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Effect of Electron Beam Irradiation on Viability of *Sarcocystis* spp. in Beef

G. Eslami [1,2], S. Peletto [3], M. Vakili [4], S. Kargar [2,5*]

1. Department of Parasitology and Mycology, School of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
2. Research Center for Food Hygiene and Safety, School of Public Health, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
3. Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Via Bologna 148 - 10154 Torino, Italy
4. Department of Community and Preventive Medicine, Health Monitoring Research Center, School of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
5. Department of Food Hygiene and Safety, School of Public Health, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

HIGHLIGHTS

- Electron beam irradiation at zero time was not effective on viability of *Sarcocystis*; but at 24 h, irradiation doses 3 and 4 kGy caused a significant reduction in *Sarcocystis* viability.
- No significant differences were observed between irradiation doses 3 and 4 kGy.
- Electron beam irradiation at dose of 3 kGy was effective as the optimal dose for the elimination of *Sarcocystis* spp. in beef.

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Acronyms and abbreviations
PCR=Polymerase Chain Reaction

ABSTRACT

Background: Sarcocystosis is one of the most distributed parasitic diseases over the world, caused by *Sarcocystis* spp. In this study, we assessed the effect of electron beam irradiation on the viability of *Sarcocystis* spp. in beef.

Methods: Experimental beef groups were irradiated by four different electron beam doses of 1, 2, 3, and 4 kGy, at intervals of 0 and 24 h after irradiation, then the samples were transferred inside a sterile microtube containing RNA*later* solution and stored at -20 °C till next steps. RNA extractions and cDNA synthesis were done using the related kit in order to detect the presence of the 18S ribosomal RNA region. Relative quantification was carried out using SYBR Green Real time Polymerase Chain Reaction. The statistical analysis was done using SPSS 16.0 by Tukey's and Kruskal-Wallis tests.

Results: Irradiation at zero time was not effective on viability of *Sarcocystis*, but at 24 h, irradiation doses of 3 (p=0.003) and 4 kGy (p=0.008) caused a significant reduction in *Sarcocystis* viability. Irradiation doses of 1 and 2 kGy had no significant (p>0.05) effect on *Sarcocystis* viability reduction. Also, no significant differences (p>0.05) were observed between irradiation doses of 3 and 4 kGy.

Conclusion: Electron beam radiation at dose of 3 kGy was effective as the optimal dose for the elimination of *Sarcocystis* spp. in beef.

Introduction

Meat is one of the most important sources of animal protein. Meat is rich in valuable proteins, which contain essential amino acids such as histidine, isoleucine, leucine, methionine, tryptophan, as well as fats. It also

* Corresponding author (S. Kargar)
✉ E-mail: samanekargar3@gmail.com
ORCID ID: https://orcid.org/0000-0002-5517-6869

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contains fatty acids such as linoleic acid, linolenic acid, and arachidonic acid; minerals, including phosphates, sulfates, iron, and zinc; vitamins, especially B vitamins; and carbohydrates (Górska-Warsewicz et al., 2018). Therefore, meat has fundamental roles in the optimal growth of the body. Possibility of meat infected with various pathogens causes important health problems; food safety risks include parasites like *Sarcocystis* spp., *Toxoplasma gondii*, *Taenia saginata*, *T. solium*, etc.

Sarcocystis spp. are considered as the most important protozoa in domestic ruminants; it can also be found in many wild animals, birds, cold-blooded animals, rodents, reptiles, and humans. This parasite has a mandatory dual host life cycle; dogs, cats, and wild carnivores such as foxes, wolves, raccoons, coyotes, etc. as the definitive hosts and cattle, sheep, goats, pigs, etc. as the intermediate host. The intermediate hosts could infect by eating food contaminated with oocysts defecated with the feces of the definitive host, where the tissue cyst forms in the muscles of the intermediate host. The definitive hosts infect with eating of intermediate host harboring the tissue cyst. There are more than 130 *Sarcocystis* spp., some of which can cause great economic losses by causing clinical or subclinical diseases. Symptoms of infection in human usually include diarrhea, abdominal pain, temporary nausea, bloating, watery diarrhea, fever, dizziness, joint pain, and anorexia (Dubey et al., 2016). In order to prevent sarcocystosis in human, it is recommended to freeze or heat the beef at 55 °C for 20 min for inactivation of the transmission (Dubey and Lindsay, 2006).

