The Journal of Veterinary Medical Science

FULL PAPER

Public Health

Effects of temperature, pH and curing on the viability of *Sarcocystis,* a Japanese sika deer (*Cervus Nippon centralis*) parasite, and the inactivation of their diarrheal toxin

Mioko HONDA[1)], Mamoru SAWAYA[2)], Kensuke TAIRA[3)], Akiko YAMAZAKI[4)], Yoichi KAMATA[5)], Hideki SHIMIZU[6)], Naoki KOBAYASHI[2)], Ryoichi SAKATA[3)], Hiroshi ASAKURA[7)] and Yoshiko SUGITA-KONISHI[2)]*

[1)]Department of Animal Nursing Science, Yamazaki University of Animal Health Technology, 4-7-2 Minami Osawa, Hachioji, Tokyo 192-0364, Japan
[2)]Department of Food and Life Science, Azabu University, 1-17-71 Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252-5201, Japan
[3)]Faculty of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252-5201, Japan
[4)]Department of Veterinary Sciences, University of Iwate, 3-18-8 Ueda, Morioka, Iwate 020-8550, Japan
[5)]Department of Food Design, Faculty of Nutrition, Koshien University, 10-1 Momijigaoka, Takarazuka, Hyogo 665-0006, Japan
[6)]Kyonan Public Health Department of Yamanashi Prefecture, 771-2 Kajikazawa, Fujikawa, Minamikoma, Yamanashi 400-0601, Japan
[7)]Division of Biomedical Food Research, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

J. Vet. Med. Sci.
80(8): 1337–1344, 2018
doi: 10.1292/jvms.18-0123

Received: 18 March 2018
Accepted: 20 June 2018
Published online in J-STAGE: 3 July 2018

ABSTRACT. Recently, the *Sarcocystis* parasite in horse and deer meat has been reported to be a causative agent of acute food poisoning, inducing nausea, vomiting and diarrhea. Compared with other causative agents, such as bacteria, viruses and other parasites, in deer meat, the *Sarcocystis* species parasite, including its stability under various conditions, is poorly understood. In this study, we assessed the viability of *Sarcocystis* spp. and the activity of their diarrhea toxin (a 15-kDa protein) in deer meat under conditions of freezing, cold storage, pH change and curing. In addition, the heat tolerance was assayed using purified bradyzoites. The results showed that the species lost viability by freezing at −20, −30 and −80°C for <1 hr, heating at 70°C for 1 min, alkaline treatment (pH 10.0) for 4 days and addition of salt at 2.0% for <1 day. Immunoblot assays showed that the diarrhea toxin disappeared together with the loss of viability. However, the parasite survived cooling at 0 and 4°C and acidification (pH 3.0 and 5.0) for more than 7 days with the diarrhea toxin intact. These results provide useful information for developing practical applications for the prevention of food poisoning induced by diarrheal toxin of *Sarcocystis* spp. in deer meat during cooking and preservation.

KEY WORDS: 15-kDa protein, deer meat, diarrhea toxin, *Sarcocystis*, various conditions

With the recent increase in the population of sika deer [13], the Japanese government has recommended the consumption of wild deer meat as a part of wild game cuisine. However, sanitation guidelines for wild deer meat have yet to be standardized. Epidemiological studies have accumulated evidence on the prevalence of pathogenic microbes such as bacteria, viruses and parasites in such game meat [12]. More than 20 relevant zoonotic pathogens in game meat, including deer meat, have been reported [30].

For bacteria, *Escherichia coli* O157:H7 and *Salmonella* outbreaks have been attributed to deer meat [22, 23]. A recent surveillance of microbiological agents for foodborne diseases in game meat revealed that *Enterobacteriaceae, Leptospira, Listeria, Campylobacter* and Shiga toxin-producing *E. coli* are detected mainly in fecal matter, suggesting that these bacteria can contaminate the meat during the slaughter process [5].

For viruses, hepatitis E virus (HEV) is a representative causative agent of foodborne disease. A surveillance of HEV in domestic

*Correspondence to: Sugita-Konishi, Y.: y-konishi@azabu-u.ac.jp

wild boar and deer in Japan confirmed the high prevalence of HEV in wild boar, suggesting that its consumption carries a high risk of acquiring HEV [17, 37]. Conversely, the prevalence of anti-HEV IgG antibody in sika deer in Japan was not higher and HEV RNA was not detected from 976 samples [27], although one case of HEV infection in human has been suspected of being transmitted by eating the deer meat in Japan [38].

For parasites, Trichinosis, Toxoplasmosis and Onchocerciacis are common transmissible parasitic diseases from wild animals, including deer, and a number of studies on their characterization and prevention have been performed [4, 11, 16]. Regarding *Sarcocystis* (Protozoa: Apicomplexa), many species have already been identified in other animals, such as *S. cruzi*, *S. hirsuta* and *S. bovihominis* in cattle; *S. miescheriana, S. porcifelis* and *S. suihominis* in pigs and *S. tenella* and *S. mihoensis* in sheep [1, 10, 15, 24, 33, 35, 36]. Among them, *S. bovihominis* and *S. suihominis* are known to be transmitted to humans as a final host [16] and cause a type of intestinal sarcocystosis after infection [14]. However, *S. fayeri* in horses [21] and *Sarcocystis* spp. in deer [2] have recently been reported to be a new food poisoning agent inducing gastrointestinal symptoms within 24 hr, such as nausea, vomiting and diarrhea.

