pmol of each primer, 3 µL of DNA template, and 15.25 µL of molecular grade water to make a final volume of 25 µL. In addition, commercially available Master-Mix Buffer (QIAGEN) was also used in some of the PCR reactions in order to eliminate unnecessary optimizations, especially with templates extracted from meatballs. Distilled water without template was included in each PCR run as a negative control. The PCR reaction was carried out in a Biometra T Model thermocycler, with an initial hot start at 94 °C for 3 min, followed by 45 cycles of denaturation at 94 °C for 45 s, annealing at 57.5 °C for 45 s, and extension at 72 °C for 1 min with a final extension of 72 °C for 10 min. All PCR products together with a 100-bp DNA ladder (Fermentas) were run on 1% agarose gels and after staining with ethidium bromide were visualized under UV light. Positive control DNA samples were produced and supplied from the previous laboratory studies by us.

2.4. Statistical analysis

The statistical significance between groups was determined by the Mann–Whitney test. SPSS 16 was used as the statistical program.

3. Results

The results of the microscopic examination and PCR analysis of the minced meat, meatball, and fermented sausage samples are presented in the Table.

In microscopic examination, sarcocysts or bradyzoites of the parasite were found in two samples of minced meat (4%) and one sample of meatballs (2%) but were not found in the sausage samples. The difference between the groups was statistically significant ($P < 0.01$). In microscopic examination, the morphological structures of the cysts were not evaluated so as to not to fall into scientific error because the shapes did not conform to the references. Overall, 2% of a total of 150 samples tested positive microscopically. Species identification was not performed on these positive samples (Figures 1A and 1B).

On the other hand, 14 of the minced meat (28%), 34 of the meatball (68%), and one of the sausage (2%) samples tested positive by PCR. That is, 32.6% of the meat products (49 samples) tested positive by PCR (Figures 2–4), with

Table. Results of the microscopic examination and PCR analysis of *Sarcocystis* spp. in samples.

Sample	n	Microscopic examination		PCR analysis		P
		Number of positive samples	%	Number of positive samples	%	
Minced meat	50	2	4	14	28	0.001
Meatballs	50	1	2	34	68	0.000
Sausage	50	0	0	1	2	*
Total	150	3	2	49	32.6	

*Could not be evaluated.