There are five *Sarcocystis* spp. in cattle, including *S. cruzi*, *S. heydorni*, *S. hirsuta*, S. *hominis*, and *S. rommeli* with their specific definitive hosts, canines for *S. cruzi*, felines for *S. hirsuta* and *S. rommeli*, and primates for *S. heydorni* and *S. hominis*. Humans can be considered as the definitive host for *S. hominis* and *S. heydorni*. Human infection with *S. hominis* has symptoms, including nausea, stomachache, and abdominal pain (Dubey et al., 2016).

Irradiation is one of the ways to sanitize and promote food safety which used in many foods to control of various parasites. The maximum allowable amounts of irradiation for red meat and poultry meat are 4.5 and 7 kGy, respectively. One of the purposes of irradiation is to delay the ripening of fruits and some vegetables (Farkas and Mohácsi-Farkas, 2011). The use of irradiation process as a food storage process by the Food and Agriculture Organization is allowed up to the dose of 10 kGy which approved from a toxicological point of view. The use of advanced processing technologies such as irradiation has excellent potential in improving the safety and quality of the food industry (Ehlermann, 2016; McFadden et al., 2016; Murray et al., 2015). Therefore, it is necessary to study the effect of irradiation for parasites control. In this study, we assessed the effect of electron beam irradiation on viability (survival) of the tissue cyst of *Sarcocystis* spp. in beef.

Materials and methods

Preparation of experimental groups

Beef was prepared from different parts of carcass of slaughtered cattle in a slaughterhouse in Yazd City (Iran) and immediately was transferred to the laboratory. Then, 1×1 cm slices were separated from different parts of the beef and randomly spread on 15 separate plates in each group of time zero and 24 as in each group of exposure; three of 15 plates were considered untreated as control group. Before the exposure, all beef samples were stored at refrigerator.

Electron beam treatment

The beef samples were sent to the Taft Radiation Center (Iran). Four different electron beam doses of 1, 2, 3, and 4 kGy were used. After exposure, the samples were inserted inside a sterile tube with RNA*Later* (Sigma-Aldrich, Taufkirchen, Germany), then were stored at -20 °C till next experiments. Finally, all beef groups were analyzed at 0 and 24 h for detection of viable *Sarcocystis* spp. All the tests were repeated in triplicate.

Detection of Sarcocystis spp.

Before any exposure, molecular detection of all samples in all plates was done to ensure of infection with *Sarcocystis* spp. For this purpose, DNA extraction was done using salting out method (Hajimohammadi et al., 2014; Moghadam Ahmadi et al., 2015). Detection of the *Sarcocystis* spp. was done using the amplification of the target of 18S ribosomal RNA by the specific primers of Sar-F 5'-TGGCTAATACATGCGCAAATA-3' and Sar-R 5'-AACTTGAATGATCTATCGCCA-3' in a 20 µl reaction with the final concentration of 0.5 µM for each primer, 1X Polymerase Chain Reaction (PCR) buffer (Ampliqon, Odense, Denmark), including 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 1 U *Taq* DNA polymerase. The thermal amplification was done using the thermal cycler (SimpliAmp, ABI, USA) by the thermal program of 94 °C for 5 min for the first denaturation and followed by 30 cycles of 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 45 s. The final extension was done at 72 °C for 5 min. The amplicons were assessed using 2% agarose gel electrophoresis alongside with 50 bp DNA ladder (Fermentas, USA). The positive and negative controls were used in all amplification by *Sarcocystis* spp. isolated from tissue cyst and ddH_2O, respectively. All the experimental assays were done in triplicate.

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Viability (survival) of Sarcocystis spp.

-RNA extraction and cDNA synthesis

RNA extraction was done using GF-1 Total RNA Extraction Kit (Vivantis, South Korea) based on the manufacturer's instruction. RNA quantification was carried out using spectrophotometer (Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer, USA). The cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) based on the manufacturer protocol. All tests were done in triplicate.

-Real time PCR

Each reaction was prepared in a volume of 20 µl containing 1× master mix (SYBR™ Green PCR Master Mix-Thermo Fisher Scientific, USA), 200 nM of each primer, and 2 µl of cDNA. The amplification for Real time PCR was performed by thermal cycler (StepOne, Life Technologies, USA). The 18S rRNA target of *Sarcosystis* spp. was detected using Sar-F and Sar-R that was mentioned in genus detection section. Amplification cycles were performed with initial heating at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s, 60 °C for 1 min. All tests were done in triplicate. Samples with amplification of at least 2 of the 3 replicates were considered positives. In order to ensuring the specific amplification, the melting point analysis was done. The data were collected based on Ct for all treated and control samples in the exponential phase of the reaction. Finally, the Relative Quantification (RQ) was calculated for the comparison and analysis.

