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Demonstration and Isolation of *Clostridium botulinum* Types from Whitefish Chubs Collected at Fish Smoking Plants of the Milwaukee Area

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A total of 1,071 whitefish chub samples were examined at eight stages of processing, including sampling aboard ship, various processing steps in the smoking plant, and display in retail cases. The frequency of *Clostridium botulinum* contamination of freshly caught and eviscerated chubs was approximately 13 to 14%. The highest percentage of contamination (20%) was noted among chubs sampled at the brining step of processing. The prevalence of contamination among chubs sampled at other processing stages prior to the smoking operation ranged from 6 to 14%. Of 858 freshly smoked chubs that had been processed at 180 F for 30 min (internal temperature of loin muscle), 10 were contaminated with *C. botulinum* (1 Type B and 9 Type E). The use of heat-shocked (60 C for 15 min) and nonheat-shocked enrichment cultures in combination yielded a greater number of positive samples than either method yielded when used alone. Each toxic enrichment culture obtained was subcultured to obtain isolation of the toxigenic organism. Toxigenic pure cultures of *C. botulinum* were obtained from 80% of the fish samples observed to produce toxic enrichment cultures.

The occurrence of *Clostridium botulinum* type E in smoked whitefish chubs caught and processed by a Lake Michigan fishery was clearly demonstrated by the tragic outbreak of human botulism in 1963 (Morbidity and Mortality Weekly Rept. 13:1, 1964). E. M. Foster and his associates at the University of Wisconsin, Madison, with inimitable dispatch, elucidated the prevalence of *C. botulinum* types in both mud samples and the intestinal contents of fish collected from the Great Lakes (3, 4, 8).

Our attention was directed toward whole and eviscerated whitefish chubs (*Leucichthys* sp.) as they occur in fish-smoking plants of the Milwaukee area. The general bacteriological quality of these fish has been reported previously by Pace and associates (Botulism Symp., Moscow, 1966).

From the onset of surveillance studies, initiated in September 1964, a particular effort has been made to recover *C. botulinum* from toxic enrichment cultures. This communication extends prevalence data, and it describes methodology developed for detection of *C. botulinum* types in raw and smoked whitefish chubs. Data are included which corroborate the detection of *C. botulinum* in toxic enrichment cultures by recovery of toxigenic isolates.

MATERIALS AND METHODS

Sampling procedure. The cooperation of three smoked-fish processors in the Milwaukee area was obtained for these investigations. Sampling was limited to whitefish chubs taken by gill net or trawl net from Lake Michigan between Port Washington, Wis., and Milwaukee, Wis. Given catches of fish were sampled at eight processing stages.

Collection bags were provided by Milprint, Inc., Milwaukee. They were constructed from a material produced by lamination of Mylar (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) and polypropylene with a bonding agent (Milseal 32051) formulated to withstand heat sterilization. These pouches were individually enclosed in sealed kraft paper bags and sterilized at 121 C for 15 min.

Five fish from each sampling stage were collected from given catches. Round chubs (sample point 1) were taken directly from the nets and placed in sterile, identified pouches by the tug master. These containers were iced during the trip back to the fishery where they were picked up by the Health Department inspector. At this time, the inspector randomly selected five groups of five fish each from the catch which had been eviscerated and usually iced during the return trip from the fishing grounds. One group was placed in a sterile collection pouch and identified as stage 2. A second group was placed in the processor's cooler [38 F (3.3 C)] and a third in his

freezer [−5 to −23 F (−20.6 to −30.6 C)] for storage of 4 and 7 days, respectively. These were identified as sample stages 3 and 4. The fourth and fifth groups were allowed to go through normal processing and were picked up the following day.

Sample stage 5 was comprised of one of these groups which had been immersed 4 to 16 hr in a brine tank of 40 to 50° salinity. Brined chubs which had been rinsed in tap water were identified as sample 6.

The handling of fresh smoked fish varied at the three plants. One processor wheeled the smoking rack from the smoke chamber back through an area where brined fish were handled. This rack was stored in a 38 F (3.3 C) refrigerator, which also housed both raw and brined fish. After a cooling period of 1 to 2 hr, the rack was moved to an area for packaging just outside the entrance to the smoke chambers. This area was also adjacent to the traffic lane used for transporting racks of brined chubs to the cooler and to the smoke chambers.