Kamata *et al*. found that a 15-kDa protein of *S. fayeri* induced diarrhea and lethal toxicity in rabbits by intravenous administration and also exhibited enterotoxicity in rabbits in an ileal loop test [21]. This toxin showed homology with the actin-depolymerizing factor of *Toxoplasma gondii* and *Eimeria tenella* [21]. Irikura *et al*. also showed that the same 15-kDa protein of *S. fayeri* induced enterotoxicity in a rabbit ileal loop test using a recombinant 15-kDa protein [20]. Aoki *et al*. isolated two *Sarcocystis* spp. (*S. sybillensis* and *S. wapiti*) from deer meat that had caused food poisoning and revealed that both had a protein that reacted with the antibody of the 15-kDa protein of *S. fayeri* on immune-histochemical staining [2]. These findings showed that the 15-kDa protein of *Sarcocystis* spp. in deer meat and *S. fayeri* of horse meat were diarrheal toxins that functioned as food poisoning agents in humans.

To prevent food poisoning caused by deer meat, it is essential to understand how to inactivate the responsible toxin. In *S. fayeri*, the freezing process has been confirmed to inactivate it [18]; however, whether or not deer meat harbors *Sarcocystis* spp. has been unclear.

In this study, we focused on *Sarcocystis* in deer meat and examined the prevalence and characteristics of *Sarcocystis* spp. in 20 samples of deer meat from animals captured in Hayakawa Town, Yamanashi, South-East of Japan. The viability and inactivation of toxin of *Sarcocystis* spp. under conditions of freezing, heating, pH change and curing were also studied to determine how to inactivate *Sarcocystis* spp. in deer meat during the cooking and preservation processes.

MATERIALS AND METHODS

Source of deer meat, identification and population of Sarcocystis spp. in deer meat

Twenty Japanese sika (*Cervus Nippon centralis*) meat blocks (diaphragm and loin) were obtained from Hayakawa Town, Yamanashi, Japan, from October 2014 to October 2015 which were obtained after the deer had been shooting. The samples were removed aseptically immediately and transported to our laboratory under chilled conditions within 2 days. They were subjected to a morphological detection assay for *Sarcocystis* spp. essentially as described previously [32]. In brief, deer muscle (2.0 × 5.0 × 0.5 cm) was cut vertically from the sample and placed on a slide glass. The cysts in the specimen were observed by stereoscopic microscope with lighting from the top.

Determination of Sarcocystis viability under various temperature and pH conditions and after curing

All meat provided for the viability and toxicity experiments were trimmed of fat and connective tissue. After the prevalence of *Sarcocystis* was checked *via* the method of Saito *et al*. [32], the meat was cut into same-sized blocks and tenderized (50 g, 5 × 4 × 3 cm).

To examine the effects of temperature at cold storage and freezing on the viability of cysts in meat, a 50-g block of meat was kept at 4, 0, −20, −30 and −80°C for various periods (1 hr to 7 days). These conditions were chosen according to the study of Harada *et al*. [18]. The meat core temperature was measured by a Data logger (SK-L00TII; Sato Keiryoki Mfg., Co., Ltd., Tokyo, Japan).

For our examination of the effect of pH, an observation range of pH 3.0 to pH 10.0 was selected based on different cooking situations. A 50 g block of meat (5 × 4 × 3 cm, tenderized) was soaked in 300 m*l* of 0.1 M citric acid buffer at pH 3.0 and pH 5.0, 0.01 M phosphate buffer at pH 7.0 and 0.1 M borate buffer at pH 10.0 for various periods (1–7 days). To examine the effect of curing on the viability of cysts, a 50 g block of meat (5 × 4 × 3 cm) was soaked or rubbed with 2.0 or 6.0% NaCl and/or nitrite-enriched curing salt (NSC, NaCl enriched with 5% sodium nitrite, 10% potassium nitrate; New Shouso, Chiyoda Industry, Tokyo) for up to 7 days according to the study of Pott *et al*. [28].

The viability of cysts was determined by the presence of live bradyzoites among purified bradyzoites in the treated meat (Fig. 1). Whether bradyzoites were alive or dead was determined by microscopy after dyeing with 0.4% trypan blue (Fig. 2). When the death damage was strong, all bradyzoites were digested by pepsin, and shapes were not detected.

In each of the experiments, the day or time for measurement was based on the preliminary experiment result.

Purification of bradyzoites

After treatment under various conditions, meat blocks were digested by pepsin, as shown in Fig. 1. In brief, a 50-g block of meat was sliced with a knife, placed in a plastic bag, and digested by 0.25% pepsin solution (pH 2.0) with shaking for 10 min at 37°C.

50 g of block of meat (5 cm × 4 cm × 3 cm)
⇩
Slice by knife
⇩
Digestion by 0.25% pepsin pH 2.0 at 37°C for 10 min. with shaking
⇩
Filtration with 40-mesh membrane
⇩
Centrifugation at 400 × *g* for 10 min at 4°C
⇩
Resuspension of the precipitates with PBS
⇩
Layer of the suspension on 90% Percoll solution
(Percoll : 9% NaCl solution = 9 : 1)
⇩
Centrifugation 400 × *g* for 10 min at 4°C
⇩
Wash of the precipitates with PBS for 3 times
⇩
Isolation of the purifed bradyzoites

Fig. 1. Preparation of purified bradyzoites.

