Journal of Zoonotic Diseases
2022, 6 (1): 39-44
doi: 10.22034/jzd.2021.13769
https://jzd.tabrizu.ac.ir/article_13769.html

Original Article

Study on the effects of the different temperatures on the viability of *Sarcocystis* bradyzoites

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(Received 3 June 2021, Accepted 4 September 2021)

Summary
Sarcocystis is one of the most typical parasites in domestic animals, affecting cattle, sheep, and goats. The meat business loses millions of dollars each year due to the eradication of *Sarcocystis*-infected carcasses. In humans, some *Sarcocystis* species induce digestive problems such as nausea, vomiting, and diarrhea. Because this parasite is usually found in skeletal and cardiac muscles, it is crucial and required to provide preventative and control strategies such as inactivating or eradicating the bradyzoites in contaminated meat. The employment of freezing and heating temperatures is one of these methods. The main objective of this study was to investigate the effects of freezing and heating temperatures on the survival rate of Sarcocystis bradyzoites in infected mutton. To verify the presence of contamination in meat, two methods of Dob smear and digestion were applied. The samples were then exposed to heat and cold, and the mortality rate was determined using a digestion method. ANOVA and LSD post hoc tests were employed to assess the association between the variables ($P < 0.05$). The results showed that temperature of 100 ºC for 10 minutes and temperature of 80 ºC and for above 20 minutes were effective in killing the parasites. Also, the parasites were completely disappeared after 24 hours at -20 ºC. The findings revealed that the bradyzoites did not have a substantial mortality rate at 4 ºC for up to six days. As a result, this parasite can be killed by heating or freezing.
***Keywords**:* Freezing, Heating, *Sarcocystis*, Temperatures, Viability

Introduction

Sarcocystis is one of the most typical parasites in domestic animals, affecting cattle, sheep, and goats. Some *Sarcocystis* species can cause sickness in intermediate hosts such as cattle, sheep, goats, and pigs, resulting in weight loss, neurological disorders, anorexia, lameness, paralysis, fever, anemia, muscle weakness, decreased milk production, miscarriage, and sometimes death (Radostits et al., 2007; Titilincu et al., 2008; Dalimi et al., 2009; Parandin et al., 2015; Berenji et al., 2019).

Sarcocystis species have a two-host life cycle, with herbivores serving as intermediate hosts and carnivores serving as definitive hosts (Dhaliwal and Juyal, 2016). Many investigations have been conducted on the prevalence of sarcocystosis in pigs, cattle, and sheep in the world and Iran (Razmi and Rahbari, 2000; Shekarforoush and Alikhani, 2003; Bonyadian and Meshki, 2006; Dalimi et al.,

2010; Dubey et al., 2015; Farhangpazhouh et al., 2020).

According to domestic and international studies, the meat business loses millions of dollars each year due to eradicating *Sarcocystis*-infected carcasses. In humans, some *Sarcocystis* species induce digestive problems such as nausea, vomiting, and diarrhea. In humans, consuming undercooked or raw beef and buffalo meat carrying *Sarcocystis bovihominis*, or eating pork carrying *Sarcocystis suihominis* cysts, causes sarcocystosis (Parandin et al., 2015).

Because this parasite is usually found in skeletal and cardiac muscles (Dubey et al., 2015), it is crucial and required to provide preventative and control strategies such as inactivating or eradicating the bradyzoites in the contaminated meat, which is one of the stages of infection and completes the parasite's life cycle. The employment of freezing and heating temperatures is one of these methods.

The main objective of this study was to investigate the effects of freezing and heating temperatures on the survival rate of *Sarcocystis* bradyzoites in infected mutton. These temperatures could be utilized to disinfect meat if the outcomes of this study are positive.

Materials and methods

Sampling

From December 2019 to March 2021, we collected samples of mutton from the slaughterhouse and transported them to the laboratory (next to the ice). To verify the presence of contamination in meat, two methods of Dob smear and digestion were applied.

Dob smear method

In this method, we took the sample with surgical forceps and pressed it on the slide several times so that a layer of it remained on the slide. The slides were numbered, then fixed with methanol, and stained by Trypan blue (Gabriele et al., 2006). There is a distinction between the staining of living and dead parasites in this sort of staining. A light microscope was used to analyze the slides. Live bradyzoites are impermeable to this color and do not stain, while dead bradyzoites are completely stained by this color. If this method was not suitable (there were not enough parasites found), the digestion method was used to prove contamination.