The second processor removed fish racks from the smoke chamber directly to an area where raw and brined chubs were handled. The smoked fish were allowed to cool at room temperature prior to packaging.

The third processing plant employed a smoke house separate from the main building in which fish were cleaned and brined. Fish racks were transported by an indoor-outdoor, overhead trolley. After the fish were smoked, the racks were returned to the main building. Fish on these racks were cooled either in a refrigerator or more usually at room temperature.

At each fishery, two packages of five smoked chubs each were collected directly from the smoking racks into retail distribution bags (low-density polyethylene). One package, identified as sample 7 (fresh smoked), was delivered to the laboratory that day. The other was placed in the processor's retail display case [36 to 42 F (2.2 to 5.6 C)], from where it was collected after 7 to 8 days of storage.

Upon receipt at the laboratory, the samples were processed immediately or stored at 4 C when there was to be a delay of a few hours. In those instances, when a delay of a day or more before examination was anticipated, the specimens were stored at −20 to −30 C.

Media. Two media were employed for enrichment purposes. One was prepared from fresh ground beef liver which had been previously skinned and freed of adhering fat (1). Screw-cap bottles of 240-ml capacity were prepared with a 1-inch (2.54-cm) depth of ground liver and a sufficient amount of liver infusion broth, usually 40 ml, to exceed the level of meat by 2 inches (5.08 cm). The bottles of cooked liver media (CLM) were sterilized at 121 C for 20 min. The CLM was used either freshly prepared and cooled to 60 C, or, when stored, it was held in a boiling water bath for 10 min and cooled to 60 C prior to inoculation. A second enrichment medium consisting of Trypticase (BBL), peptone, and glucose (TPG) was prepared following a published formula (13) which was modified by the addition of 0.1% soluble starch. TPG medium was always freshly prepared the day it was to be used.

Two solid isolation media were employed. One was prepared from 5% sheep-blood agar (BBL Trypticase Soy Agar base), with 20 units of Penase (Difco) per ml plus 2.5 mg of *p*-aminobenzoic acid per 100 ml; the other was prepared from pork infusion-pea infusion agar (2).

Enrichment procedure one. Five fish per sample were sectioned into head, abdominal, and dorsal portions. The like parts from individual samples were combined and passed through sterile, home-type food choppers. The ground material was collected into appropriately identified sterile beakers which were sealed and set aside until eight samples were processed. Approximately 10 g of ground flesh from each beaker (head, abdominal, and dorsal) were inoculated into identified jars of CLM equilibrated to 60 C. After re-equilibration, cultures were held at that temperature for 15 min. These heat-shock enrichments were rapidly cooled in flowing tap water to 25 C.

Enrichment procedure two. The following procedure was used to determine whether detection methodology could be improved. Remaining portions of fish, from which the 10 g had been removed for enrichment procedure one, were combined to make a composite of head, dorsal, and abdominal materials from five fish. This composite was placed into 1-liter flasks containing 600 ml of TPG with 0.1% soluble starch previously equilibrated to 60 C. Each flask was re-equilibrated to 60 C after inoculation, heat-shocked, and cooled as above.

Enrichment procedure three. To determine whether methodology could be further improved, five whole fish per sample were comminuted through food choppers. The resulting material was thoroughly mixed and divided into approximately two equal portions. One portion was heat-shocked and cultured as described in enrichment procedure two above. The second portion was inoculated into TPG plus 0.1% soluble starch having a temperature of 20 to 25 C and was incubated directly. This enrichment culture was identified as nonheat shock.

Incubation of all enrichment cultures was carried out in biochemical oxygen demand incubators at 28 C for 7 days.

