APPLIED MICROBIOLOGY, Jan. 1968, p. 133-137
Vol. 16, No. 1

Survival and Outgrowth of *Clostridium botulinum* Type E Spores in Smoked Fish

LEE N. CHRISTIANSEN, JANET DEFFNER, E. M. FOSTER, AND H. SUGIYAMA

Department of Bacteriology and the Food Research Institute, University of Wisconsin, Madison, Wisconsin 53706

Received for publication 5 September 1967

Chub injected in the loin muscle with 10^6 *Clostridium botulinum* type E spores were smoked to an internal temperature of 180 F (82.2 C) for 30 min, sealed in plastic bags, and incubated at room temperature (20 to 25 C) for 7 days. Viable type E spores were found in practically all such fish. Toxin formation by the survivors in the smoked fish was dependent on the brine concentration of the smoked fish. A brine concentration of 3% or higher, as measured in the loin muscle, inhibited toxin formation. Six different type E strains gave similar results. Only a few hundred of the million spores in the inoculum survived the smoking. Moisture in the atmosphere during smoking did not reduce the incidence of fish with type E survivors.

Clostridium botulinum type E is part of the natural flora of the fish of the Great Lakes. The incidence of fish harboring the organism in the gastrointestinal tract varies from 1% for those of Lake Superior to 90% in parts of the Green Bay arm of Lake Michigan (4). The potential botulism hazard inherent in this distribution of the organism has been demonstrated by the smoked fish type E botulism outbreaks of 1960 and 1963 (2, 7, 11).

The percentage of fish carrying type E spores increases during stages of processing prior to smoking (12). From a public health viewpoint, any fish caught in the Great Lakes and readied for smoking should be assumed to carry the organism. Thus, the prevention of botulism from smoked fish depends on destruction of all *C. botulinum* type E spores during smoking or on inhibition of germination and outgrowth of the spores that may be present in the smoked product. The objectives of the present work were to determine whether the presently recommended heating at 180 F (82.2 C) for 30 min during smoking (2) would result in a product free from viable type E spores and to study the effect of NaCl on outgrowth of type E spores in smoked fish.

The generally accepted low heat tolerance of type E spores would indicate an adequate safety margin in the fish smoking procedure. The D_{176} (time of heating at 176 F required to reduce the viable spore population by 90%) of two type E strains heated in 0.067 M phosphate buffer of *p*H 7 was 3.3 and 0.4 min., respectively (10); that of the Minneapolis strain was 1.78 min in Trypticase-peptone-glucose (TPG) and 2.3 min in phosphate buffer (14). The *D* values of aqueous spore suspensions of nine strains heated at 176 F ranged from 0.33 to 1.25 (13). Assuming logarithmic destruction, 180 F for 30 min should destroy about 10^{10} spores of the most heat-resistant of these strains.

Exceptions have been noted. Survivors were found when 10^{11} spores were heated at 100 C for 30 min (5). Heating for 120 min at 85 C did not sterilize a phosphate buffer suspension of 10^7 type E spores/ml (J. T. Graikoski and L. L. Kempe, Bacteriol. Proc., p. 3, 1964), and toxin was formed in minced herring inoculated with 10^5 spores/g before heating to 80 C for 110 min (1).

Germination and outgrowth of *C. botulinum* type E spores are inhibited by lower concentrations of NaCl than are spores of type A or B. At incubation temperatures of 16 to 30 C, 5% NaCl in TPG prevented outgrowth of 2×10^6 spores of three type E strains for 1 year (16). Toxin was not formed when Robertson's chopped meat medium containing 4.5% NaCl was inoculated with 10^5 spores and incubated at 30 C for 90 days or at 15 C for 160 days (1).

MATERIALS AND METHODS

Spore suspension. VH, Alaska E43, Minneapolis (from two sources), Beluga, Can, and Detroit strains of *C. botulinum* type E were maintained in TPG (16) supplemented with 0.1% yeast extract. Spores were produced in this TPGY medium by inoculating a 10% volume of an actively growing culture and incubating it for 3 to 5 days at 25 C. Spores were harvested by centrifugation, washed several times, and suspended in sterile deionized water.