Digestion method

In this method, about 50 grams of the appropriate tissue was crushed with a meat grinder and inserted in 100 mL of digestive solution. After one hour at 37 ºC, the solution was strained. After that, the solution was transfered to the test tube and centrifuged for 5 minutes at 2500 rpm (Hamidinejat et al., 2010). Then, we prepared a slide from the sediment in the bottom of the test tube. The prepared slide was then treated with methanol (Merck, Germany) and dyed with Trypan blue after drying. Under a microscope, we examined the stained slide with immersion oil. To continue the research, the samples that were deemed positive were maintained at 4 °C (Honda et al., 2018). To make the pepsin solution (digestive solution), 2.5 g of pepsin powder (Sigma Aldrich, USA) was dissolved in 100 mL of PBS, then 10 mL of choloridric acid was added (Dubey et al., 1989).

Heating and freezing

Samples that were considered positive were affected by different temperatures. For this, samples were incubated for 10 and 20 minutes at 40, 50, 60, 70, 80, 90, and 100 ºC. Also, some samples were refrigerated at temperatures of -2,-4, and -20 ºC for one and two days. Three replications were used in the tests, and some samples were held at 4 °C as controls.

Investigating the mortality rate of Sarcocystis bradyzoites

The digestion method and staining by Trypan blue, as previously noted, were used to assess parasite survival, and their mortality rate was then recorded. Trypan blue stained the dead bradyzoites fully, while the surviving bradyzoites were impenetrable to the dye (Honda et al., 2018).

Statistical analyses

The SPSS software version 21 was used to analyze the data by ANOVA and LSD post hoc testto assess the association between the variables ($P < 0.05$).

Results

The effect of heating

The results showed that 40° C had the least effect after 10 minutes (8.8%), while at 100° C, no parasites survived after 10 minutes. After 20 minutes at 80, 90, and 100ºC, all parasites were dead (100%). The results of statistical analysis showed that there was a significant associateion between bradyzoite mortality and temperature ($P < 0.05$). The results are presented in Table 1.

Table 1- The effects of heating on the mortality rate of *Sarcocystis* bradyzoites in mutton

Time (min) / Temperature (ºC)	10 min	Average	20 min	Average
	Percent (%)			
40	6.8	8.8[a]	40.3	34[a*]
40	11.5		21.9	
40	8.1		39.8	
50	24.9	29.2[b]	69	63.8[b]
50	25.4		57.5	
50	37.3		64.9	
60	47.1	49.5[c]	97.1	93.7[c]
60	53.5		92.8	
60	47.9		91.2	
70	83	83.8[d]	100	97.43[c]
70	81.9		94	
70	86.5		98.3	
80	91.4	89.8[d]	100	100[c]
80	87.4		100	
80	90.6		100	
90	94.8	96.7[e]	100	100[c]
90	100		100	
90	95.3		100	
100	100	100[e]	100	100[c]
100	100		100	
100	100		100	
P-value	0.00		0.00	

*Values with different letters (a–e) are statistically different.

The effect of freezing

According to the data, after one day at 2 °C, bradyzoites had the lowest mortality rate (39.3%). The -20 °C temperature was far more effective than the other two temperatures, and no survived bradyzoite was found after one day at this temperature (Table 2). The bradyzoites mortality rate was significantly affected by temperature ($P < 0.05$). Several samples were maintained at 4 °C for additional examination, and the mortality rate of bradyzoites on days 1, 2, 3, 4, 5, and 6 was investigated. The findings revealed that the bradyzoites did not have a considerable mortality rate at this temperature for up to 6 days (Table 3).

Discussion

Sarcocystis is a parasite that affects the skeletal and cardiac muscles of various animals. This parasite is found all across the world, with a significant incidence in Iran. Humans can become infected by consuming raw or undercooked meat that has been contaminated (Dubey et al., 2015). This disease is significant in terms of both economics and human health. As a result, implementing proper preventative strategies to disable it is critical.

According to the previous studies, heating, freezing, irradiation, and marinating in NaCl and acetic acid were all used to inactivate this parasite, and each had a unique impact (Franssen et al., 2019). According to the FAO, one of the most efficient parasite control techniques is heat treatment. Several studies demonstrate that heating duration is equally as essential as temperature and should be set such that optimal temperatures are attained, sustained, and distributed uniformly throughout the meat (Gajadhar, 2015).