Toxicity testing and neutralization procedures. Samples of 10 to 25 ml of broth were withdrawn from the 7-day old cultures and centrifuged in a Sorvall RC-2 machine at 12,100 × *g* for 30 min at 1 C. Supernatant fluids were harvested and stored in sterile test tubes immersed in ice baths. Each supernatant fluid was divided into three portions. One was heated in a boiling water bath for 10 min. A second was adjusted to *p*H 5.5 to 6.5, when necessary, and was treated with a final concentration of 1.0% Trypsin (Difco, 1:250) for 45 min at 37 C (7). The third portion was not heated. Each portion was diluted 1:10 in cold gel-phosphate buffer, *p*H 6.2 (7). Paired white mice were injected intraperitoneally (ip) with 0.5 ml of the diluted supernatant fluids. The animals were kept under observation for 4 days. Death of mice within 2 to 24 hr after injection of trypsinized or unheated supernatant fluid, and survival of the pair of mice that received boiled extract, designated the material as presumptively positive for botulinal toxin.

All toxic enrichment cultures, both presumptive

and those apparently nonspecific, were re-examined as follows: Individual paired mice were protected with monovalent *C. botulinum* antitoxins (supplied by A. R. Prévot, Pasteur Institute, Paris) of types A, B, C, D, and E. These were diluted to contain 100 French units per ml (1 French unit neutralizes 100 MLD of homologous toxin). On those occasions when its use was warranted, type F antitoxin (National Communicable Disease Center, Atlanta, Ga.) was employed along with the above five types in repeat neutralization tests. Each pair of monovalently protected mice received 0.5 ml of diluted antitoxin by an ip route. These five pairs of mice were rested 1 to 2 hr before being challenged, ip, with 0.5 ml of either a 1:10 or 1:5 dilution of extract in gel-phosphate buffer. The animals were observed through 4 days. Four unprotected mice were used as controls in each neutralization test. Two received boiled extract, and two, trypsinized or untreated (diluted) extract as indicated by the presumptive test. Those extracts which failed to kill a single pair of mice protected with a monovalent antitoxin, but which killed all other mice, except the pair receiving boiled extract, were considered to contain *C. botulinum* toxin homologous to the protecting antitoxin and were designated as confirmed positives.

Isolation procedures. Recovery of toxigenic clostridia was attempted from all toxic enrichment cultures regardless of classification (presumptive, confirmed, or nonspecific). To effect isolation, 2 ml of enrichment culture sediment was transferred to sterile screw-cap test tubes containing 2 ml of absolute ethyl alcohol (10). These sediments were held at room temperature for 1 hr, with occasional shaking. A loopful of sediment was then streaked on the surface of a 5% blood-agar plate, and a second loopful, on the surface of a plate of freshly prepared pork infusion-pea infusion agar. Four such plates were enclosed in phosphorus ignition jars according to a prescribed procedure (14), except that a small amount of $CaCO_3$ was included in the phosphorus container. The jars were placed in a 28 C incubator and held for 18 to 24 hr. Immediately upon removal from the anaerobic jars, the plates were examined under a magnification of 7 with a stereoscopic microscope. Colonies similar in appearance to *C. botulinum* (6, 9) were picked and transferred to the bottom of 20 by 150 mm screw-cap tubes of previously boiled and cooled CLM. Between 4 and 20 colonies per plate were subcultured, and the entire picking operation was completed within 20 min. These CLM cultures were incubated 7 days at 28 C and were examined for toxicity, and neutralization if warranted, as described above. Toxic enrichment cultures from which toxigenic *C. botulinum* was isolated were identified as completed positives.

RESULTS

Enrichment cultures. It became apparent early in our surveillance studies that *C. botulinum* contamination appeared to be randomly distributed between head, abdominal, and dorsal portions of the fish examined, as is indicated in Table 1. Furthermore, *C. botulinum*, when detected even in eviscerated fish, was found only once to be present in all three sections from a given sample and only occasionally in two sections.

These data also suggest that *C. botulinum* was present in or on fish in very low numbers. As a means of determining whether the examination individually of 10-g portions of head, abdominal, and dorsal sections of fish was sufficient to detect low numbers of *C. botulinum*, a comparative study was performed. Remaining portions of fish prepared as in enrichment procedure one were combined. The composite sample was then cultured as described in enrichment procedure two. A total of 164 packages of fish, 160 collected from 20 catches and 4 from another catch, were examined. Table 2 shows that a confirmed demonstration of the presence of *C. botulinum* was obtained in 14 packages of fish. Twelve of the 14 were obtained by culturing the entire mass of five fish remaining after the removal of the 10-g portions. In one instance (S-23,2), a positive result was obtained from a 10-g portion as well as the corresponding five fish composite sample. In two instances, (S-21,3 and S-24,3) positive results were obtained only in 10-g portions (dorsal and abdominal materials, respectively).

