Gamma-ray Sterilization and Residual Toxicity Studies of Ground Beef Inoculated with Spores of *Clostridium botulinum*[1]

LLOYD L. KEMPE AND JOHN T. GRAIKOSKI

Departments of Chemical and Civil Engineering, The University of Michigan, Ann Arbor, Michigan

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ABSTRACT

KEMPE, LLOYD L. (University of Michigan, Ann Arbor), AND JOHN T. GRAIKOSKI. Gamma-ray sterilization and residual toxicity studies of ground beef inoculated with spores of *Clostridium botulinum*. Appl. Microbiol. **10**:31–36. 1962.—Inoculated packs of cooked and raw ground beef were sterilized with gamma radiation from cobalt-60. With inocula of 5,000,000 *Clostridium botulinum* 213B spores per g of cooked ground beef, 3.8 megarad were required for sterilization; in raw ground beef, 3.72 megarad sterilized the meat when inocula of 1,700,000 *C. botulinum* 213B spores were used per g. Using *C. botulinum* 62A spores, cooked ground beef inoculated with 5,200,000 spores per g was sterilized with 3.85 megarad; raw ground beef, inoculated with 2,670,000 spores per g, was sterilized with 3.6 megarad. Cans of meat that were considered sterile by lack of culture growth after incubation for at least 6 months and, in some instances, as long as 5 years, were tested for the presence of botulinus toxin. No toxin was found in any meat taken from inoculated packs prepared from *C. botulinum* 213B spores; however, all cans of meat that had been inoculated with more than 2,670,000 *C. botulinum* 62A spores per g of meat, contained type A toxin. It was shown that these latter inocula of heat-shocked spores, by themselves, contained sufficient toxin to kill mice. However, more toxin appeared to be present than could be ascribed to the unirradiated spores alone. This finding is discussed.

The amount of gamma radiation required to sterilize canned ground beef inoculated with 40,000 spores of *Clostridium botulinum* per g has been previously determined by Kempe, Graikoski, and Gillies (1954). The present paper reports sterilization levels required for both precooked and raw ground beef inoculated with as many as 6,600,000 such spores per g.

In addition, the possibility that radiation-sterilized meat may still contain or develop botulinus toxin even after radiation processing has been a matter of concern. Possible sources of such toxin include at least three. For example, such toxin may have formed during growth of a *C. botulinum* culture in the meat before radiation processing. Since the amount of radiation needed to inactivate botulinus toxin is considerably more than that needed for sterilization, such toxin could remain after radiation processing (Wagenaar and Dack, 1960). Alternatively, *C. botulinum* spores may have been inactivated by irradiation but still be capable of germination and growth through one or two vegetative cycles. In this case, the question arises whether toxin would result and, if so, whether enough toxin would be formed to be detectable. As a third possibility, the spore inocula themselves may have contained detectable toxin even after heat shocking.

To examine these possibilities, inoculated cans of ground beef that had previously been sterilized by gamma radiation and then incubated in our laboratory were tested. Some of this meat had been radiation processed as early as 1953.

MATERIALS AND METHODS

Sterilization Studies

Spores. The spores of *C. botulinum* used in this study were originally obtained from the Hooper Foundation for Medical Research at the University of California. *C. botulinum* 213B spore suspensions were prepared and counted according to procedures described by Reed, Bohrer, and Cameron (1951) except that Difco Bacto-casitone[2] was substituted for casein digest in their medium. The *C. botulinum* 62A spores were grown in the liver broth described by these authors. Stock spore suspensions were prepared in sterile distilled water, frozen, and then stored at −40 C until needed. Identity of the spores was verified by toxin neutralization tests of the culture media, as well as by heat-resistance studies of the spores and the usual staining and microscopic techniques. Appropriate dilutions for inoculation into canned meat were prepared counting the viable spores present in the stock suspension. Additionally, for this purpose, 0.1% soluble

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[2] Difco Laboratories, Inc., Detroit, Mich.

starch was incorporated into the pork agar medium to aid germination of the spores (Wynne and Foster, 1948).

