Survival Studies with Spores of *Clostridium botulinum* Type E in Pasteurized Meat of the Blue Crab *Callinectes sapidus*

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Received for publication 16 November 1973

*Clostridium botulinum* type E studies reported in this paper include the incidence of the organism in selected Chesapeake Bay areas, growth and toxin production in crabmeat homogenates, and the effect of pasteurization upon varying levels of spores in crabmeat. Type E spores were detected in 21 of 24 bottom mud samples taken at locations from which blue crabs were being harvested. Sterilized crabmeat homogenates inoculated with as little as five spores per 10 g became toxic after 8 days at 50 F, 2 days at 75 F, and 1 day at 85 F. Growth at 50 F and above was accompanied by gas production and a slightly sour odor. Growth and toxin production at 40 F required 55 days or longer and inocula of $10^3$ spores or higher per 10 g of homogenate. At 40 F gas production was usually not apparent and no off odors could be detected. A recommended minimum pasteurization of 1 min at 185 F internal meat temperature reduced type E spore levels in inoculated packs of crabmeat from $10^4$ spores per 100 g to 6 or less spores per 100 g, and the pasteurized meat remained nontoxic during 6 months of storage at 40 F.

*Clostridium botulinum* type E was first reported by Gunnison et al. in 1935 (4). The strains studied were isolated by Kushnir (7) in Russia from the intestines and muscles of sturgeon. At about the same time, E. Hazen (5, 6) isolated two new strains from outbreaks of botulism caused by smoked salmon and canned sprats, and it became evident that her isolates were type E.

Subsequently, numerous outbreaks of type E botulism have occurred in many parts of the world with fish, fish products, or other marine species identified as the major sources of poisoning. In the United States the organism has been isolated from marine muds, waters, and marine animals from the Pacific Northwest (3), the Gulf of Mexico (14), the Atlantic Coast (15), Lake Cayuga (2), and the Great Lakes (1).

The ability of *C. botulinum* type E spores to germinate, multiply, and produce toxin at refrigeration temperatures has been well documented. In 1961 Schmidt et al. (11) showed toxin production by four type E strains in a beef strew medium at 38 F after 31 to 45 days. Lerke and Farber (8) in 1971 reported toxin formation by type E in dungeness crabmeat at 40 F after 30 to 40 days. Outgrowth at these low temperatures apparently is substrate dependent; the more suitable the substrate the lower the temperature at which spores will germinate and produce toxin.

This paper describes type E botulinum toxin production in meat of the blue crab, *Callinectes sapidus*, at temperatures of 40 F and above and the effects of a recommended minimal pasteurization (185 F for 1 min) upon varying concentrations of *C. botulinum* type E spores in crabmeat. Reported also are the results of a brief survey to determine the incidence of type E spores in bottom mud samples taken from areas where crabs are obtained for processing plants in Crisfield, Md.

**MATERIALS AND METHODS**

**Cultures.** Four strains of *C. botulinum* type E were used, no. 17786 from the American Type Culture Collection, and Beluga, 070, and G21-5 strains from the Food and Drug Administration, U.S. Department of Health, Education, and Welfare.

**Antisera.** Types A, B, and E antisera were supplied by Matteo Cardella at Fort Detrick, Frederick, Md. An additional supply of types A and B antisera was obtained from W. S. Hammond of Lederle Laboratories, Pearl River, N.Y. For toxin typing, antisera A and B were diluted 1:10 and antisera E was diluted 1:100 in 0.85% saline solution.

**Media.** Cooked meat medium (Difco) was used for culture maintenance and for determination of the
most probable numbers (MPN) of viable spores. The spore production medium consisted of 5% Trypticase, 0.5% peptone, 0.4% glucose, 2% yeast extract, and 0.1% sodium thioglycollate (TPGY). TPGY containing 0.1% filter-sterilized trypsin (TPGTY), an improved medium for the isolation of type E reported by Lilly et al. (9), was used to detect type E spores in the mud samples. Gel-phosphate buffer (0.2% gelatin in 0.4% Na₂HPO₄, in distilled water) at pH 6.2 served as a diluent for toxin and spores.