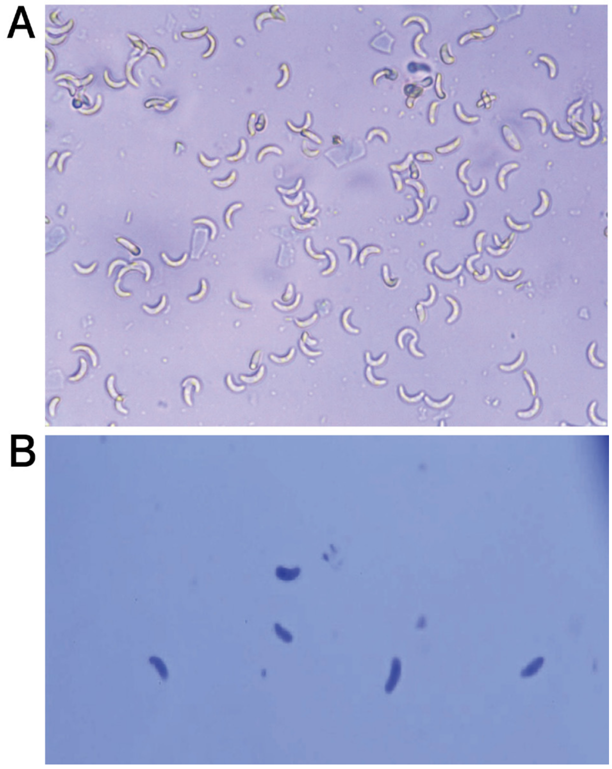

Fig. 2. Typical figures of bradyzoites. A: Live stage, B: Dead stage. Live bradyzoites are indicated by a glossy banana shape.

After filtering the solution through a 40-mesh membrane, the filtrate was centrifuge at 400 × *g* for 10 min at 4°C. The precipitate was then dissolved in 1 m*l* of phosphate-saline buffer (PBS) and layered on 90% Percoll solution with 9% NaCl (Percoll; GE Healthcare Life Sciences, Chicago, IL, U.S.A.). The solution was then centrifuged at 400 × *g* for 10 min at 4°C, and the precipitate was collected and washed with PBS 3 times.

Determination of bradyzoite viability after heating treatment

Bradyzoites were purified from fresh deer meat (diaphragm) *via* the method described above. Approximately 5×10^5 bradyzoites/m*l* in 200 μl of PBS were sealed in microcaps (Drummond Scientific, Broomall, PA, U.S.A.), followed by soaking in a water bath at 55, 60, 65 and 70°C for 1 min. The microcaps were then cooled immediately by placing on crushed ice. The bradyzoites were removed from the microcaps and counted in a Burker-Turk chamber. The live or dead bradyzoites were counted by microscopy using trypan blue stain. The survival rate of bradyzoites was calculated as follows:

$$\text{Survival rate}(\%) = \frac{\text{number of live bradyzoite}}{\text{number of live bradyzoite} + \text{number of dead bradyzoite}} \times 100$$

Western blot detection of a 15-kDa protein

The toxic activity of 15-kDa protein in the parasites of the meat samples after the treatments was evaluated by an immunoblot assay for the detection of a 15-kDa protein. The bradyzoites were purified from each meat sample, and approximately 1×10^7 bradyzoites were then boiled in sample buffer (Atto Corp., Tokyo, Japan) at 96°C for 3 min. Proteins were loaded and separated on 15% acrylamide gels, followed by transfer onto a polyvinylidene fluoride (PVDF) membrane. Proteins on the membrane were blocked with EzBlock Chemi (Atto Corp.) and then incubated with Rabbit anti-15-kDa protein of *S. fayeri* antibody (polyclonal antibody) in XL-enhancer solution 1 (Apro Life Science Institute, Inc., Naruto, Japan) (TBS, pH 7.6). The membrane was washed with 0.1% Tween 20 in 0.05 M TBS and incubated with horseradish peroxidase-labeled anti-rabbit IgG antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, U.S.A.) in XL-enhancer solution 2 (Apro Life Science Institute, Inc.) . The immune-reacted proteins were detected by using Ez West Blue (Atto Corp.).

Table 1. Summary for the detection of *Sarcocystis* spp. in deer meat from animals captured in Yamanashi prefecture (n=20)

Species	*S. sybillensis*	*S. wapiti*	*S. hofmanni*	Others	Total
Numbers of cysts (%)	190 (30.5)	396 (63.7)	6 (1.0)	30 (4.8)	622 (100)

Table 2. Viability of *Sarcocystis* in cold storage

Temperature (°C)	Day 1	Day 3	Day 5	Day 7
4	+	+	+	+
0	+	+	+	+

+: Live bradyzoites were detected from the sample by microscopic assay. The sample size was 50 g (5 × 4 × 3 cm).

Table 3. Viability of bradyzoites of *Sarcocystis* spp. in deer meat under frozen conditions

Freezing temperature (°C)	1 hr	2 hr
−20	-	-
−30	-	NA
−80	-	NA

-: No live bradyzoites were detected from the sample by microscopic assay. NA: not analyzed.

Table 4. Viability of *Sarcocystis* in deer meat under various pH conditions

pH range	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
3.0	+	+	+	+	+	+	+
5.0	NA	NA	NA	NA	NA	NA	+
7.0	NA	NA	NA	NA	NA	NA	+
10.0	+	+	+	-	NA	NA	NA

+: Live bradyzoites were detected from the sample by microscopic assay. -: No live bradyzoites were detected from the sample by microscopic assay. NA: not analyzed.

RESULTS

The population of Sarcocystis spp. in deer meat

A morphological detection assay for *Sarcocystis* spp. in deer meat samples showed that all 20 samples obtained in Yamanashi prefecture, Japan were contaminated (Table 1). The morphological characterization of the *Sarcocystis* spp. detected in deer meat was performed according to the methods of Dubey *et al*. [6, 9]. The parasites were classified into *S. wapiti*, *S. sybillensis*, *S. hofmanni* and others, and the population was found at rates of 63.7, 30.5, 1.0 and 4.8%, respectively (Table 1). These results demonstrated that *Sarcocystis* spp. in deer meat samples obtained from Yamanashi pref. were predominantly *S. wapiti* and *S. sybillensis* dominantly.