These results clearly indicate the advisability of culturing the entire mass from five fish for maximal detection of *C. botulinum*.

An effort was made to compare the frequency with which *C. botulinum* might be detected by heat-shock enrichment (60 C, 15 min) as opposed to nonheat-shock enrichment in TPG. Thus, 659 specimens of five fish each were ground, and one-half of each sample was cultured by heat shock and its counterpart by nonheat shock. The results (Table 3) do not allow one to conclude that exposing enrichment cultures to 60 C for 15 min is either advantageous or deleterious to detection of *C. botulinum*. One must consider the possibility that 38 samples harbored the organism in only one of two halves of the ground material. The data do indicate, however, that the combined use of heat-shocked and nonheat-shocked samples generally yield a greater number of positive samples for *C. botulinum* than either system yields when employed alone.

Distribution and prevalence. Initially, individual catches of fish were sampled at eight stages of processing, six prior to smoking and two after smoking of the whitefish chubs. Table 4 shows that *C. botulinum* occurred in all processing stages prior to smoking. The presence of *C. botulinum* was demonstrated to range from 6% in freezer-stored chubs to 21% in brine-tank chubs. One of 66 packages of fresh smoked chubs, collected directly from smoke racks on the day of processing, was found to contain *C. botulinum*. In continuing surveillance studies,

TABLE 1. *Distribution of Clostridium botulinum in or on whitefish chubs*[a]

Section(s) yielding toxic cultures	No. of confirmed toxic cultures at each of 8 processing stages[b]								Total confirmed/ no. cultured
	1	2	3	4	5	6	7	8	
Head	1				2	2			5/416
Abdominal	1	1	2		1				5/416
Dorsal		1	2		1				4/416
Head and abdominal				1		1			2/416
Head and dorsal									0/416
Abdominal and dorsal									0/416
Head, abdominal, and dorsal			1						1/416

[a] Catches of fish, 52; 8 sample points/catch.
[b] CLM cultures, equilibrated to and held at 60 C for 15 min prior to rapid cooling and incubation. Culture supernatant fluids specifically neutralized by monovalent antitoxin.

sampling of fish was reduced to: whole chubs, brine-tank fish, and fresh smoked fish. Surveillance was later limited to fresh smoked chubs only, in order to gain prevalence information from a sufficiently large sample. Data from this portion of the study are presented in Table 5. In 39 catches (brine-tank chubs unavailable from three catches), *C. botulinum* was present in 17 to 42% of the fish collected at stages prior to smoking. Of 472 packages of fresh smoked fish, 6 contained *C. botulinum*. This rate of about 1% agrees well with that observed with the more limited sample depicted in Table 4. Consolidation of surveillance data from Tables 4 and 5 provides the following *C. botulinum* prevalence values: round chubs, 13/90 (14%) positive; fresh eviscerated chubs, 12/78 (13%); and brine-tank chubs, 20/102 (20%).

In the conduct of experiments designed to assay the sensitivity of heat-shock versus nonheat-shock enrichment, 320 individual fresh smoked fish were examined; 4 of these produced toxic cultures and 3 (1%) were confirmed to contain *C. botulinum*. From Tables 4 and 5, it is seen that 538 packages (five fish per package) of fresh smoked fish were examined. These, in addition to the 320 packages of single fish, provided 858 packages of fresh smoked fish; 10 (1%) were confirmed to contain *C. botulinum*, 9 of which provided isolates. The tenth confirmed culture was inadvertently discarded before an isolation attempt was made. These smoked fish were collected from

TABLE 2. *Demonstration of Clostridium botulinum in or on fish by two culture methods*[a]

Sample no.	Inoculum			
	Head[b]	Abdominal[b]	Dorsal[b]	Entire mass of ground fish[c]
M-16,5				+
M-17,5				+
M-18,4				+
M-18,6				+
M-24,4				+
S-17,6				+
S-18,5				+
S-21,3			+	
S-21,6				+
S-22,3				+
S-23,2		+		+
S-24,3		+		
S-25,5				+
S-26,2				+
Totals	0	2	1	12

[a] Confirmed by neutralization of enrichment culture supernatant fluids.
[b] CLM culture, heat shock at 60 C for 15 min.
[c] TPG culture, heat shock at 60 C for 15 min.