**Mud samples.** Mud samples were taken from those areas of the Chesapeake Bay adjacent to Crisfield that were being actively fished with crab pots. Bottom mud samples, taken with a Ponar grab sampler, were placed in cans with press-type lids and stored at 36 F until they were examined. For detection of type E spores, 15 g of well-mixed mud were placed in wide-mouth bottles containing glass beads and 15 ml of gel-phosphate diluent. The bottles were shaken vigorously and then allowed to settle for 10 min. The supernatant was decanted into flasks of TPGTY and incubated for 3 to 5 days at 85 F. Broth (10 ml) was then transferred to a screw-capped test tube and frozen for toxin typing. For detection of heat-labile toxin, 0.5 ml of a 1:10 dilution of unheated culture fluid was inoculated intraperitoneally into mice weighing 19 to 24 g, and a second group of mice was inoculated with a 1:10 dilution of growth fluid that had been heated for 10 min in boiling water. The mice were observed for 48 h. Survival of the mice receiving heated sample and death of those receiving unheated sample suggested the presence of botulinum toxin. Botulinum toxin was confirmed and typed with specific antisera. The toxic broth was thawed, and separate 0.25-ml portions of a 1:10 dilution were mixed with 0.25 ml of type A, B, or E antiserum. The mixtures were held at room temperature for 1 h and then inoculated into mice.

**Spore preparation.** The type E stock cultures were inoculated into cooked meat medium and incubated at 85 F for 24 h. TPGY medium (500 ml) was inoculated from the 24-h cooked meat tube and incubated at 85 F for 5 to 7 days. The broth was then centrifuged for 30 min, and the sediment was suspended in gel-phosphate diluent at 2% of the original volume. The concentrated spore suspensions were centrifuged and washed twice in diluent, and the MPN of viable spores was determined by inoculating serial 10-fold dilutions into cooked meat tubes, three tubes per dilution. In all MPN determinations, trypsinized culture fluids from those positive growth tubes defining the end point were inoculated into unprotected mice and mice protected with type E antiserum for toxin identification.

**Growth in crabmeat and crabmeat homogenate.** Freshly picked crabmeat was packed tightly into screw-capped test tubes, approximately 10 g per tube. Crabmeat homogenate, prepared by blending 1 part crabmeat with 2 parts 1% saline in a Waring blender for 90 s, was dispensed in 10-ml samples to screw-capped test tubes. The tubes with whole meat and homogenate were autoclaved for 15 min at 250 F, cooled rapidly in cold water, and inoculated with 0.1 ml of spore suspensions. Inoculum per tube was up to a maximum of 10⁸ spores. The inoculated tubes with caps loosened were placed in Gas Pak (BBL) jars to assure anaerobiosis and incubated at 85 F, room temperature (approximately 75 F), 50 F, and 40 F. The tubes were examined periodically for gas production, off odors, and for toxin by mouse inoculation. The procedures used for detection of toxin followed those outlined in Chapter 2 of the Bacteriological Analytical Manual (13). Whole meat was homogenized with an equal weight of gel-phosphate diluent and centrifuged to separate the extract. Similarly, the contents of the crabmeat homogenate tubes were compressed, and extract was obtained by removal of the separated liquid. Filter-sterilized extracts were mixed 1:1 with 1% trypsin, held for 45 min at 37 C, and inoculated into mice without further dilution. Toxin type was verified by inoculation of the trypsinized extracts into mice protected with 0.5 ml of type E antiserum.

**Pasteurization experiments.** One-pound (375.24-g) quantities of crabmeat were inoculated by thorough mixing with 4.5 ml of spore suspensions adjusted to give an inoculum range of from 10⁵ to 10⁸ spores per 100 g of meat. The inoculated meat was packed into one-pound cans (401 × 301), hermetically sealed, and heated in a 190 F water bath. One can of crabmeat in each pasteurization run was fitted with a thermocouple passing through the side of the can to the geometric center for determination of internal meat temperatures. The thermocouple was connected to a potentiometer from which the temperature readings were made. Those cans receiving full pasteurization remained in the water bath until the internal meat temperatures had reached 185 F. They were held at 185 F for 1 min and transferred to an ice bath for rapid cooling (12). When the internal meat temperatures had dropped to 100 F, the cans were moved to refrigerated storage at 40 F.

During four of the six pasteurization runs, three cans were removed at regular intervals from the hot water bath. These were immediately tested for type E spores by the MPN method. Of processed cans stored at 40 F, one from each pasteurization run was removed monthly and tested for toxin.

**RESULTS**

**Incidence of C. botulinum type E in crab fishing areas.** The results of the survey are shown in Table 1. Twenty-four samples were analyzed, and C. botulinum type E was detected in 21. The presence of type E was suspected in the other three since they contained a heat-labile toxin that killed mice within 16 h, but the results of toxin typing were inconclusive in that the mice were protected by both A and E antisera. Possibly, the broth contained both types of toxin, each at a level too low to kill the mice independently.