The effect of temperature, pH conditions and salt on the viability of Sarcocystis

In experiments performed at chilled temperatures and different pH conditions, the samples were stored for 7 days to avoid bacterial spoilage. Table 2 shows the viability of *Sarcocystis* in 50-g blocks of meat kept at 4 and 0°C. *Sarcocystis* survived for at least 7 days at both temperatures. In the experiments at freezing temperatures (−20, −30 and −80°C), the time to taken to reach the set temperature at the core of the meat was 2 hr for −20°C, 7 hr for −30°C, and 8 hr for −80°C. Taking into account the time needed to reach the set temperature, the cysts were killed as soon as the set temperature was reached in all cases (Table 3).

Regarding the effects of pH on the viability of *Sarcocystis*, the neutral (pH 5.0 and 7.0) and acidic (pH 3.0) conditions seemed to have no effect after at least 7 days at 4°C; however, under an alkaline condition (pH 10.0), it took 4 days for viability to be lost at 4°C, as shown in Table 4. For all pH conditions in this study, the pH variation of soaked solution during each experiment was ± 1 at 4°C (data not shown). Acidic and alkaline conditions did not effectively reduce the viability of the parasites.

Table 5 showed the effects of curing on the viability of *Sarcocystis*. The *Sarcocystis* parasites were killed within 1 day by the combination of 6.0% salt and 2.0% NCS (Sample ID: A), the combination of 2.0% salt and 0.25% NCS (Sample ID: B), 6.0% salt only (Sample ID: C) and 2.0% salt only (Sample ID: D). However, <1.0% NCS alone failed to kill organisms within 7 days. These findings indicate that more than 2.0% salt and NCS were effective in reducing the viability of *Sarcocystis*.

The effect of heating on the viability of bradyzoites

Since *Sarcocystis* spp. in deer was non-infective parasite, we could not use bioassay like that used for *Toxoplasma gondii* to determine of viability [8]. To assess the heat tolerance of these parasite, the viability of bradyzoites was observed directly. The purified bradyzoites were sealed in glass capillary tubes and heated for 1 min at various temperatures. The survival rates of the bradyzoites were as follows: 55°C, 94.44%; 60°C, 77.62%; 65°C, 22.02%; and 70°C, 0.00% (Fig. 3). The viability of *Sarcocystis* was completely lost by heating to 70°C for 1 min.

Table 5. Viability of bradyzoites of *Sarcocystis* spp. in deer meat with addition of salt

Sample ID	NaCl (%)	NCS (%)	Day 1	Day 3	Day 5	Day 7
A	6.0	2.0	-	NA	NA	NA
B	2.0	0.25	-	NA	NA	NA
C	6.0	-	-	NA	NA	NA
D	2.0	-	-	NA	NA	NA
E	-	2.0	-	NA	NA	NA
F	-	1.0	+	+	+	+
G	-	0.5	+	+	+	+
H	-	0.25	+	+	+	+

+: Live bradyzoites were detected from the sample by microscopic assay. -: No live bradyzoites were detected from the sample by microscopic assay. NA: not analyzed. NCS: nitrite-enriched curing salt.

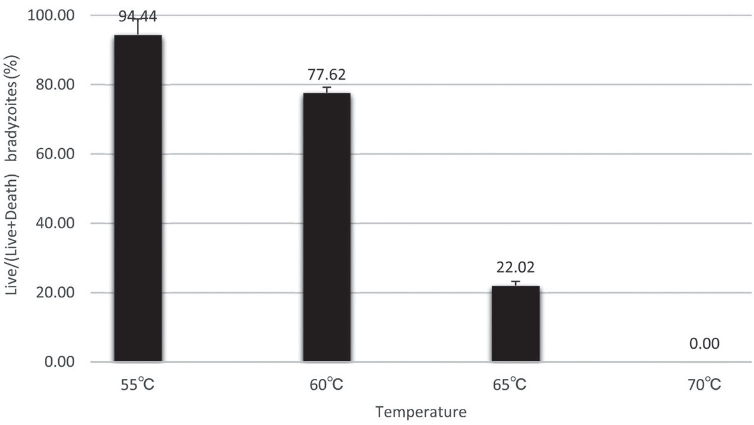

Fig. 3. The effects of heating on the viability of bradyzoites of *Sarcocystis* spp. in deer meat. The experiments were performed triplicate and calculated as the average and standard error.

The effects of temperature and pH conditions on the activity of diarrheal toxin (15-kDa protein) of Sarcocystis in deer meat

To assess the toxicity of *Sarcocystis* in various conditions, we confirmed the presence of 15-kDa protein after digestion by pepsin, which was detected Western blotting using the anti-15-kDa of *S. fayeri*. Because bradyzoites and 15-kDa protein in live cyst was not digested by pepsin in the condition used in this study [18].

The 15-kDa protein would express diarrheal toxicity when intact conformation was maintained after pepsin digestion in stomach. Thus, to determine the inactivation of the 15-kDa protein by various conditions, we confirmed the presence of the 15-kDa protein after pepsin digestion by Western blotting using the anti-15-kDa of *S. fayeri* antibody. Figure 4 shows the Western blotting patterns of bradyzoites purified from treated meat maintained at various temperatures (A) and pH conditions (B). The bradyzoites purified from the meat kept at 0°C and 4°C for 7 days retained the immune-reactive band located at 15-kDa, just like the positive control for *S. fayeri*. However, there was no band in the bradyzoites purified from the samples kept at −20°C for 2 hr or −30°C and −80°C for 1 hr. In addition, the bradyzoites purified from the meat soaked in pH 3.0, 5.0 and 7.0 buffers for 7 days and in pH 10.0 buffer for 3 days still had a detectable 15-kDa band that reacted with the antibody; however, the band disappeared in the meat samples that were soaked for 4 days at pH 10.0 (Fig. 4A and 4B).

