APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1981, p. 179-183
0099-2240/81/010179-05$02.00/0
Vol. 41, No. 1

Persistence of *Clostridium botulinum* Type B on a Cattle Farm After an Outbreak of Botulism

S. NOTERMANS,* J. DUFRENNE, AND J. OOSTEROM

Laboratory for Zoonoses and Food Microbiology, National Institute of Public Health, 3720 BA Bilthoven, The Netherlands

On farms involved in botulism outbreaks, cycles of *Clostridium botulinum* have occurred. The cycles were initiated by feeding brewers' grains contaminated with proteolytic *C. botulinum* type B to the cows. Spreading of manure containing feces of these cows increased the contamination of the pastures. In grass silages prepared with wilted grass from these pastures the number of *C. botulinum* type B organisms increased, and toxin type B was produced. Feeding cows with the contaminated silage fodder completed the cycle. Besides contamination of human foodstuffs (milk and meat), further contamination of the environment occurred. It was demonstrated that fowl may be important vectors in spreading *C. botulinum*.

In 1976 and 1977 outbreaks of botulism followed by the death of many animals occurred on about thirty dairy farms in The Netherlands. The disease was caused by feeding the animals brewers' grains (malt). Proteolytic *Clostridium botulinum* type B cells as well as botulinum toxin type B were found to be present in the brewers' grain (1). Toxin type B was detected in only 4.5% of the sera of the animals affected (4). In the feces of the cows large numbers of *C. botulinum* type B organisms (10^5 to 10^7/g) were detected (8). Also, after termination of feeding the contaminated fodder, *C. botulinum* was present in the feces for more than 8 weeks. Consequently, the environment of the farms, especially the grass pastures, was probably contaminated by manure containing *C. botulinum*. Whether cows would be reinfected would be determined by the persistence of such contamination and the possibility that *C. botulinum* multiplied in this environment.

From results of Notermans et al. (9) it has become clear that proteolytic strains of *C. botulinum* are able to produce toxin with grass as a substrate. In grass silages prepared with wilted grass, circumstances are favorable for multiplication of *C. botulinum*. However, until now only a few reports describe botulism caused by grass silage (3, 5).

In the present investigation the persistence of *C. botulinum* on cattle farms affected by botulism was studied; also, the significance of grass silages prepared with wilted grass in the multiplication of *C. botulinum* was investigated under natural conditions.

MATERIALS AND METHODS

History of botulism on the farm investigated. On 4 June 1977 a severe outbreak of botulism occurred on the dairy farm. The outbreak was caused by feeding the cows a supplement of brewers' grain containing *C. botulinum* type B as well as botulinum toxin type B. Immediately after recognition of the disease, the feeding with brewers' grain was stopped. The morbidity among the cows was 74%, and the mortality amounted to 42%. Toxin type B was detected in two serum samples of cows before death. In the feces of affected cows high numbers of *C. botulinum* organisms were detected (ca. 10^5/g). To replenish the herd, new cows were purchased from local markets 1 month after the outbreak.

Cows. On the investigated farm 52 dairy cows were present. In the wintertime (October to May) they were housed in a free stable with cubicles. In the summertime they were outside on the pastures. During the housing period the cows were fed with grass silage (ca. 15 to 20 kg of fodder per animal daily) and concentrates. Occasionally, they were fed with maize silage. The maize crop used for preparing the silage was obtained from elsewhere. Starting in October 1978, at regular intervals, feces of 10 cows were sampled randomly. From these samples the presence of *C. botulinum* was determined quantitatively.

In April 1979 and April 1980 sera from 10 cows selected randomly were screened for antibodies against botulinum toxin type B by the serum neutralization test.

Besides this study, feces of cattle from four other farms with a comparable history of botulism were sampled and investigated for the presence of *C. botulinum*. Feces was sampled in January 1979 and January 1980.

Grass pastures. The grass pastures were situated around the farm and had an area of about 200,000 m^2. The pastures were surrounded by ditches and a small lake. As a consequence, waterfowl were often foraging on the pastures. The pastures were fertilized during the winter and the early spring with manure from the cows as well as with chemical nitrogen fertilizer. Starting in August 1977 soil samples were obtained randomly from the pastures, and the number of *C. botulinum* organisms present was determined. Further-

more, the feces of fowl (predominantly coots) foraging in the pastures were sampled, and the presence of *C. botulinum* was investigated. These feces were gathered in the neighboring pastures.