The stock suspensions, stored frozen or at 4 C,

were sonically treated for 30 sec at maximal activity of an MSE ultrasonic disintegrator (Measuring and Scientific Equipment Ltd., London, England) immediately before use to disrupt clumps. Viable-spore counts were based on colony counts in deep tubes of beef infusion agar (infusion fortified with 1% Proteose Peptone, 0.2% Na_2HPO_4, 0.1% soluble starch, 0.5% NaCl, and 0.05% sodium thioglycolate; *p*H 7.4).

Experimental pack studies with fish. Eviscerated, medium-sized chub (*Leucichthys hoyi*) obtained from a commercial fishery were frozen until needed. The thawed fish were placed in 30 C salometer brine (7.8% NaCl) for 4 hr or in 45 C brine (11.7%) for 8 or 20 hr. The hanging of the brined fish on nails arranged on wooden strips and inoculation of 10^6 type E spores (0.1 ml) into the right loin muscle were done outside the smoke chamber. Smoke processing was to a final temperature of 180 F for 30 min (the time required to reach this temperature was about 3.5 hr). Temperatures during the entire course of the smoking operation were monitored by thermocouples inserted into representative fish and connected to a temperature-recording instrument. The procedure and apparatus for brining and for smoking have been described (18).

The fish were cooled in a cold room (4 C) for 1 hr immediately after smoking and then placed individually in triple laminated plastic pouches (6.5 × 8.5 inch Flex-Vac; Standard Packaging Corp., Clifton, N.J.). In the laboratory, the left loin muscle was removed aseptically for determination of brine level from water (heating in vacuum oven to constant weight) and NaCl (3) contents. The brine level was calculated as follows: per cent brine = [grams of NaCl/(grams of water + grams of NaCl)] × 100. The remainder of the fish was vacuum packaged and incubated at room temperature (20 to 25 C) for 7 days, at which time the spore-inoculated loin muscle was ground up with 10 ml of gelatin-phosphate buffer (6). Half of the slurry was subcultured in 10 ml of TPGY; the rest was refrigerated overnight at 4 C. The refrigerated slurry was centrifuged, and 0.25 ml of the supernatant fluid was injected intraperitoneally into a 20-g white mouse. Mice protected with type E antitoxin were used, as necessary, to establish that deaths were due to the specific neurotoxin. Tryptic activation of the extract was not used; preliminary experiments showed that trypsin treatment of the extracts did not increase the number of samples which killed mice.

When type E toxin was absent from the fish extract, the corresponding TPGY subculture incubated for 3 days at 30 C was tested for toxin. Toxin formation in the incubated smoked fish indicated survival of type E spores through the smoking process and subsequent outgrowth; no toxin in the fish extract but in the subculture indicated survival but inhibition of outgrowth in the packaged smoked fish.

Heating of fish in moist atmosphere. Fish injected in the loin muscle with 10^6 type E spores were heated in the smoke chamber by introduction of steam, hot air, or a combination of the two. The apparatus permitted essentially the same time sequence of temperature rise during these procedures as during the normal smoking of fish. In these experiments, the fish were heated on individual aluminum sheets set on a wire screen, since the texture change of the fish did not permit hanging on nails. After 30 min at 180 F, the fish were put into separate Flex-Vac pouches which were heat-sealed after the addition of 50 ml of TPGY. Tests for type E toxin were made on 0.25 ml of the liquid after incubation at 25 C for 7 days.

In a variation of the experiment, fish were heated in sealed plastic pouches (6.5 × 10 inches). A horizontal midline seal with a gap of about 2 inches gave two interconnected compartments. The fish was placed in the top compartment, separated from 50 ml of TPGY in the bottom compartment by the midline seal. The pouches were closed above the fish and heat-processed in the smoking chamber. Thermocouples inserted into some of these fish showed that heat penetration was similar to that of unpouched fish. The bags were inverted after "smoking" and incubated with the fish bathed in TPGY.

RESULTS

The development of type E toxin in fish inoculated with 1 million spores and heated to 180 F for 30 min during the smoking procedure depended on the brine level in the fish. Above 2.75% brine, none of the fish became toxic; greater percentages of fish were toxic with decreasing salt concentrations. In contrast, viable type E spores were present in nearly all of the smoked fish, regardless of the brine level. The consolidated results of 14 separate experimental pack experiments with six different type E strains are given in Table 1.