These results showed that live bradyzoites retained the 15-kDa protein after pepsin digestion, while the dead parasites did not despite maintaining their overall shape. This suggests that the toxicity of the 15-kDa protein was associated with the viability of *Sarcocystis* in deer meat.

DISCUSSION

Parasitic foodborne disease is mainly caused by zoonotic parasites, such as *Trichinella spiralis* and *T. gondii*, as most such parasites are transmissible between multiple hosts. Thus, risk assessments and the preventative measures designed for these parasitic food borne diseases should be performed to evaluate their infectious activities.

However, in recent years, parasitic foodborne diseases have also been induced by non-transmissible parasites, such as *S. fayeri*, which can cause food poisoning via a diarrheal toxin associated with the consumption of horse meat [21]. Indeed, Aoki *et al*. recently reported that the same type of food poisoning was caused by the consumption of Japanese sika deer meat (venison) in

Japan [2]. We must therefore make an effort to reduce the risk of parasite contamination in order to prevent food poisoning by non-transmissible parasites.

In the present study, we first examined the frequency of *Sarcocystis* spp. contamination and its population in deer meat samples. This study revealed the high prevalence of *Sarcocystis* spp. infection in deer meat captured from Yamanashi prefecture, Japan. Among the *Sarcocystis* spp. detected in deer meat, *S. wapiti* and *S. sybillensis* were the predominant species.

Matsuo *et al*. surveyed *Sarcocystis* infections in 64 Japanese sika deer (*C. Nippon centralis*) caught in Hyogo prefecture, in the middle of Honshu island, Japan, and found that *Sarcocystis* spp. was detected in 81.3% of individual muscles [26]. Matsuo *et al*. [25] surveyed *Sarcocystis* spp. infections in 63 Japanese sika deer captured in Gifu prefecture (also in the middle of Honshu island), Japan, and reported that 95.2% of samples were infected. Saito *et al*. reported that 124 of 136 Japanese sika deer (*C. Nippon centralis*) and wild sika deer (*C. Nippon yesoensis*) were positive for *Sarcocystis* spp. infection (91.2%) [36]. Dubey *et al.* reported that *S. wapiti* and *S. sybillensis* were detected in North American elk [7]. Saito *et al*. reported that *S. wapiti* and *S. sybillensis* were detected in 98% and 100% of all of infected samples from Japanese sika deer, respectively [34]. These other surveillance studies also supported our results and indicated that Japanese sika deer were infected with *Sarcocystis* spp.*,* predominantly *S. wapiti* and *S. sybillensis*.

Next, we examined the conditions that induced a loss of parasite viability and the inactivation of the diarrheal toxin of *Sarcocystis* spp. in meat samples, with different conditions reflecting aspects of the preparation and cooking processes. Kamata *et al*. previously identified a 15-kDa protein as a diarrheal toxin and causative component of acute food poisoning in *S. fayeri* [21]. Saito *et al*. found that *S. wapiti* and *S. sybillensis* originating from Japanese sika deer expressed the 15-kDa protein, which also induced enterotoxicity in a rabbit ileal loop test [31].

Our data showed that the inactivation of toxins in deer meat was observed as soon as the set temperature was reached at −20, −30 and −80°C (Fig. 3), demonstrating that the effectiveness of freezing for *Sarcocystis* spp. was in agreement with the findings of a previous study in which the expression of the 15-kDa protein by *S. fayeri* in horse meat tended to decrease after freezing [18]. The *Sarcocystis* spp. found in the deer meat samples in the present study might be more susceptible to freezing than the *S. fayeri* in horse meat, as the inactivation of *S. fayeri* toxin required holding for 48, 36, or 12 hr at −20, −30, or −60°C, respectively [18]. On the other hand, the *Sarcocystis* spp. in the deer meat indicated resistant to pH treatment except an alkaline condition (pH 10.0) (Fig. 4). A previous study showed that the infectious activity of *T. gondii* cysts was maintained for 26 days under a neutral condition (pH 5.0) [28]. These findings suggested that pH adjustment might not be adequate for controlling contamination by these parasites in meat.