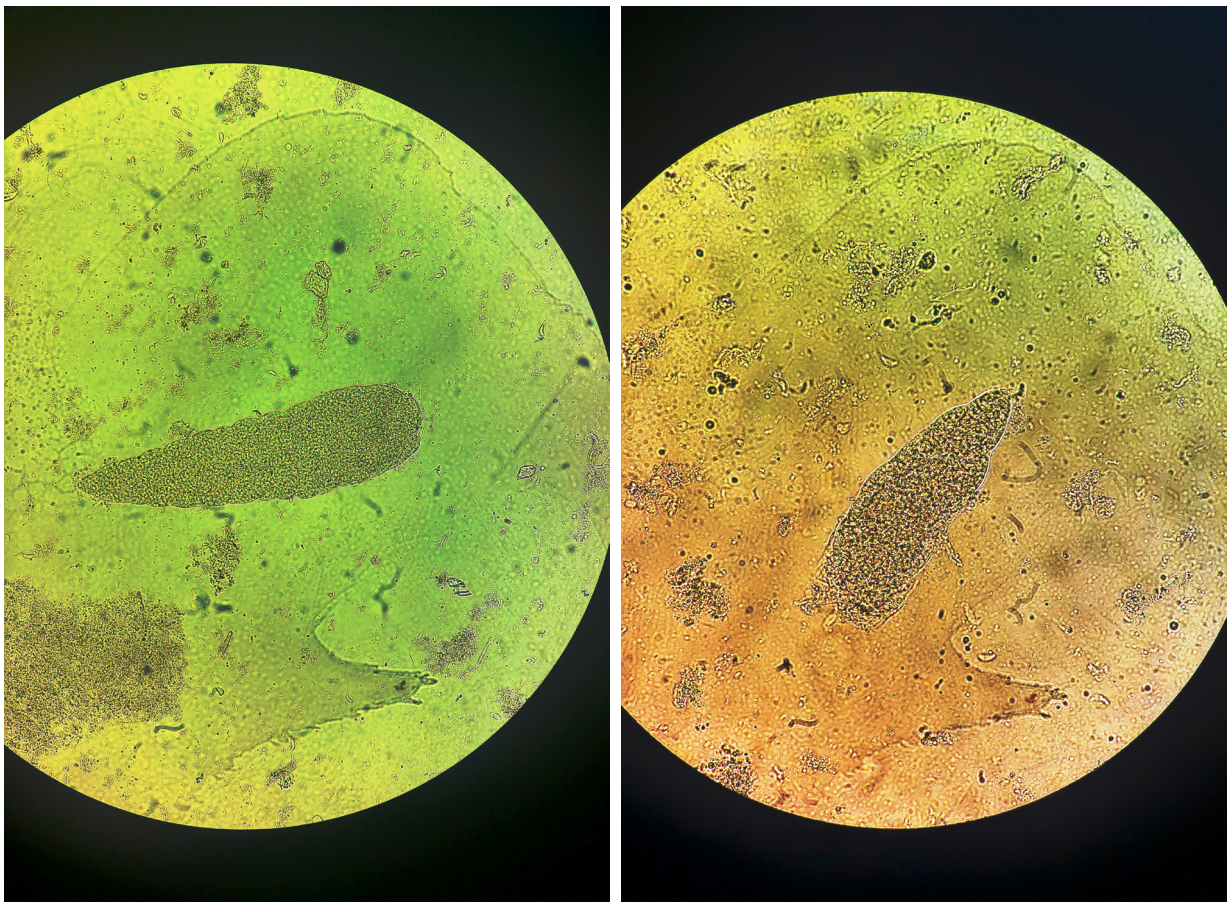

Figure 1. *Sarcocysts* visible in minced meat (A) and meatball (B) samples under microscopic examination at 40×.

the highest level of positivity in the meatball samples and the lowest in the sausage samples. The difference between the groups was statistically significant ($P < 0.01$) in PCR analysis.

4. Discussion

Studies on sarcocystosis, both in meat carcasses and in meat products, have been undertaken in many countries worldwide, including Turkey (2). Although studies were done worldwide (1), human sarcocystosis cases were not identified in Turkey. In research conducted in Turkey, the prevalence of the disease was found to be 94.8% in cattle in Ankara (14). Macroscopic and microscopic cysts of *Sarcocystis* are commonly found. The prevalence of sarcocystosis in cattle in Turkey has been shown to be 93.4% (2). The presence of *S. hominis*, *S. bovicanis*, and *S. bovifelis* species has been established in meat carcasses in the abattoirs of Kars and Erzurum, with the prevalence of

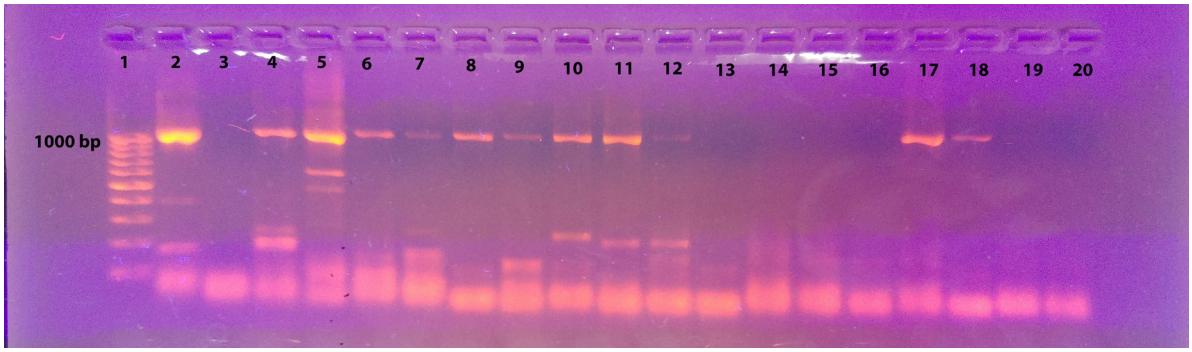

Figure 2. Agarose gel electrophoresis of PCR products amplified from minced meat samples. 1: Marker; 2: positive control; 4–12, 17, 18: positive samples; 3, 13–16, 19: negative samples; 20: negative control.