Statistical analysis

Statistical analysis of data was done using SPSS software v. 16.0. Comparison of viability of *Sarcocystis* spp. between each group exposure with electron beam and the control group (untreated) was performed by the Tukey's test. The comparisons between the treated groups in different doses in each time were done using Kruskal-Wallis test. In all cases, a significance level was considered as 0.05.

Results

Molecular detection showed that the beef we used in this study were infected with *Sarcocystis* spp. (Figure 1). The expected amplicon size was 150 to 200 for *Sarcocystis* spp. detection.

SYBR Green Real time PCR analyses showed that no significant difference (p>0.05) was observed in the viability of the *Sarcocystis* spp. before and after irradiation at any of the doses at time zero (Figure 2).

Significant differences were observed in the viability of *Sarcocystis* spp. before and after irradiation at doses of 3 (p=0.003) and 4 (p=0.008) kGy at time 24. According to statistical analysis, doses of 1 and 2 kGy had no effect on the viability of the parasite (p>0.05; Figure 3).

Discussion

In this study, the effect of electron beam irradiation was assessed on the viability of *Sarcocystis* spp. in beef using SYBR Green Real time PCR. We showed that at least 3 kGy after 24 h destroyed *Sarcocystis* parasite. Due to the fact that the consumption of half-cooked meat is common in some parts of the world, the beam electron irradiation processing can be used as one of the ways to make meat safe.

Electron beam is one of the most significant methods for microbial inactivation. In this process, high-energy electrons with the acceleration close to light speed are used, therefore, this energy penetrate in food matrices. This method is useful for elimination of parasites, viruses, insects, bacteria, yeasts, and molds in food. Irradiation also extends the shelf life of raw materials to avoid wasting food resources (Lung et al., 2015; McFadden et al., 2016; Murray et al., 2015).

The results of this study showed that electron beam had an appropriate efficiency in inactivation of *Sarcocystis* spp. in meat. Although, the irradiation at zero time had no effect on the viability of the parasite but the doses 3 and 4 kGy at time of 24 h reduced significantly the *Sarcocystis* viability (survival). No significant difference was observed between doses of 3 and 4 kGy after 24 h. Therefore, considering 3 kGy may be suitable as the optimal dose for the destruction of *Sarcocystis* parasites. There are some studies for using electron beam against microorganisms. Javanmard et al. (2006) showed that doses of 2 and 3 kGy are effective in reducing *Salmonella* in poultry. It is proved that 2 kGy has the ability of killing *Entamoeba histolytica* cysts (Rahman, 2007). In some other studies, doses from 0.3 to 1 kGy are suitable for killing *Cysticercus bovis* and *Trichinella spiralis*, respectively (Farkas, 1998). Also, Al-Farisi et al. (2013) showed that doses of 0.5, 1, and 2 kGy are effective in destroying of *Orizafilus surinamensis*. In the study by Ooi et al. (1993), the dose of 2 kGy is effective in killing *Angiostrangillus cantonensis*. Collins et al. (2005) examined the effect of electron beam on *Cryptosporidium parvum* in oysters, their data shows that although doses of 1 and 1.5 kGy are effective in its destruction, but a dose of 2 kGy destroys all inoculated parasites. However, Yu and Park (2003) showed that the doses from 1 to

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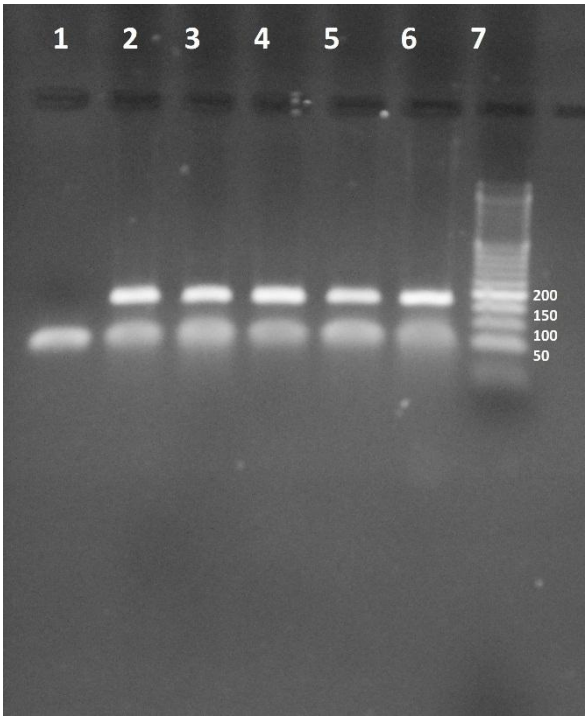