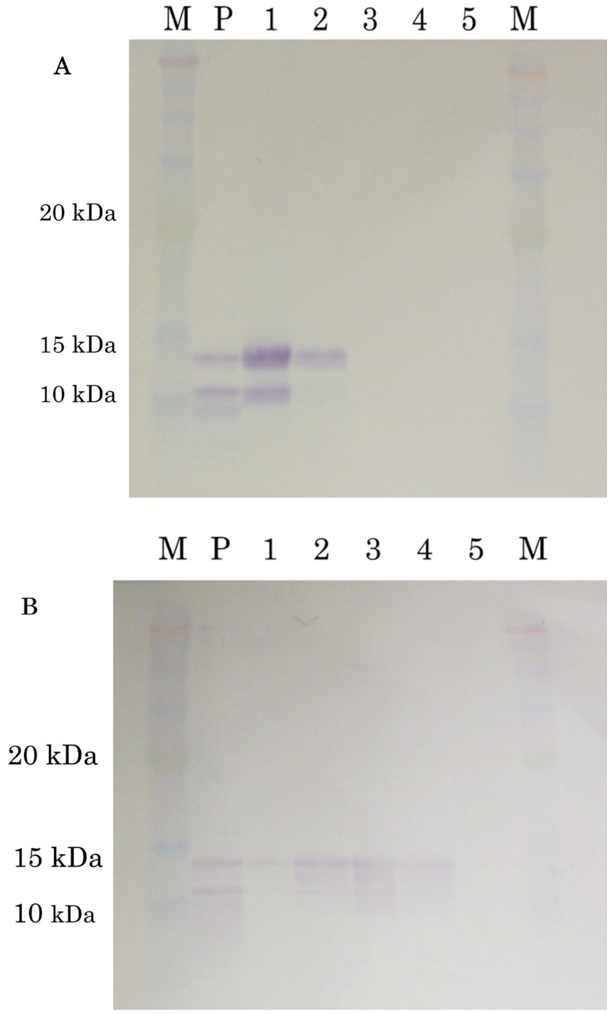

Fig. 4. Immunoblotting pattern of the 15-kDa protein in *Sarcocystis* spp. in deer meat under various temperature (A) and pH (B) conditions. The 15-kDa protein was detected by an immunoblot assay using anti-15-kDa protein of *Sarcocystis fayeri* cysts. A) M: Marker, P: *Sarcocystis fayeri* cysts, 1: 4°C at Day 7, 2: 0°C at Day 7, 3: −20°C at 2 hr, 4: −30°C at 1 hr, 5: −80°C at 1 hr post-incubation. B) M: Marker, P: *Sarcocystis fayeri* cysts, 1: pH 3.0 on Day 7, 2: pH 5.0 on Day 7, 3: pH 7.0 on Day 7, 4: pH 10.0 on Day 3, 5: pH 10.0 on Day 4.

The manufacturing of sausage and bacon requires the addition of sodium chloride to the meat during processing. Our data demonstrated that the sodium concentration affected the viability of *Sarcocystis* spp. in deer meat. It is likely that 2.0% NaCl eliminated the infectivity of *T. gondii* cysts within 8 hr [19]. *Sarcocystis* viability in deer meat may therefore have shown a similar tendency on exposure to NaCl. Indeed, the Canada Food Inspection Agency recommends the use of >3.3% NaCl for the manufacturing of dry-cured fermented sausage in order to control parasites, based on the sensitivity of *Trichinella* in pork [3].

Heating is another method suitable for killing pathogenic parasites. In our study, the viability was examined using purified bradyzoites and the lethal condition for *Sarcocystis* parasites was at 70°C for 1 min. Dubey *et al*. reported that *T. gondii* cysts in a small pork sample (2-mm thick) retained their infective ability after 9 min at 52°C, but that they lost their infectivity after 3.5 min at 61°C [8]. Compared with the infective ability of *Trichinella* spp.*, Sarcocystis* spp. is relatively resistant to heat. Purslow *et al*. recently expressed concern about the United States Department of Agriculture (USDA) recommendation to reduce the minimum core temperature of pork while cooking from 71.11°C (160°F) to 63.33°C (145°F) unless using an extended cooking time [29].

The authors pointed out that the risk assessment data for achieving a complete loss of *Trichilla*, *Toxoplasma* spp. and other parasite infectivity should be taken into account when drafting recommendations or guidelines even though many people prefer the increased sensory qualities of pork [39]. We feel that the data obtained in the present study will be useful for establishing guidelines concerning the preparation of game meat.

Taken together, we found that meat from Japanese sika deer was frequently infected *Sarcocystis* spp., mainly with *S. sybillensis* and *S. wapiti*. Since these *Sarcocystis* spp. are non-transmissible parasites, to prevent the food poisoning caused by these parasites, we focused on the determination of the conditions that caused a loss parasite viability of parasite and toxin inactivation. Our data also clearly indicated that the *Sarcocystis* in deer meat was highly tolerant to cold temperatures and pH changes (pH 3.0 to pH 10.0), and adequate freezing, heating and curing conditions are recommended for the cooking and storage of deer meat in order to efficiently reduce the risk of parasite contamination of game meat. Although further studies will be necessary to clarify whether or not the 15-kDa protein is responsible for the enterotoxicity induced by *Sarcocystis* detected in deer meat, the 15-kDa protein might be a useful indicator for determining the possibility of food poisoning via non-transmissible *Sarcocystis* spp.

ACKNOWLEDGMENT. This study was financially supported in part by the grant from Ministry of Health, Labour and Welfare, Japan (H27-shokuhin-ippan-011).