Cooked ground beef. Samples for irradiation were prepared from lean beef that was kept refrigerated during grinding and until used. For a run, the ground beef was placed in shallow enameled pans and cooked for 30 min in an autoclave at 15-lb steam pressure. Mushroom-type (202×202) cans were then filled within ¼ in. of the top with hot meat, covered loosely by can lids, and sterilized in an autoclave at 121 C for 60 min. Individual cans were removed from the autoclave as needed; their covers were aseptically lifted, and 1 ml of a properly diluted spore suspension was injected into the geometrical center of the meat. This method of inoculation did not result in a uniform spore distribution throughout the meat, but concentrated the spores in the center of the can. Finally, the cans were sealed in a Western-type of closing machine. Since the meat was still at a temperature of approximately 95 C, the cans were immersed in running tap water for 30 min; this cooled the meat to an average temperature of 20 C and produced a vacuum in the cans. The cans to be irradiated were then either cooled in a refrigerator to about 4 C or immediately placed in the radiation room. Here the meat cooled to 5 C, or below, during irradiation. Control cans were placed in an incubator at 29 C.

Raw ground beef. Lean ground beef was spread into shallow enameled plans and placed in an evacuation chamber; then dissolved metabolic gases and oxygen were removed by evacuation to 25 in. of Hg. The evacuation procedure was repeated three times, after which the meat was packed into mushroom-type (202×202) cans, inoculated, and sealed in a commercial-type, vacuum closing machine under a vacuum of 29 in. of Hg. The meat was kept below 5 C throughout this process. Experimental cans were then either irradiated, temporarily stored under refrigeration, or incubated at 29 C as indicated.

For irradiation, the cans of meat were placed in the center well of the large cobalt-60 gamma-radiation source at the Fission Products Laboratory of The University of Michigan. During these experiments, the radiation dosage rate averaged about 120,000 rad[3] per hr at the center of the cans. The radiation dosage was determined by ferrous sulfate dosimetry as previously described by Kempe et al. (1954). This involved dosimetry in cans of meat placed at the same positions in the center well as those used for the experimental cans. Also, the meat was kept below 5 C during irradiation. This was accomplished in the summer by an ice bath; in the winter this precaution was unnecessary because the source is located in an unheated room having a temperature slightly below 5 C.

Following irradiation, the cans were incubated at 29 C. Some of those that swelled were aseptically opened and subcultured to verify the *C. botulinum* culture growth. This verification also included both toxin presence and toxin neutralization tests in mice; it was carried out on the meat from selected swollen cans and on media from the subcultures as previously described by Kempe et al. (1954).

TABLE 1. *Dosages of gamma radiation from cobalt-60 required to sterilize cooked ground beef containing spores of clostridium botulinum 213B*

Run no.: C-1
Can size: Mushroom (202×202)
Product: Cooked ground beef
Inoculum: 104,000 *C. botulinum* 213B spores per g
Incubation temperature: 29 C

Dosage	Can no.	Days-to-gas formation
megarad		
2.36	1	—
	2	—
	3	—
	4	—
	5	12
2.79	6	—
	7	13
	8	12
	9	—
	10	12
3.29	11	—
	12	—
	13	—
	14	—
	15	—
3.72	16	—
	17	—
	18	—
	19	—
	20	—
Noninoculated controls	NI-1	—
	NI-2	—
	NI-3	—
	NI-4	—
Inoculated controls	IC-1	5
	IC-2	5
	IC-3	5
	IC-4	5

Conclusion: Under these conditions, cooked ground beef was sterilized with between 2.79 and 3.29 megarad of gamma radiation.

[3] One rad is the dose of ionizing radiation capable of producing an energy absorption of 100 ergs per g in the material irradiated.

The radiation sterilization dose for any particular run was established as a range of dosages. The upper limit of this range was set by the group of cans at the lowest dosage level that produced no spoilage upon incubation for 6 months at 29 C; the lower limit, by the next lower dosage level tested. Obviously, at the lower level, one or more cans spoiled. The results from one run are shown in Table 1 to illustrate typical data and the method by which the sterilization range was established.

Noninoculated controls were included to be sure that the cooked meat was initially sterile; inoculated controls tested viability of the spores as well as their ability to grow and produce toxin under conditions of the experiments.

Toxicity Determinations

Canned ground beef for analysis was selected from among those cans of inoculated packs that had been stored the longest; of these, cans remaining unswollen from groups that received approximately the minimal radiation sterilization dose were taken. For any particular experiment, the radiation sterilization dose is defined as the dose at which none of the cans processed at that or any higher dosage swelled during subsequent incubation. In some instances, cans of meat remained unswollen from groups that received just less than the sterilizing dose: some of these were also tested.

For examination, the cans were first scrubbed with a water-detergent mixture; then, they were dried and placed in an enameled pan on a towel soaked in a phenol solution. The depression in the top of the can was then filled with 95% ethyl alcohol which was ignited and allowed to burn off; this sterilized the can lid. At the same time, the sides of the can were warmed with the flame from a Bunsen burner to neutralize any remaining vacuum. At this point, care was taken not to overheat the can since botulinus toxin is heat labile. A sterile pad of cotton was next aseptically placed over the can in preparation for finally relieving any vacuum that remained. This was accomplished by punching a small hole in the can cover with a sterile ice pick, the tip of which was pushed through the pad and then through the cover. The can cover was next removed with a sterile can opener.