TABLE 3. *Detection of Clostridium botulinum by heat-shock versus nonheat-shock enrichment of halved portions of ground fish*[a]

Type of enrichment culture[b]	No. positive[c] from 659 specimens
Heat shock	13
Nonheat shock	25
Heat shock and nonheat shock	5
Total	43

[a] Five fish per sample.
[b] TPG medium.
[c] Neutralization of toxic supernatant fluid with monovalent antitoxin or isolation of *C. botulinum*.

TABLE 4. *Distribution and prevalence of Clostridium botulinum at eight stages of smoked fish processing by confirmed and completed tests (1964–65)*

Stage no.	Processing stage	No. of toxic cultures/ no. of specimens		No. of specimens containing *C. botulinum*, confirmed and completed
		Confirmed[a]	Completed[b]	
1	Round chub		5/63	5/63 (7.9%)
2	Fresh eviscerated	1/66	4/66	5/66 (7.6%)
3	Cooler stored		9/66	9/66 (13.6%)
4	Freezer stored	1/65	3/65	4/65 (6.2%)
5	Brine tank	1/66	13/66	14/66 (21.2%)
6	Brined and washed	1/66	7/66	8/66 (12.1%)
7	Fresh smoked		1/66	1/66 (1.5%)
8	Display case			0/66 (0.0%)

[a] Neutralization only of toxic enrichment culture supernatant fluids with monovalent antitoxin.

[b] Isolation of toxigenic *C. botulinum* from toxic enrichment culture.

three processors in the Milwaukee area. All had been processed in smoke chambers in which an internal temperature of a single fish reached at least 180 F (82.2 C) and maintained for a minimum of 30 continual min. These values have been attested by tapes produced by recording thermometers employed at the time of smoking.

Isolation. Not all confirmed positives were corroborated by isolation of *C. botulinum* from toxic enrichment cultures (Tables 4 and 5). Of 46 confirmed positives, 42 yielded isolates of *C. botulinum* (Table 4). Similarly, data in Table 5 indicate 15 of 25 confirmed cultures provided toxigenic isolates. This was, in fact, not the case. The data in Tables 4 and 5 were derived from enrichment culturing of 1,071 packages of fish collected from the previously described sampling stages. Of these packages, 71 produced toxic cultures, the supernatant fluids of which, at a 1:10 dilution in a dose of 0.5 ml, killed mice with symptoms of botulism (4); 60 of these 71 packages (85%) were shown to be positive for *C. botulinum* by confirmed or confirmed and completed tests. Eleven of the 71 yielded toxigenic isolates, to conform with the completed category; yet, supernatant fluids of the respective toxic enrichment cultures had not produced neutralization patterns which permitted identification of the causative organism. Most of the enrichment culture supernatant fluids from these 11 samples lost toxicity upon storage at 4 to 6 C. A few became nonspecifically toxic upon storage at the same temperature. A total of 57 of these 71 samples (80%) yielded isolates of *C. botulinum*.

The toxin types of *C. botulinum* detected by enrichment culture neutralization and isolation are shown in Table 6. These distribution data are derived from the same 1,071 cultures which form the subject matter for Tables 4 and 5. Type C was detected on two occasions, once in an eviscerated sample and once in a brine-tank sample. Type B was encountered twice, once in a brine tank sample and once in a freshly smoked fish. Neither of the confirmed type C enrichment cultures yielded a toxigenic isolate; however, both type B enrichment cultures conformed to the completed category. *C. botulinum* type E was detected most frequently in these surveillance studies. Its

TABLE 5. *Distribution and prevalence of Clostridium botulinum at four stages of smoked-fish processing by confirmed and completed tests (1965–66)*

Stage no.	Processing stage	No. of toxic cultures/ no. of specimens		No. of specimens containing *C. botulinum* confirmed and completed
		Confirmed[a]	Completed[b]	
1	Round chub	6/27	2/27	8/27 (29.6%)
2	Fresh eviscerated	2/12	3/12	5/12 (41.7%)
5	Brine tank	2/36	4/36	6/36 (16.7%)
7	Fresh smoked	—	6/472	6/472 (1.3%)

[a] Neutralization only of toxic enrichment culture supernatant fluids with monovalent antitoxin.