REFERENCES

1. Ashford, R. W. 1977. The fox, Vulpes vulpes, as a final host for Sarcocystis of sheep. *Ann. Trop. Med. Parasitol.* **71**: 29–34. [Medline] [CrossRef]
2. Aoki, K., Ishikawa, K., Hayashi, K., Saito, M., Sugita-Konishi, Y., Watanabe, M. and Kamata, Y. 2013. An outbreak of suspected food poisoning related to deer meat containing *Sarcocystis* cysts. *Jpn. J. Food Microbiol.* **30**: 28–32. [CrossRef]
3. Canadian Food Inspection Agency Meat Hygiene Manual of Procedures. 2017. Annex A: Curing –Approved curing methods to ensure the destruction on *Trichinella* in sausages and other meat. Products containing striated pork muscle tissues. http://www.inspection.gc.ca/food/meat-and-poultry-products/manual-of-procedures/chapter-4/annex-a/eng/1370451068611/1370451132888 [accessed on March 6, 2018].
4. Davies, P. R. 2011. Intensive swine production and pork safety. *Foodborne Pathog. Dis.* **8**: 189–201. [Medline] [CrossRef]
5. Díaz-Sánchez, S., Sánchez, S., Herrera-León, S., Porrero, C., Blanco, J., Dahbi, G., Blanco, J. E., Mora, A., Mateo, R., Hanning, I. and Vidal, D. 2013. Prevalence of Shiga toxin-producing *Escherichia coli, Salmonella* spp. and *Campylobacter* spp. in large game animals intended for consumption: relationship with management practices and livestock influence. *Vet. Microbiol.* **163**: 274–281. [Medline] [CrossRef]
6. Dubey, J. P. and Kreiler, J. P. 1977. *Toxoplasma, Hammondia, Babesia, Sarcocytis,* and other tissue cyst-forming coccidian of man and animals. pp.101–237. In: Parasaitic Protozoa, Vol III. (Kreiler, J. P. ed.), Academic Press, New York.
7. Dubey, J. P., Jolley, W. R. and Thorne, E. T. 1983. *Sarcocystis sybillensis* sp, nov from North American elk (*Cervus elaphus*). *Can. J. Zool.* **61**: 737–742. [CrossRef]
8. Dubey, J. P., Kotula, A. W., Sharar, A., Andrews, C. D. and Lindsay, D. S. 1990. Effect of high temperature on infectivity of Toxoplasma gondii tissue cysts in pork. *J. Parasitol.* **76**: 201–204. [Medline] [CrossRef]
9. Dubey, J. P., Saville, W. J., Sreekumar, C., Shen, S. K., Lindsay, O. S., Pena, H. F., Vianna, M. C., Gennari, S. M. and Reed, S. M. 2002. Effects of high temperature and disinfectants on the viability of *Sarcocystis neurona* sporocysts. *J. Parasitol.* **88**: 1252–1254. [Medline] [CrossRef]
10. Dubey, J. P., Streitel, R. H., Stromberg, P. C. and Toussant, M. J. 1977. *Sarcocystis fayeri* sp. n. from the horse. *J. Parasitol.* **63**: 443–447. [Medline] [CrossRef]
11. Endeshaw, T., Taye, A., Tadesse, Z., Katabarwa, M. N., Shafi, O., Seid, T. and Richards, F. O. Jr. 2015. Presence of *Wuchereria bancrofti* microfilaremia despite 7 years of annual ivermectin monotherapy mass drug administration for onchocerciasis control: a study in north-west Ethiopia. *Pathog. Glob. Health* **109**: 344–351. [Medline] [CrossRef]
12. Erickson, M. C. 2017. Overview: Foodborne in wildlife populations. pp. 1–30. *In*: Food Safety Risks from Wildlife (Jay-Russell, M. and Doyle, M. P. eds.), Springer International Publishing AG, Cham.
13. Environment Agency The population estimation of nihon deer and wild boar by statistical method. http://www.env.go.jp/press/files/jp/26914.pdf [accessed on February 22, 2018].
14. Fayer, R. 2004. *Sarcocystis* spp. in human infections. *Clin. Microbiol. Rev.* **17**: 894–902. [Medline] [CrossRef]
15. Fayer, R., Esposito, D. H. and Dubey, J. P. 2015. Human infections with *Sarcocystis* species. *Clin. Microbiol. Rev.* **28**: 295–311. [Medline] [CrossRef]
16. Guo, M., Dubey, J. P., Hill, D., Buchanan, R. L., Gamble, H. R., Jones, J. L. and Pradhan, A. K. 2015. Prevalence and risk factors for *Toxoplasma gondii* infection in meat animals and meat products destined for human consumption. *J. Food Prot.* **78**: 457–476. [Medline] [CrossRef]
17. Hara, Y., Terada, Y., Yonemitsu, K., Shimoda, H., Noguchi, K., Suzuki, K. and Maeda, K. 2014. High prevalence of hepatitis E virus in wild boar (*Sus scrofa*) in Yamaguchi Prefecture, Japan. *J. Wildl. Dis.* **50**: 378–383. [Medline] [CrossRef]
18. Harada, S., Furukawa, M., Tokuoka, E., Matsumoto, K., Yahiro, S., Miyasaka, J., Saito, M., Kamata, Y., Watanabe, M., Irikura, D., Matsumoto, H. and Sugita-Konishi, Y. 2013. [Control of toxicity of Sarcocystis fayeri in horsemeat by freezing treatment and prevention of food poisoning caused by raw consumption of horsemeat]. *Shokuhin Eiseigaku Zasshi* **54**: 198–203. [Medline] [CrossRef]
19. Hill, D. E., Benedetto, S. M., Coss, C., McCrary, J. L., Fournet, V. M. and Dubey, J. P. 2006. Effects of time and temperature on the viability of *Toxoplasma gondii* tissue cysts in enhanced pork loin. *J. Food Prot.* **69**: 1961–1965. [Medline] [CrossRef]
20. Irikura, D., Saito, M., Sugita-Konishi, Y., Ohnishi, T., Sugiyama, K. I., Watanabe, M., Yamazaki, A., Izumiyama, S., Sato, H., Kimura, Y., Doi, R. and Kamata, Y. 2017. Characterization of Sarcocystis fayeri's actin-depolymerizing factor as a toxin that causes diarrhea. *Genes Cells* **22**: 825–835. [Medline] [CrossRef]
21. Kamata, Y., Saito, M., Irikura, D., Yahata, Y., Ohnishi, T., Bessho, T., Inui, T., Watanabe, M. and Sugita-Konishi, Y. 2014. A toxin isolated from *Sarcocystis fayeri* in raw horsemeat may be responsible for food poisoning. *J. Food Prot.* **77**: 814–819. [Medline] [CrossRef]
22. Keene, W. E., Sazie, E., Kok, J., Rice, D. H., Hancock, D. D., Balan, V. K., Zhao, T. and Doyle, M. P. 1997. An outbreak of *Escherichia coli* O157:H7 infections traced to jerky made from deer meat. *JAMA* **277**: 1229–1231. [Medline] [CrossRef]
23. Madar, C. S., Cardile, A. P., Cunningham, S., Magpantay, G. and Finger, D. 2012. A case of *Salmonella* gastroenteritis following ingestion of raw venison sashimi. *Hawaii J. Med. Public Health* **71**: 49–50. [Medline]