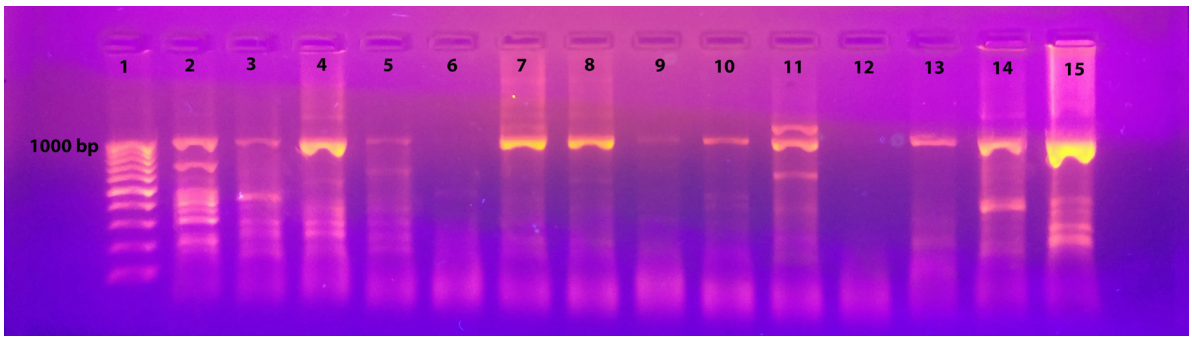

Figure 3. Agarose gel electrophoresis of PCR products amplified from meatball samples. 1: Marker; 2: positive control; 3–5, 7–11, 13–15: positive samples; 6, 12: negative samples.

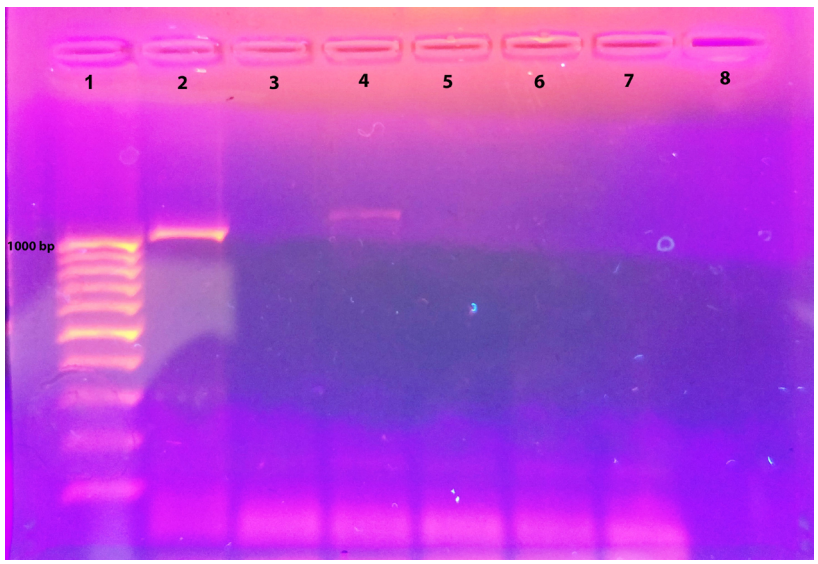

Figure 4. Agarose gel electrophoresis of PCR products amplified from sausage samples. 1: Marker; 2: positive control; 4: positive sample; 3, 5–7: negative samples.