Figure 1: Agarose gel electrophoresis-Amplification with the specific primer pair of Sar in order to molecular detection of *Sarcocystis* spp. in meet. Lane 1: negative control (ddH_2O), lanes 2 to 5: samples with *Sarcocystis* spp., lane 6: positive control (*Sarcocytis* spp. isolated from the tissue cyst), lane 7: 50 bp DNA ladder. The expected amplicons size was around 150 to 200 bp.

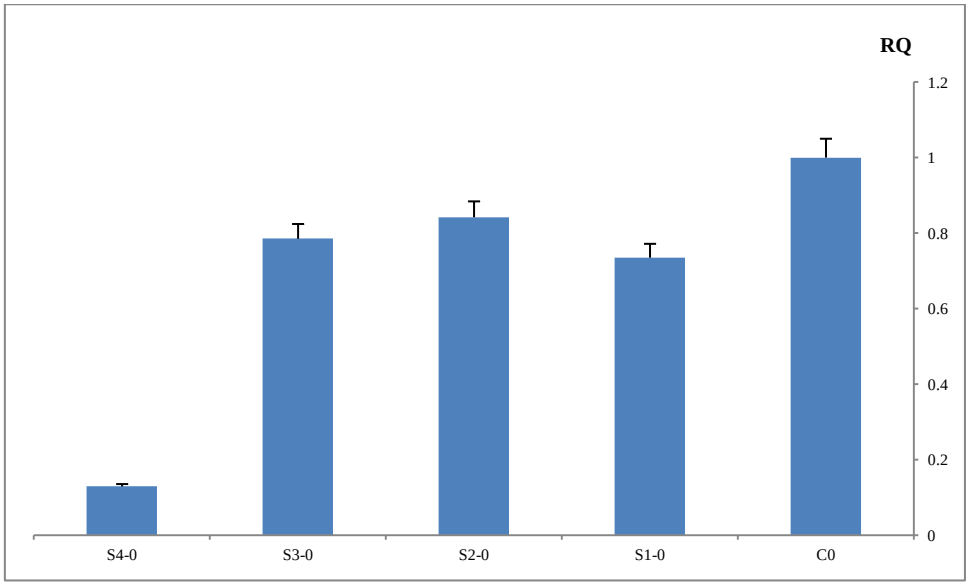

Figure 2: Comparison of the effect of the studied electron beam irradiation doses (1 to 4 kGy) at time zero on *Sarcocystis* spp. in meat. RQ is relative quantification.

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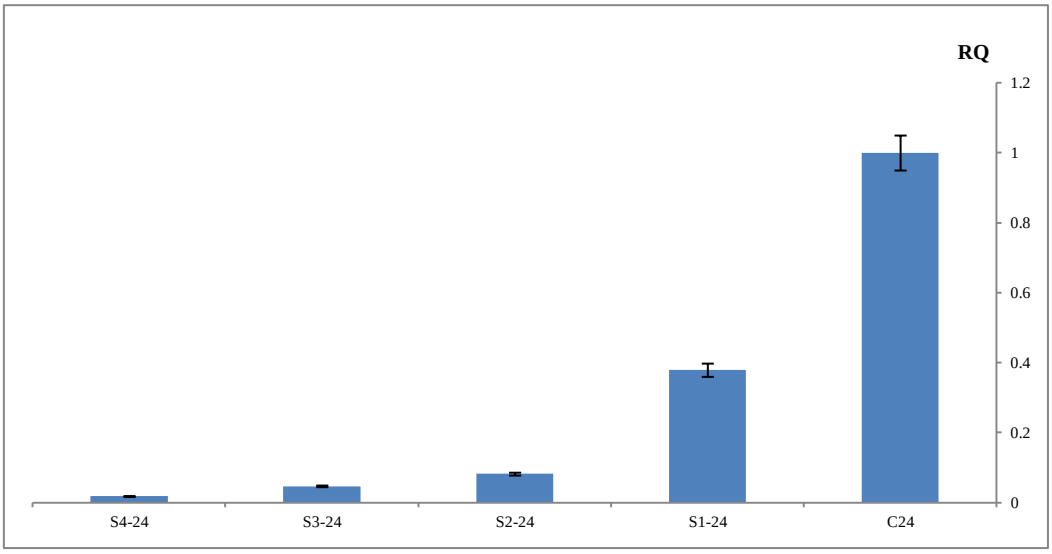

Figure 3: Comparison of the effect of the studied electron beam irradiation doses (1 to 4 kGy) after 24 h on *Sarcocystis* spp. in meat. RQ is relative quantification.