[b] Isolation of toxigenic *C. botulinum* from toxic enrichment culture.

TABLE 6. *Clostridium botulinum types detected and isolated from seven processing stages of whitefish chubs*

Stage no.	Processing stage[a]	No. of specimens which contained *C. botulinum*					
		Confirmed (enrichment culture neutralized)			Completed (isolated)		
		Toxin type			Toxin type		
		B	C	E	B	C	E
1	Round chub			14			7
2	Fresh eviscerated		1	8			7
3	Cooler stored			7			9
4	Freezer stored			4			3
5	Brine tank	1	1	10	1		16
6	Brined and washed			7			7
7	Fresh smoked	1		6	1		6
	Totals	2	2	56	2		55[b]

[a] *C. botulinum* was not demonstrated in any fish obtained from processing stage eight (retail display cases).

[b] Eleven isolated from nonconfirmable toxic enrichment cultures.

presence has been corroborated by isolation from 55 of 71 presumptively positive enrichment cultures.

DISCUSSION

The occurrence of *C. botulinum* in fish of the Great Lakes has been abundantly demonstrated by others (3, 4, 8). Data contained herein reveal the minimal extent to which *C. botulinum* contaminated whitefish chubs occur along processing lines of smoked-fish producers. Failure to develop toxic enrichment cultures cannot, as yet, be taken as evidence that *C. botulinum* was not present in fish (4). The sensitivity of procedures employed for detection of *C. botulinum* has yet to be evaluated. Such studies will have to consider the antagonistic effect of substances produced by concomitantly occurring microorganisms (11).

In the design of surveillance studies for detection of *C. botulinum* in environmental and food samples, one finds numerous enrichment procedures in the literature from which to select. These have been discussed in a recent review (5). This laboratory has undertaken a comparison of three enrichment methods during the course of the surveillance studies reported herein. These data reveal that the development of toxic enrichment cultures is greatly increased when the entire mass of five ground fish is used rather than the culturing of a presumably representative 10-g portion of an homogenate derived from five fish.

Although the use of TPG medium supplemented with 0.1% soluble starch as an enrichment substrate for detection of *C. botulinum* organisms has not previously been evaluated, data in this report indicate it is quite efficient. Quantitative studies are in progress in this laboratory to determine with more certainty the efficiency of TPG enrichment with and without heat shock.

The use of heat to select for *C. botulinum* has been already discussed with respect to spore survival of the various toxin types (5). The comparative susceptibility to heat inactivation of type E spores in contrast to spores of types A and B has been documented at temperatures of 70, 75, and 80 C (12). However, exposure to 60 C for as long as 30 min has been reported to have no appreciable effect on viable counts of type E spores (12). More recently, it has been suggested that heat treatment at 60 C for 30 min destroyed *C. botulinum* in mud samples from which type E organisms grew without the use of heat (4). Our data suggest that unequal distribution of *C. botulinum* in a given sample accounts for one of two portions failing to develop toxic cultures. It is clear that the advantage or disadvantage of mild heat (60 C for 15 min) in selecting for *C. botulinum* remains to be quantitatively evaluated.

In the present study, 26 nonspecific cultures developed from specimens prepared without heat treatment, whereas only 12 developed in heated cultures. The problem of nonspecific cultures occurred most frequently with raw fish samples, the total bacterial load of which, as previously reported by Pace and associates (Botulism Symp., Moscow, 1966), was found to be of considerable magnitude.

Microscopic slide preparations of heat-shock enrichment cultures revealed few to no gram-negative bacteria, yet they were abundant in companion nonheat-shock cultures. It is probable that mild heat treatment provides a competitive advantage to gram-positive bacilli and cocci, as evidenced by their predominance in the heat-shock enrichment cultures.