S. bovihominis reported to be 63.2% (13). In the province of Van, the prevalence of sarcocystosis in cattle has been reported to be 97%, with the prevalence of *S. bovihominis* identified as 21% (15).

The fact that the parasite *S. bovihominis* is so common in animals means that it is important to determine the extent to which it is present in meat products. In a study by Maskar et al. (23), cysts of *Sarcocystis* were found in

95% of beef, 75% of fermented sausage, 88% of pastrami, 65% of salami, and 50% of sausage products. It is thought that the high levels reported in that study reflect the fact that it was carried out at a time when efforts to control the parasite were not yet widespread. In Van, the prevalence of sarcosporidia was determined to be 73.1% in beef, 51.2% in mutton, 84.1% in goat's meat, 73.3% in sausage, and 78.5% in ready mince samples (24).

In a study carried out in Iran, cysts of *Sarcocystis* were found in 56% of hamburgers, 20% of sausages, and 8% of fermented sausages and, given this rate of incidence, the significance of meat and meat products in relation to sarcocystosis in humans was highlighted (25). A further study in the same country investigated the presence of *Sarcocystis* species in 100 traditional and 90 industrial samples of raw hamburger. The results of the study established that *S. cruzi* was present in 39%, *S. hirsuta* in 61%, and *S. hominis* in 54% of the traditional hamburger samples, while *S. cruzi* was present in 67.8%, *S. hirsuta* in 58.9%, and *S. hominis* in 57.8% of the industrial hamburger samples (12). Meistro et al. (26) conducted a study to identify the *Sarcocystis* species present in 25 samples of minced beef and used PCR to establish that the level of *S. hominis* was 68%. Likewise, in a study done by Pritt et al. (17), it was found that the positivity level was 40% by histological methods and 54.4% by PCR.

The presence of *Sarcocystis* cysts in meat and meat products is of importance in relation to public health. People may become infected with *Sarcocystis* by consuming such meat either raw or undercooked. Since the parasite is zoonotic, this study was undertaken in the belief that it would be of potential benefit in relation to public health. This is the first study to use the molecular analysis technique of PCR to investigate the prevalence of *Sarcocystis* species in the minced meat, meatballs, and fermented sausages made from beef marketed in Kars. The level of risk posed by these products in terms of public health was established.

The study found that *Sarcocystis* spp. was most often present in meatballs, and then in minced meat. The reason why the parasite was found more often in meatballs than in minced meat is believed to stem from the fact that meatballs contain tissue from the more active muscles, such as the esophagus, diaphragm, heart, and masseter, in which sarcocysts are more frequently encountered. The reason why so few sarcocysts were found in the case of sausage is thought to be related to differences in its composition, to the use of large amounts of fat, to the emulsifying process, and to the likelihood that it has been heat-treated even though it is marketed as fermented. Indeed, Ghisleni et al. (8) found very low levels of sarcocysts in cooked products. Moreover, it is believed that the method used for preparing the specimens may not be suitable for sausages and that a different procedure might be required in the preparation of samples for PCR. Nonetheless, sequence analysis should be performed on positive samples.

The study established that the prevalence of this parasite, which is at a high level in animals in Turkey, is also high in meat products (68% in meatballs). Therefore, it is advisable that meat and meat products that test positive for sarcocysts should be kept in storage for at least 1 day at –20 °C or for 2 days at –4 °C (25). An alternative method to ensure that meat and meat products are safe is to apply heat treatment at over 70 °C (27).

Since *S. hominis* is a zoonotic agent, it is of concern in relation to public health and therefore its diagnosis is important in meat offered for human consumption, as well as in live animals. The currently available methods for detecting the genus *Sarcocystis* microscopically are limited to enzymatic digestion methods and examination of histological sections. As is clear from the present study and from much previous research, these methods may fail to provide definite results or may prove inadequate in differentiating between species. The results of this study confirm that molecular techniques, such as PCR, may provide a more reliable alternative method of diagnosis.

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