24. Markus, M. B. 1978. Sarcocystis and sarcocystosis in domestic animals and man. *Adv. Vet. Sci. Comp. Med.* **22**: 159–193. [Medline]
25. Matsuo, K., Uretsu, H., Takashima, Y. and Abe, N. 2016. High occurrence of *Sarcocystis* infection in Sika deer *Cervus Nippon centralis* and Japanese wild boar *Sus acrofa leucomystax* and molecular characterization of *Sarcocystis* and *Hepatozoon* isolated from their muscles. *Jpn. J. Zoo Wildl. Med.* **21**: 35–40. [CrossRef]
26. Matsuo, S., Morita, T., Imai, S. and Ike, K. 2014. Prevalence of *Sarcocystis* in Japanese Sika Deer (*Cervus Nippon centralis*) in Hyogo prefecture, Japan. *J. Vet. Epidemiol.* **18**: 124–129. [CrossRef]
27. Matsuura, Y., Suzuki, M., Yoshimatsu, K., Arikawa, J., Takashima, I., Yokoyama, M., Igota, H., Yamauchi, K., Ishida, S., Fukui, D., Bando, G., Kosuge, M., Tsunemitsu, H., Koshimoto, C., Sakae, K., Chikahira, M., Ogawa, S., Miyamura, T., Takeda, N. and Li, T. C. 2007. Prevalence of antibody to hepatitis E virus among wild sika deer, Cervus nippon, in Japan. *Arch. Virol.* **152**: 1375–1381. [Medline] [CrossRef]
28. Pott, S., Koethe, M., Bangoura, B., Zöller, B., Daugschies, A., Straubinger, R. K., Fehlhaber, K. and Ludewig, M. 2013. Effects of pH, sodium chloride, and curing salt on the infectivity of Toxoplasma gondii tissue cysts. *J. Food Prot.* **76**: 1056–1061. [Medline] [CrossRef]
29. Purslow, P. 2016. Parasitic zoonoses present some risks with low-temperature cooking of pork. *Meat Sci.* **119**: 14–15. [Medline] [CrossRef]
30. Ruiz-Fons, F. 2017. A Review of the Current Status of Relevant Zoonotic Pathogens in Wild Swine (Sus scrofa) Populations: Changes Modulating the Risk of Transmission to Humans. *Transbound. Emerg. Dis.* **64**: 68–88. [Medline] [CrossRef]
31. Saito, M., Arai, Y., Kamata, Y., Sugita-Konishi, Y. and Hashimoto, K. 2013. Entetotoxicity of extract from *Sarcocystis* Cysts with Canine Final Host in Japan. *J. Jpn. Vet. Assoc.* **66**: 725–727. [CrossRef]
32. Saito, M., Hachisu, K., Iwaski, K., Nakazima, T., Watanabe, A., Moriya, H. and Itagaki, H. 1984. A new simple method for detection of bovine *Sarcocystis* cysts. *Nippon Juishikai Zasshi* **37**: 158–162.
33. Saito, M., Shibata, Y., Kobayashi, T., Kobayashi, M., Kubo, M. and Itagaki, H. 1996. Ultrastructure of the cyst wall of *Sarcocystis* species with canine final host in Japan. *J. Vet. Med. Sci.* **58**: 861–867. [Medline] [CrossRef]
34. Saito, M., Shibata, Y., Kobayashi, T., Kubo, M. and Itagaki, H. 1998. *Sarcocystis* spp. from Sika Deer, *Cervus Nippon yesoensis*. *J. Jpn. Vet. Assoc.* **51**: 683–686. [CrossRef]
35. Saito, M., Shibata, Y., Kubo, M. and Itagaki, H. 1997. Sarcocystis mihoensis n. sp. from sheep in Japan. *J. Vet. Med. Sci.* **59**: 103–106. [Medline] [CrossRef]
36. Saito, M., Shibata, Y., Ohno, A., Kubo, M., Shimura, K. and Itagaki, H. 1998. Sarcocystis suihominis detected for the first time from pigs in Japan. *J. Vet. Med. Sci.* **60**: 307–309. [Medline] [CrossRef]
37. Sonoda, H., Abe, M., Sugimoto, T., Sato, Y., Bando, M., Fukui, E., Mizuo, H., Takahashi, M., Nishizawa, T. and Okamoto, H. 2004. Prevalence of hepatitis E virus (HEV) Infection in wild boars and deer and genetic identification of a genotype 3 HEV from a boar in Japan. *J. Clin. Microbiol.* **42**: 5371–5374. [Medline] [CrossRef]
38. Tei, S., Kitajima, N., Takahashi, K. and Mishiro, S. 2003. Zoonotic transmission of hepatitis E virus from deer to human beings. *Lancet* **362**: 371–373. [Medline] [CrossRef]
39. United States Department of Agriculture Food Safety and Inspection Service Fresh Pork from Farm to Table. https://www.fsis.usda.gov/wps/portal/fsis/topics/food-safety-education/get-answers/food-safety-fact-sheets/meat-preparation/fresh-pork-from-farm-to-table/ct_index [accessed on March 6, 2018].