Four samples of meat were now taken with a sterile tube-and-plunger-type sampler. The samples were removed from the interior contents of the can, which could not have been warmed during the opening proceedings. The three samples for subculturing weighed approximately 15 g each; the sample for toxin analysis was slightly less than twice as large. At this time, Gram-stained preparations were made from meat in the cans. These preparations were examined with particular reference to residual ungerminated spores and vegetative cells of *C. botulinum*. Also the color and odor of the meat in the cans along with other characteristics were noted to discover any evidence of the growth of *C. botulinum*.

For subculturing, each of the 15-g samples was pushed into individual tubes containing 50 ml of liver broth medium (Read et al., 1951) and a strip of pure iron. These tubes of broth had been previously exhausted in a hot water bath. The broth and meat were now incubated at least 2 weeks at 29 C unless visible growth occurred before this time elapsed. Following either the evident development of a culture or the 2-week interval, the liquid in the tube was tested for the presence of bacteria. For this purpose, Gram stains were prepared and examined. If any growth was evident, the liquid was further tested for the presence of toxin by injecting 1 ml intraperitoneally into each of four 10- to 15-g mice. A tentatively positive finding was indicated by death of the mice within 72 hr. If only one mouse died out of four, the sample was rechecked using a series of three more mice. If further confirmations were necessary, they were carried out with antitoxin. This involved intraperitoneal injection into mice of portions of the sample which had first been incubated over night in a refrigerator with the specific-type botulinus antitoxin.

For determination of the presence of toxin in the meat, the larger, approximately 25-g, sample was pushed into a sterile test tube and an approximately equal volume of physiological saline added. Also, for some of the tests, instead of 25 g, all of the meat remaining after samples were removed for subculturing was used. This provided about 75 g instead of 25 g of meat for analysis. The meat and saline solution were aseptically mixed and allowed to infuse in a refrigerator for a few hours. Then the supernatant liquid was aseptically filtered through a glass-wool pad and ½-ml portions of the filtrate were injected intraperitoneally into each of four 10- to 15-g mice. If no mice died within 72 hr, the sample was assumed to be nontoxic. If one or more mice died within this interval, a portion of the filtrate was mixed with the specific-type botulinus antitoxin and this mixture again injected into mice for the final determination of toxigenicity of the filtrate.

Results and Discussion

Sterilization Dosage

Data showing variation of the sterilization dose of gamma radiation for cooked ground beef as a function of spore concentration are summarized in Table 2, and plotted in Fig. 1. As would be expected from results previously reported by Kempe et al. (1954), the sterilizing dosage varied directly with the logarithm of the number of. *C botulinum* 213B spores per g of meat: the

line also shows a D value[4] of 0.34 megarad for these spores. A sterilization dose of 3.8 megarad of gamma radiation from cobalt-60 is indicated for cooked ground beef containing approximately 5,000,000 *C. botulinum* 213 spores per g. *C. botulinum* 213B spores were previously found to be slightly less resistant to gamma radiation than *C. botulinum* 62A spores (Kempe et al., 1954), so it is reasonable to expect the sterilization dose for the latter spores to be somewhat higher.

In Fig. 2 and Table 2, it will be noted that the radiation sterilization dose for canned, raw, ground beef also varies with the logarithm of the number of spores present, and that with a spore concentration of approximately 1 million *C. botulinum* 213B spores per g of raw meat, the sterilization dose is indicated as 3.6 megarad of gamma radiation from cobalt-60. It will also be observed that the data for raw ground beef are less precise than those shown for cooked beef. This is probably due to the native bacterial flora of raw ground meat.

The radiation sterilization dose for both cooked and raw ground beef, inoculated with *C. botulinum* 62A spores, is also summarized in Table 2. These data indicate that cooked ground beef, inoculated with approximately 5,000,000 *C. botulinum* 62A spores per g, was sterilized by 3.85 megarad of gamma radiation. Similar results were obtained with raw ground beef under these conditions.

These studies show that the amount of radiation required to sterilize canned ground beef was greater for an inoculated spore concentration of 5,000,000 than for 40,000 spores per g of meat. Data for the latter concentration had previously been reported (Kempe et al., 1954). It is also evident that higher spore concentrations than 5,000,000 per g should require even more than 3.85 megarad of gamma radiation to produce sterility.