Variation of salt uptake among fish (18) is reflected in the range of brine concentrations in the loin muscle obtained by three brining procedures (Table 1). However, the brine level of the contralateral loin muscle should be an accurate representation of the salt concentration of the muscle injected with the spores. The number of fish in the critical range was limited, but none of 16 samples with 2.75 to 2.99% brine was lethal for mice. Making allowances for experimental errors in brine analysis, 3.0% can be taken as the minimal concentration inhibiting germination and outgrowth of the surviving spores.

On the basis of the available data, fish inoculated intramuscularly with 10^6 type E spores after they had been smoked do not become botulinogenic if the brine concentration exceeds 3%. As expected, viable type E can be found in subcultures of these fish (Table 2).

Only a small percentage of the 10^6 type E spores inoculated into the fish seems to survive the smoking. Based on a five-tube most probable number determination, 2×10^5 of the 10^6 spores could be enumerated immediately after injection into the loin muscle. After smoking, about 50

TABLE 1. *Survival of Clostridium botulinum type E spores (10⁶/fish) heated to 180 F for 30 min during smoking and increasing inhibition of outgrowth of survivors by higher brine concentrations of the fish*

Per cent brine	No. of fish	No. of fish with viable spores			
		Toxin in fish[a]		Total with survivors	
		No.	Per cent	No.	Per cent
<1.0	39	33	85	39	100
1.0 -1.49	139	93	67	130	94
1.5 -1.99	95	55	58	88	93
2.0 -2.49	85	26	31	75	88
2.50-2.74	41	5	12	38	93
2.75-2.99	16	0	0	15	94
3.0 -3.24	21	0	0	19	90
3.25-3.50	6	0	0	6	100
>3.50	46	0	0	43	93
Totals	488			453	93

[a] Type E toxin in smoked fish upon incubation for 7 days at room temperature.

[b] Toxic fish plus nontoxic fish which produced type E toxin in subculture.

TABLE 2. *Toxin formation in smoked fish of differing brine concentrations*[a]

Per cent brine	Incubation (days)	Toxin	
		Fish	Subculture
1.17	12	+	+
1.23	8	+	+
1.36	8	−	+
1.55	12	+	+
1.64	8	−	+
2.38	9	+	+
2.39	13	−	
2.73	9	+	+
2.93	29	−	+
3.31	9	−	+
4.18	29	−	+
4.22	9	−	+
4.34	9	−	+
5.06	9	−	+

[a] The loin muscle was inoculated with 10^6 type E spores (VH strain) after the fish had been smoked. Incubated at 25 C.

viable spores/fish could be demonstrated. Correcting for the approximate 20% recovery by the counting method, about 250 spores would have survived the smoke treatment.

The intramuscular injection of 0.1 ml of spore suspension results in some leakage back onto the surface. The skin of fish reaches a relatively low moisture level early in the smoking process (Table 3), so that spores on the surface would be subjected to dry heat. Since dry heat is less effective than moist heat as a sterilizing agent, the possibility that this combination of factors contributed to the results of the experimental pack studies was investigated.

TABLE 3. *Per cent water of skin of fish at different times in the smoking process*

Time (min)	Temp (F)[a]	Per cent water of skin[b]
0	72	62
30	96	62
60	118	36
90	140	29
120	153	30
150	162	28
180	173	30
210	187	32
240	192	22

[a] Average temperature in loin muscle of 10 fish.

[b] Average of four fish.

Unbrined fish inoculated in the loin muscle with type E spores were heated in the smoking chamber in atmospheres of different moisture levels (Table 4). The experiment was necessarily done in three different runs, but the records of the temperature come-up were similar for all heatings. The results show that additional moisture in the heating atmosphere had no significant effect on the percentages of fish with type E spore survivors.

The "smoking" of fish sealed in plastic pouches also resulted in the processing of the fish in a moist environment. Viable type E spores were present in 68% of the experimentally inoculated fish (Table 4).