**Growth and toxin production in crabmeat and crabmeat homogenate.** Over a 2-month period at room temperature, only 2 out of 84 tubes of autoclaved whole crabmeat inoculated
TABLE 1. Incidence of C. botulinum type E in marine bottom samples from the Crisfield area of the Chesapeake Bay

| Location         | Description | Sampling date (9/71 11/71 5/72) |
|------------------|-------------|----------------------------------|
| Little Annemessex| Black mud   | + + + + + + + + + + + + + + + + |
| Little Annemessex| Black mud   | + + + + + + + + + + + + + + + + |
| Big Annemessex   | Shells, mud | + + + + + + + + + + + + + + + + |
| Jones Creek      | Black mud   | + + + + + + + + + + + + + + + + |
| Pocomoke Sound   | Sand, mud   | + + + + + + + + + + + + + + + + |
| Pocomoke Sound   | Black mud   | + + + + + + + + + + + + + + + + |
| Tangier Sound    | Sand        | + + + + + + + + + + + + + + + + |

* + Indicates the presence of type E spores.
* ? Indicates that the results of typing were inconclusive.

with $2.4 \times 10^4$ spores became toxic after 12 and 17 days. The reason for the low incidence of toxin development in the whole meat is not understood. Perhaps autoclaving altered the substrate in some way, adversely affecting growth or toxin production by type E spores. Four tubes of unautoclaved crabmeat inoculated at the same spore level spoiled rapidly at room temperature, and after 3 days all four contained type E toxin. It is suggested that the growth of the natural flora in these tubes produced anaerobic conditions necessary for growth and toxin production by the type E spores.

Table 2 shows the number of days incubation at 50, 75, and 85 F required before toxin could be detected in homogenates inoculated with 100 type E spores. The same time periods were required for toxin development in homogenate containing as little as a calculated five spores per tube. Below five spores, not all homogenates became toxic, and toxin development was usually though not always delayed by 24 to 48 h. At 50 F and above, toxin development was invariably accompanied by gas production and a slight sour odor which could easily remain undetected.

Toxin development in homogenates at 40 F was considerably delayed, and more highly concentrated inocula were required as shown in Table 3. Toxin was not detected in any tube receiving less than 1,000 spores. In the majority of cases, there was no visible gas production nor were any odors detectable to indicate growth and toxin formation by the type E spores.

**Pasteurization experiments.** The six pasteurization tests are summarized in Table 4. The data show the surviving spores when meats containing $10^4$ to $10^5$ spores per 100 g are given the recommended minimal minimal pasteurization of 1 min at 185 F. All samples remained free of toxin during 6 months of storage. No storage data are reported for pasteurization no. 6, since all the cans were used to determine the MPN of surviving spores during the pasteurization cycle. In pasteurizations three through six the Beluga strain was used since it was the only spore suspension sufficiently concentrated to

TABLE 2. Days required for toxin production by 100 spores of C. botulinum type E in crabmeat homogenate at 50 F and above

| Strain         | No. of days* |
|----------------|--------------|
|                | 85 F* | 75 F | 50 F |
| No. 17786, ATCC| 1     | 2    | 8    |
| Beluga 070     | 1     | 2    | 8    |
| G21-5          | 1     | 2    | 8    |

* Number of days of incubation before toxin was first detected.
* Incubation temperatures.

TABLE 3. Days required for toxin production by C. botulinum type E spores in crabmeat homogenate at 40 F

| Strain         | No. of days |
|----------------|-------------|
|                | 10<sup>6</sup> | 10<sup>5</sup> | 10<sup>4</sup> | 10<sup>3</sup> | 10<sup>2</sup> |
| No. 17786, ATCC| >120<sup>a</sup> | >120 | 68 | 68 | 55 |
| Beluga 070     | >120 | 88 | 75 | 60 | 55 |
|                | >120 | 82 | 80 | 75 | 58 |

* Spore concentrations per tube.
* No toxin detected through 120 days at 40 F, the longest period tested.