[4] The D value is the time in minutes required to reduce the number of viable spores by 90%, using only the linear portion of the curve for calculation.

TABLE 2. *Summary of dosages of gamma radiation from cobalt-60 required to sterilize ground beef containing spores of Clostridium botulinum*

Run no.	No. of spores per g of meat	Radiation sterilization range
		megarad
A. *C. botulinum* 62A spores in cooked ground beef		
AC-2	4,800,000	3.50–3.80
AC-1	5,200,000	3.40–3.85
B. *C. botulinum* 62A spores in raw ground beef		
A-2	2,670,000	3.20–3.60
A-3	3,200,000	Slightly more than 3.80
C. *C. botulinum* 213B spores in cooked ground beef		
C-11	1.42	1.80–2.00
C-7	4.00	>2.66
C-10	16.7	1.77–2.00
C-4	570	2.14–2.42
C-3	1,220	2.10–2.46
C-2	8,600	2.79–3.29
C-1	104,000	2.79–3.29
C-6	3,880,000	<3.71
C-8	4,000,000	<3.88
C-5	4,900,000	2.74–5.21
C-9	6,600,000	3.42–3.86
D. *C. botulinum* 213B spores in raw ground beef		
S-4	10.9	1.70–1.75
S-7	311	2.00–2.65
S-5	790	2.80–2.90
S-3	17,000	2.90–3.53
S-1	632,000	2.79–3.72
S-6	1,440,000	2.65–3.30
S-2	1,700,000	3.29–3.72

Toxicity

A total of more than 130 cans of meat were tested, including both cooked and raw ground beef; these had been irradiated over the past 6 years and incubated since irradiation. When opened, all of the cans showed the presence of some vacuum and the meat lacked any evidence of putrefaction.

Inocula as high as 6,600,000 *C. botulinum* 213B and 5,200,000 *C. botulinum* 62A spores per g of meat were included in the tests. Subcultures from all the meat inoculated with *C. botulinum* 213B spores were nontoxic for mice. With strain 62A spores, however, all of the meat and all of the subculture broths from the meat contained demonstrable botulinus toxin when the meat had been inoculated with 2,670,000 or more such spores per g. Even with strain 62A spores, however, when 40,000 or fewer spores were inoculated per g of meat, neither the meat nor the subculture broths were toxic. It should be emphasized that neither with strain 213B nor 62A inocula did the meat itself, or the

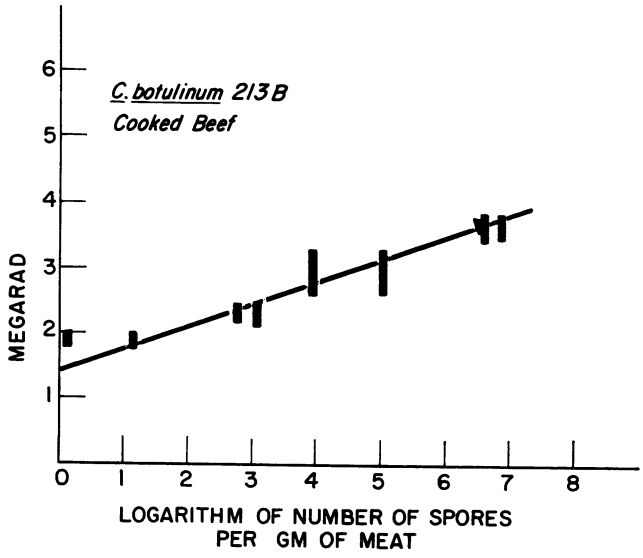

FIG. 1. *Dosage of gamma radiation from cobalt-60 required to sterilize cooked ground beef containing spores of Clostridium botulinum 213B.*

subcultures, show evidence of the growth of *C. botulinum* bacteria; but in all of the meat, residual spores from the original inocula were demonstrable.

Some points in connection with this finding of type A toxin in radiation-sterilized meat need emphasis here.

1) The spores used for inoculation were all *C. botulinum* 62A, grown in liver extract medium. These spores were washed at least 12 times with distilled water before storage in distilled water at 4 C. They were taken from different spore crops.

2) Before inoculation into cans of meat, all spore suspensions were heat shocked at 85 C for 15 min. This inactivated any toxin that may have "leaked" from the spores during storage.