Table 2- The effects of freezing on the mortality rate of *Sarcocystis* bradyzoites in mutton

Time (h) / Temperature (ºC)	24h	Average	48h	Average
	Percent (%)			
-2	48.1	39.3[a]	68.7	62.4[a*]
-2	38.7		57.3	
-2	31.1		61.2	
-4	73.2	68.5[b]	93.7	89.1[b]
-4	61.9		81	
-4	70.4		92.6	
-20	100	100[c]	100	100[c]
-20	100		100	
-20	100		100	
P-value	0.00		0.00	

*Values with different letters (a–c) are statistically different.

Table 3-The control samples in 4 ºC

Time (day) / Temperature (ºC)	1	2	3	4	5	6	*P-value*
	Percent (%)						
4	0	1.3	0.1	1.9	4.1	2.5	0.06
4	0	0	3	2.8	1.5	2.9	
4	0.3	1.4	2.7	1.6	0.7	4.8	
Average	0.1[a*]	0.9[a]	1.93[ab]	2.1[ab]	2.1[ab]	3.4[b]	

*Values with different letters (a–b) are statistically different.

The current results showed that at 100 °C, no parasites survived after 10 minutes. After 20 minutes at 80, 90, and 100 °C, all parasites were dead (100%). Furthermore, -20 ºC was significantly more effective than other temperatures, as no parasites survived even after a single day at this temperature. The procedure used in the current study differs significantly from the procedures used by others. Here, the mortality rate was determined using the digestion method, whereas other previous similar studies have employed the bioassay method. The digestion method allows for a more meaningful and understandable comparison of data.

There are very little published studies on the effects of heating and freezing on parasite mortality. Meat subjected to 70 ºC for 15 minutes and 100 ºC for 5 minutes was no longer infected (Franssen et al., 2019). In support of our findings, Franssen et al. (2019) found that freezing buffalo heart muscle at -4 °C for 48 hours inactivated the bradyzoites. They also discovered that heating *Sarcocystis* to 65 to 75 °C for 20 to 25 minutes inactivated it (Franssen et al., 2019). Also, it was reported that infected beef could be frozen at -20 °C for 4-6 days and -6 °C for one day, similar to the present results (Franssen et al., 2019). Collins and Charleston (2011) found that cooking mutton at 60 °C for 20 minutes should render it non-infective for cats.

Honda et al. (2018) have demonstrated that temperatures ranging from -20 ℃ to -80 ℃ for more than an hour could inactivate *Sarcocystis* bradyzoites.

The present findings showed that maintaining contaminated mutton at 4 ℃ for six days did not affect *Sarcocystis* bradyzoites. Similar to our findings, Honda et al. (2018) reported that consuming tainted meat that had been held at this temperature for seven days induced disease. Several studies have demonstrated the effectiveness of microwave heating in destroying parasites such as Anisakis. Heating in microwave ovens, on the other hand, may not penetrate all portions of the meal, resulting in hot and cold zones, and so some parasites may elude inactivation (Vidacek et al., 2011). Toxoplasma cysts in mutton steaks cooked in a microwave oven at 65 ℃ remained infectious (Franssen et al., 2019). Anisakis spp. were inactivated in fish by freezing in a blast freezer at 35 ℃ for 15 hours or at 20 ℃ for at least 24 hours (McClelland., 2002). Although, few studies have been conducted, trematode metacercariae appear to be more resistant to freezing temperatures. *Clonorchis sinensis* in fish and fisheries products is thought to be inactivated after 5–20 days at -10 ℃ to -20 ℃ (EFSA, 2010).

Conclusion

Because this parasite is usually found in skeletal and cardiac muscles, it is crucial and required to provide preventative and control strategies such as inactivating or eradicating the bradyzoites in contaminated meat, which is one of the stages of infection and completes the parasite's life cycle. The employment of freezing and heating temperatures is one of these methods. Heating and freezing have a substantial impact on this parasite, according to our findings, and a temperature of -20 ºC for 24 hours can be utilized to inactivate it. Temperatures above 90 ºC for 10 minutes and temperatures above 60 ºC for 20 minutes are also suitable for its deactivation.

Acknowledgment

Not applicable.

Conflict of interest statement

The author has declared no competing interest.

Ethical approval

Not applicable.

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