50 kGy have no effect on *C. parvum* cyst. It means that the dose of 50 kGy just can inactivate the parasite. Therefore, it seems that the minimum and maximum lethal dose varies depending on the morphological characteristics, structure of the parasite, and the establishment of the parasite. On the other hand, the effectiveness of ionizing irradiation is highly dependent on the stage of development of a parasite (Franssen et al., 2019; Hallman, 2013). For instance, for sterilization of *T. spiralis*, dose of 0.012 kGy is necessary, while for maturation inhibition of *T. spiralis*, doses of 0.020 to 0.030 kGy are efficient and for its destruction, doses of 1.4 to 6.3 kGy are needed (Munir and Federighi, 2020).

It seems that *Sarcocystis* spp. needs higher dose of irradiation to lose its viability. On the other hand, the doses from 2 to 6 kGy have positive effects on the shelf life of camel meat and increase it from two to six weeks (Al-Bachir and Zeinou, 2009). Murrell (1995) reported that the 6 kGy dose kills *Anisakis* larvae. The dose of 0.25 kGy is sufficient to eliminate *T. gondii* and *Entamoebea histolytica* from food. It seems that lower doses have no sufficient effects for the inactivation of some parasites because doses from 0.05 to 0.1 kGy have no enough power to inactivate metacestodes of *Echinococcus multilocularis* (Pohle et al., 2011).

Depending on the *Sarcocystis* species, tissue cysts differ in size and shape. They range from a few micrometers to several millimeters in length, range from narrow to wide in circumference, and have a great variety of wall structures that differ in thickness and in patterns of peripheral protrusions called cytophaneres. Seven morphologically unique wall structures were described in early reports of *Sarcocystis* (Fayer et al., 2015). Some tissue cysts have internal septa that form compartments, while in others, no septa are apparent. The septa and cytophaneres may be difficult to distinguish by light microscopy and are best seen by electron microscopy. *Sarcocystis* spp. can be found in the muscles of limbs, tongue, esophagus, diaphragm, and heart but also in neural tissue in the brain, spinal cord, and Purkinje fibers.

To prevent intestinal sarcocystosis, meat must be thoroughly cooked or frozen to kill the bradyzoites in the sarcocysts. Thorough cooking make bradyzoites noninfectious, as demonstrated in a volunteer study involving *S. suihominis* (Fayer et al., 2015). *S. meischeriana* in pork was rendered noninfectious for dogs after cooking meat at 60 °C for 20 min, 70 °C for 15 min, and 100 °C for 5 min; or frozen at -4 °C for 48 h and at -20 °C for 24 h (Fayer et al., 2015). In addition, the suitable irradiation dose of electron beam could apply to make inactivate *Sarcocystis* spp. Doses below 10 kGy are the permitted and recommended for foods (IAEA, 2015). Our results showed that 3 kGy after 24 h is adequate for inactivation of the studied parasite. However, further studied are necessary to determine meat quality and undesirable changes on the sensory characteristics of meat after exposing with irradiation.

This work also showed the usefulness of the Real time PCR for assessing viability of *Sarcocystis* spp. in meat. This method can be considered as sensitive, specific, and

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fast in comparison with bioassay methods. In addition, we cannot use bioassay for each cattle after slaughtering but it is possible to set up the SYBR Green Real time PCR in the main centers of meat monitoring organization especially after any treatment on meat. On the other hand, unlike the bacteria, there is no culturing method for many food-borne parasitic agents such as *Sarcocystis* spp.

Conclusion

Electron beam irradiation at dose of 3 kGy was effective as the optimal irradiation dose for elimination of *Sarcocystis* spp. in beef. As a result, due to the sensitivity of the *Sarcocystis* spp. to low doses of electron beam, the irradiation process could be widely applied to reduce the risk of this parasite in meat and meat products.

Author contributions

G.E. designed the study; G.E. and S.P. wrote the manuscript; M.V. analyzed the data; S.K. did the experimental work. All the authors read and approved the final manuscript.

Conflicts of interest

All the authors stated that there was not any conflict of interest.

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