Data, reported herein, indicate duplicate enrichment cultures, heat shock and nonheat shock, of surveillance samples will result in maximal detection of *C. botulinum* in whitefish chubs. Of 84 *C. botulinum* enrichment cultures encountered, 8 were detected only with trypsin-treated extracts, and, on two occasions, toxin was detected only in nontrypsin-treated extracts. Accordingly, it appears advisable that trypsin activation be employed concomitantly with nontrypsin-treated enrichment supernatant fluids in surveillance studies.

Isolation data reported in this paper are not intended to reflect any particular skill in selecting toxigenic colonies. They are included only to corroborate the occurrence of *C. botulinum* as detected by neutralization of toxic enrichment cultures. An important observation to be noted is that 11 specimens produced toxic enrichment cultures which could not be specifically neutralized. These cultures did provide isolates of *C. botulinum*. Thus, it is worth the effort to attempt isolation from all toxic enrichment cultures, whether or not *C. botulinum* is confirmed to be present, not only to complement but also to supplement surveillance data. Ninety-one samples of fish, tested by both heat-shock and nonheat-shock methods, were studied for toxigenic isolates regardless of whether they had been demonstrated to be toxic or not. At no time was *C. botulinum* recovered from a nontoxic enrichment culture.

Therefore, one might conclude that the expenditure of money and labor required for isolation of *C. botulinum* is not warranted when confronted with nontoxic enrichment cultures. This may be particularly true when dealing with surveillance specimens. However, for a thorough analysis it would be desirable to include such a

practice in the examination of outbreak specimens.

As many as 20 colonies have been selected from single plates. At times, all have been found to be toxigenic; however, in some attempts, only 2 or 3 of 20 have so rewarded the effort. Consequently, it appears advisable to pick at least four suspicious colonies or more per plate until reliable criteria for selection of toxigenic *C. botulinum* colonies are established.

All toxigenic isolates have been tested in complete neutralization tests. To date, all isolates from a given specimen have been of the same toxin type and have never failed to be homologous with toxin types indicated by corresponding enrichment culture neutralization, if available. The relative infrequency with which toxin types other than E were observed is probably responsible for failure as yet to encounter fish samples contaminated with multiple toxin types of *C. botulinum*.

The fact that 1% of the freshly smoked chubs [180 F (82.2 C) for 30 min] tested were shown to harbor *C. botulinum* is both distressing and encouraging. One cannot intelligently define the botulinogenic potential of these 10 fish with data at hand. Similarly, the data do not clearly demonstrate why 1% contamination was observed. It is apparent this study was not designed to differentiate between spores capable of resisting the smoking procedure and those that may have gained entry to the product as postprocessing contaminants. It was a common practice of processors to remove smoked fish from smoking chambers directly back into an area where raw fish are handled. Therefore, one cannot rule out postprocessing contamination, even though exposure in such an area was brief. Consequently, three possibilities for the occurrence of *C. botulinum* containing smoked fish in this study must be considered. (i) persistence of *C. botulinum* in or on fish heated to an internal temperature of at least 180 F (82.2 C) for at least 30 min; (ii) failure to obtain this temperature and exposure time uniformly in all fish processed in a given smoke house; (iii) postprocessing contamination.

Although the process of smoking at 180 F for 30 min remains to be definitively evaluated for its ability to render smoked fish free from *C. botulinum*, its adoption is recommended for the following reasons: (i) *C. botulinum* contamination of 20% of fish, at a stage immediately prior to smoking, was reduced to 1% in the finished product, which is a step in the right direction in reducing a public health hazard; (ii) some 3 years of compliance by Milwaukee area fisheries attests to the commercial feasibility of the process.

Encouraging as these data may be, they serve only to reinforce a conclusion that smoked whitefish chubs processed at 180 F for 30 min must be considered not only as highly perishable (Pace et al., Botulism Symp., Moscow, 1966), but also as potentially dangerous. Therefore, it appears advisable, to further assure consumer safety, that fish processed under these conditions be limited to a shelf life not to exceed 7 days at 40 F (4.4 C) or below.

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