Fish not injected with type E spores were included as controls in these experiments. Comparatively low, but significant, numbers of these fish often became toxic (Table 4); this happened even when sterilized (autoclaved) fish were used as controls.

The relationship between the spore inoculum and the incidence of smoked fish containing viable spores is given in Table 5. Fish were surface-inoculated with 0.1 ml of suspensions containing different numbers of spores and were smoked; the inoculated skin was then removed and cultured for type E in TPGY for 3 days at 30 C.

DISCUSSION

Heating of chub inoculated with 1 million *C. botulinum* type E spores to an internal tempera-

TABLE 4. *Survival of type E spores in fish heated at different moisture levels for 30 min at 82.2 C*[a]

Heat source	Fish	No. of toxic fish/ no. of fish tested	Comments
Hot air + steam	Control	0/10	Humidity higher than in hot air but fish dry after heating
	Inoculated	35/40	
Steam	Control	4/10	Humidity very high; fish "mushy" after heating
	Inoculated	40/40	
Hot air	Control	3/10	Fish dry after heating
	Inoculated	39/40	
Hot air	Control	0/27	Heated while sealed in plastic bags; fish "mushy" after heating
	Inoculated	23/34	

[a] Inoculum of 10^6 Alaska E 43 spores in loin muscle.

ture of 180 F for 30 min during smoke processing comparable to a commercial operation did not result in a smoked-fish product free from viable type E spores. Survivors were found in nearly all such fish.

Some control smoked fish, including those autoclaved before being placed in the smoke chamber, had viable type E spores in spite of all precautions to prevent postsmoking contamination. This is not surprising, since the fish are exposed to the air during and for a short time after smoking. However, contamination could not have influenced significantly the results with the type E spore-injected fish. These fish would have had type E survivors regardless of contamination. The correlation between the size of the spore inoculum and the incidence of fish containing viable type E spores (Table 5) would not exist if contamination was the determining factor; the random nature of the contamination should have resulted in approximately equal numbers of positive fish in all six groups. That the heating during the smoking procedure is inadequate to sterilize the spore inoculum is emphasized by the high percentage of fish with type E survivors after "smoking" in sealed plastic pouches, an experimental procedure that prevents contamination from an external source during and after smoking.

Indigenous type E spores probably account for some of the positive control smoked fish (Table 4). Survivors could be expected in the processed product if the original natural spore load of the fish is sufficiently high.

The failure of the smoke processing of fish to destroy all of the 10^6 type E spores inoculated and the possibility of postsmoking contamination show the necessity of additional means to control the hazards of botulism in this product. A brine concentration of 3% or higher would satisfy this requirement, since germination of the viable type E spores present on the smoked fish would be inhibited. The observation that

TABLE 5. *Relationship between size of type E spore inoculum (surface inoculation) and number of fish containing viable type E spores after smoking*

Inoculum/fish	Fish with viable spores/fish tested
0	1/8
10	2/9
100	3/9
1,000	6/9
10,000	9/9
1,000,000	9/9

chub smoked to 170 F or higher may not be a good substrate for toxin formation by type E (17) may be mentioned.

In the present experiments, brine concentrations are those of the loin muscle, the part of the fish with the lowest NaCl uptake. To attain at least 3% brine in all parts of the smoked product, the average brine concentration for the whole fish must be higher than this figure. Many factors affect salt uptake, and brine concentrations of chub from the same brining tank may vary 200% (19). To assure the inhibition of outgrowth of spores present on the smoked fish would require a brine safety factor which considers these variations.

The smoked fish were incubated at room temperature to give conditions of gross temperature abuse. Holding at temperatures closer to the minimal growth temperatures for type E could add to the safety of the product, since a salt concentration which is subinhibitory at optimal temperature can prolong outgrowth times at minimal temperatures (16).

Survival of type E spores through the smoking process is contrary to the generally accepted low heat resistance of these spores. The heat tolerance of type E spores can be altered by control of the water activity at which the spores are heated (8, 9). However, heating of the spore-inoculated

fish in an atmosphere moist enough to give a cooked appearance to the fish did not change the results significantly.

ACKNOWLEDGMENTS

This investigation was supported by the U.S. Bureau of Commerical Fisheries under contract BCF 14-17-004 and by Public Health Service Grant EF-00790.