TABLE 4. Effect of a minimum pasteurization upon C. botulinum type E spores in crabmeat and toxicity tests during 6 months at 40 F

| Run | Strain         | Spores/100 g of meat |
|-----|----------------|----------------------|
|     | Before pasteurization | After pasteurization |
|     | Toxinity during 6 mo at 40 F |
| 1   | No. 17786, Beluga | 1.1 x 10<sup>4</sup> | <3<sup>a</sup> | Negative |
| 2   | No. 17786, Beluga | 1.8 x 10<sup>4</sup> | <3<sup>a</sup> | Negative |
| 3   | Beluga           | 2.4 x 10<sup>4</sup> | <3<sup>a</sup> | Negative |
| 4   | Beluga           | 1.8 x 10<sup>5</sup> | 6       | Negative |
| 5   | Beluga           | 1.8 x 10<sup>4</sup> | <3<sup>a</sup> | Negative |
| 6   | Beluga           | 1.8 x 10<sup>3</sup> | 3       | Not tested |

* The lowest spore level detectable by the procedure used was 10<sup>0</sup> g.
Table 5. Viable C. Botulinum type E spores and internal meat temperatures at various time intervals during pasteurization

| Time (min) | Pasteurization 3 | Pasteurization 4 | Pasteurization 5 | Pasteurization 6 |
|------------|------------------|------------------|------------------|------------------|
|            | Temp (F) | Survival* | Temp (F) | Survival | Temp (F) | Survival | Temp (F) | Survival |
| 0          | 50      | $1.8 \times 10^7$ | 33      | $2.4 \times 10^7$ | 58      | $1.8 \times 10^8$ | 66      | $1.8 \times 10^8$ |
| 30         | 100     | $1.4 \times 10^7$ | 76      | $2.1 \times 10^7$ | 104     | $1.8 \times 10^7$ | 110     | $1.1 \times 10^7$ |
| 60         | 160     | $7.8 \times 10^4$ | 148     | $5 \times 10^4$   | 160     | $2.4 \times 10^4$ | 162     | $1.8 \times 10^4$ |
| 90         | 180     | 160          | 175     | 60               | 182     | 430         | 178     | 260 |
| 100        | 182     | 3.6          | 180     | 22               | 183     | 30          | 182     | 100 |
| 110        | 185     | $<3$         | 184     | 7.2              | 184     | 30          | 184     | 30 |
| Final*     | 185     | $<3$         | 185     | 6                | 185     | 3           | 185     | 3 |

* MPN/100 g of meat of viable spores.
* After 1 min at 185 F.

deliver spore levels of $10^7$ per 100 g and above.

The recommended minimum pasteurization process for crab meat by the water bath method is, by definition, the heating of hermetically sealed containers in a 190 to 192 F water bath to an internal meat temperature of 185 F at the geometric center of the container and holding for 1 min at that temperature (12). The lethality of the process is determined by the cumulative effect of time and temperature; therefore, the come-up time to pasteurization temperature contributes significantly to the effectiveness of the over-all process. The relationship of time and temperature to spore survival throughout the pasteurization cycle is shown by the data in Table 5. Although an initial temperature of 60 to 65 F is recommended (12), a range of initial temperatures from 33 to 66 F did not significantly affect the final spore survival.

**DISCUSSION**

Fresh crab meat is a highly perishable commodity and is rendered unusable by the normal flora after 7 to 12 days of refrigeration. However, pasteurization kills most of the normal flora and extends the refrigerated shelf life to 6 months or more, well beyond the time required for toxin development at 40 F in crab meat homogenates. Although the homogenates did become toxic at 40 F after 55 days or longer, inocula of $10^8$ spores per tube or higher were required. Pasteurization reduced spore loads as high as $10^4$ spores per 100 g to 6 or less spores per 100 g, and the meat remained nontoxic during 6 months of storage at 40 F.

It is apparent from the limited survey of bottom mud samples reported that *C. botulinum* type E is commonly found in the environment from which blue crabs are harvested. It would follow that the organism is likely to be present on the crabs as they are brought into the processing plants. Though the spores would undoubtedly be killed by a recommended cooking of 250 F for 10 min (12), it is possible that the cooked crabs or picked meat could become contaminated by spores present in the plant. Preferably, crab meat is pasteurized immediately after picking (12); however, regulations of the Maryland State Department of Health and Mental Hygiene (10) allow the meat to be refrigerated for up to 24 h prior to pasteurizing. Unless the meat is grossly mishandled, it appears highly unlikely that more than a few chance contaminants would be present at the time the meat is ready for pasteurization.

Pasteurized meat of the blue crab has been available to the consumer for over 20 years and, to date, no case of botulism has been attributed to its consumption, nor has any commercially available pasteurized meat been found toxic. This excellent record coupled with the data in this report indicate that pasteurized meat of the blue crab is a safe product provided the prescribed procedures of cooking, handling, pasteurization, and storage are adhered to rigorously.

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