3) An occasional Gram-variable bacillus occurred in stained preparations from the incubated meat. These rods looked like old *C. botulinum* vegetative cells but similar cells were also found in the raw ground beef with essentially the same frequency. Also the spore inocula contained a few vegetative cells which were killed by the heat-shocking treatment but still stained positively by the Gram technique. Consequently, the observation of an occasional Gram-variable cell in meat from incubated cans had no meaning in these studies. More critical experiments would be needed to rule out the unlikely possibility that such cells resulted from partially aborted germination of radiation-inactivated spores during incubation of the canned meat.

4) No viable spores were found as was evidenced by lack of growth in the subculture media.

5) All the cans containing toxin had been inoculated with more than 10^6 *C. botulinum* 62A spores per g of meat. This means that more than 10^8 spores were used per can in these instances.

6) Approximately 75 g of the original 90 g of meat in the can were analyzed.

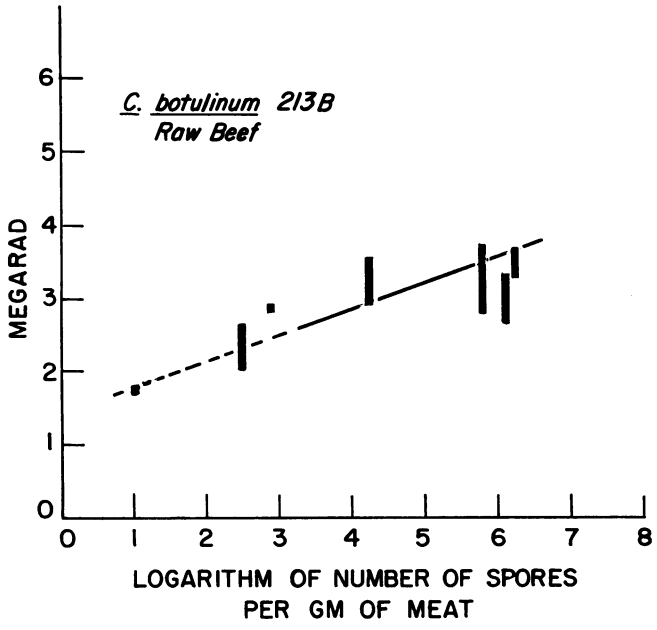

FIG. 2. *Dosage of gamma radiation from cobalt-60 required to sterilize raw ground beef containing spores of Clostridium botulinum 213B.*

From previous experience it was known that mice can often be killed with heat-shocked *C. botulinum* spores. We therefore inoculated mice with varying concentrations of *C. botulinum* 62A spores that had been heat shocked for 15 min at 85 C. The results are shown in Table 3. For this experiment, 0.5 ml of a suspension, containing the number of spores indicated per ml, was used. Lethality of the suspensions were recorded as the number of mice dying within 72 hr.

The data in Table 3 show that 10^9 spores, treated in the way that they were used for inocula, killed mice; 10^8 spores were marginally fatal, and 10^7 spores were tolerated. These data also suggest that spores irradiated in the canned meat may have contained more active toxin than the original spores, since only about 10^8 spores were inoculated into each can. Even if all of the inoculated spores had been recovered, the results shown in Table 3 indicate that they would have been only barely able to kill a mouse; actually, the lesser number recovered killed mice quickly. The question might therefore be asked, can irradiation increase the amount of toxin derived from the spores? Although it is known that ionizing radiations slowly destroy botulinus toxin (Wagenaar and Dack, 1960), recent studies suggest that conditions which increase cell permeability or degrade protein may enhance the toxicity of botulinus toxin (Bonventre and Kempe, 1960). It would seem possible, therefore, that ionizing radiations could accomplish both of the aforementioned results that would otherwise seem to be contradictory.

Furthermore, it was observed that the toxin recovered from the incubated cans was heat labile. This means that the condition of the toxin had changed from the time when it was inoculated into the meat, since the spores were heat shocked before injection. This observation suggests that modification of the toxin occurred while it was present in the canned meat

TABLE 3. *Effect of intraperitoneal inoculation of Clostridium botulinum 62A spores into mice*

No. of spores inoculated into each mouse	Lethality*	Remarks
3.4×10^9	3/3	—
3.4×10^8	1/3	—
3.4×10^7	0/3	—
Controls:		
3.4×10^9	0/3	Autoclaved 30 min
3.4×10^9	4/4	Irradiated, 3.25 megarad

* In other similar experiments, neutralization tests showed that death of mice under these conditions was due to *C. botulinum* toxin type A.

because the toxin was heat stable when it was present in the spores before they were injected into the meat.

In any event, the finding of botulinus toxin in cooked and raw ground beef that has been sterilized with ionizing radiations is a matter of concern. In this instance, it was shown that the toxin was present only when large spore inocula were used and that the toxin probably came from the spores themselves.

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