Special appreciation is due K. G. Weckel and D. Wosje of the Department of Food Science and Industries, University of Wisconsin, for help in brining of fish and for the use of the smoking facilities. Technical assistance of F. Alvares, R. Deffner, and G. Wolfe is gratefully acknowledged.

LITERATURE CITED

1. ABRAHAMSSON, K., B. GULLMAR, AND N. MOLIN. 1966. The effect of temperature on toxin formation and toxin stability of *Clostridium botulinum* type E in different environments. Can. J. Microbiol. **12**:385–394.
2. ANONYMOUS. 1964. Botulism outbreaks from smoked whitefish. Food Technol. **18**:71–74.
3. ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS. 1960. Official methods of analysis, 9th ed., p. 234, Sec. 18.008. Association of Official Agricultural Chemists, Washington, D.C.
4. BOTT, T. L., J. S. DEFFNER, E. MCCOY, AND E. M. FOSTER. 1966. *Clostridium botulinum* type E in fish from the Great Lakes. J. Bacteriol. **91**:919–924.
5. DOLMAN, C. E., AND H. CHANG. 1953. The epidemiology and pathogenesis of type E and fish borne botulism. Can. J. Public Health **44**:231–244.
6. DUFF, J. T., G. G. WRIGHT, AND A. YARINSKY. 1956. Activation of *Clostridium botulinum* type E toxin by trypsin. J. Bacteriol. **72**:455–460.
7. KAUTTER, D. A. 1964. *Clostridium botulinum* type E in smoked fish. J. Food Sci. **29**:843–849.
8. MURRELL, W. G., AND W. J. SCOTT. 1957. Heat resistance of bacterial spores at various water activities. Nature **179**:481–482.
9. MURRELL, W. G., AND W. J. SCOTT. 1966. The heat resistance of bacterial spores at various water activities. J. Gen. Microbiol. **43**:411–425.
10. OHYE, D. F., AND W. J. SCOTT. 1957. Studies in the physiology of *Clostridium botulinum* type E. Australian J. Biol. Sci. **10**:85–94.
11. OSHEROFF, B. J., G. G. SLOCUM, AND W. M. DECKER. 1964. Status of botulism in the United States. Public Health Rept. U.S. **79**:871–878.
12. PACE, P. J., E. R. KRUMBIEGEL, R. ANGELOTTI, AND H. J. WISNIEWSKI. 1966. The distribution of *Clostridium botulinum* types in fish processed by smoking plants of the Milwaukee area, p. 40–48. *In* M. Ingram and T. A. Roberts [ed.], Botulism 1966. Chapman and Hall, Ltd.
13. ROBERTS, T. A., AND M. INGRAM. 1965. The resistance of spores of *Clostridium botulinum* type E to heat and radiation. J. Appl. Bacteriol. **28**:125–138.
14. SCHMIDT, C. F. 1964. Spores of *Clostridium botulinum*: Formation, resistance, germination, p. 69–82. *In* K. H. Lewis and K. Cassel, Jr. [ed.], Botulism. U.S. Public Health Service, Cincinnati, Ohio.
15. SCHMIDT, C. F., W. K. NANK, AND R. V. LECHOWICH. 1962. Radiation sterilization of food. II. Some aspects of the growth, sporulation, and radiation resistance of spores of *Clostridium botulinum* type E. J. Food Sci. **27**:77–84.
16. SEGNER, W. P., C. F. SCHMIDT, AND J. K. BOLTZ. 1966. Effect of sodium chloride and *p*H on the outgrowth of spores of type E *Clostridium botulinum* at optimal and suboptimal temperatures. Appl. Microbiol. **14**:49–54.
17. SHENEMAN, J. M. 1965. Prevention of type E *Clostridium botulinum* toxin formation in smoked whitefish chubs with tylosin lactate. J. Food Sci. **30**:337–343.
18. WECKEL, K. G., AND D. WOSJE. 1966. Brine salting of Great Lakes chub (*Leucichthys hoyi*) for smoking. Res. Rept. 24, Ext. Service, Coll. Agr., Univ. Wisconsin, Madison.