Grass silages. On the cattle farm investigated, grass silages were prepared with wilted grass from the pastures and covered with plastic sheets. The stability of this type of silage results from its low oxygen content, a low water activity (a_w), and partially from lactic acid fermentation (11). During the housing period of the winter of 1978 to 1979, cows were fed with four different grass silages. One silage (no. 1) was prepared in 1977 before the outbreak of botulism and was not fed in the winter period of 1977 to 1978. The other three silages (no. 2, 3, and 4) were prepared in 1978, 1 year after the outbreak. During the housing period of the winter of 1979 to 1980 cows were fed with two grass silages (no. 5 and 6). These silages were prepared in the spring of 1979 under not ideal weather conditions (high humidity), resulting in an average a_w higher than that of the silage prepared in the spring of 1978 (see below).

The grass silages were sampled at the time of feeding. Samples were taken by using a depth corer (vertical stab samples) or by hand (center and edge samples from the open side). From these samples the pH and a_w were determined. Furthermore, the presence of *C. botulinum* and botulinum toxin was investigated quantitatively.

a_w. The a_w was determined at 20°C by using the a_w measurement device with a dew point hygrometer described by Northolt et al. (7). The accuracy of the measurement was ±0.005.

pH. The pH of the samples of grass silage was determined by mixing 100 g of grass with 200 ml of distilled water. After shaking for 1 h the pH of the mixture was determined.

Detection of *C. botulinum*. In grass silage samples the presence of *C. botulinum* was determined by macerating 100 g of silage is a Waring blender. Serial 10-fold dilutions of macerate were made, and 1 ml of each dilution was transferred into each of two tubes containing 30 ml of freshly prepared fortified egg meat medium (150 g of egg meat [Difco Laboratories, Detroit, Mich.], 10 g of glucose, and 10 g of ammonium sulfate per liter, sterilized at 120°C for 15 min). One tube of the pair was heated at 70°C for 20 min. The heated and the unheated tubes were incubated anaerobically at 30°C. After 5 days of incubation, the culture fluids were examined for botulinum toxin.

For the quantitative assessment of *C. botulinum* in feces, 50 g of feces of each cow were shaken with 450 ml of physiological saline solution. Serial 10-fold dilutions of the mixtures were made, and 1 ml of each dilution was transferred into each of four tubes containing fortified egg meat medium. Before incubation two tubes of the four were heated at 70°C for 20 min.

To detect *C. botulinum* in the soil samples of the pastures, 25-g portions of 20 soil samples obtained randomly from the pastures were mixed and homogenized. Serial 10-fold dilutions of the mixtures were made, and 1 ml of each dilution was transferred into each of 5 tubes containing fortified egg meat medium. Before incubation all tubes were heated at 70°C for 20 min.

The presence of *C. botulinum* in the other samples (concentrates, maize silage, feces of cows, and feces of birds) was determined by transferring 0.5 g of each sample into each of two tubes containing fortified egg meat medium. One tube of the pair was heated at 70°C for 20 min.

After incubation of the tubes at 30°C for 5 days the culture fluids were examined for the presence of botulinum toxins.

Toxicity. To determine botulinum toxins in culture fluids, 2-ml portions were diluted with 6 ml of sterile 0.05 M phosphate buffer (pH 6.0) containing 2 g of gelatin (Difco), 1 mg of streptomycin, and 10^6 U of penicillin per liter. Activation of the diluted samples was performed by adding trypsin (Sigma type III; Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 0.2 mg/ml and incubating at 37°C for 30 min. Samples of 2 ml of the diluted and trypsinized fluids were mixed with 0.5 ml of antibotulinum serum (Institut Pasteur, Paris) of types homologous to the toxin expected. Two mice (18 to 20 g) were injected intraperitoneally with 0.5 ml of each preparation and observed for 4 days.

To determine botulinum toxins in grass silage, samples of 200 g were mixed with 200 ml of sterile 0.05 M phosphate buffer (pH 6.5) containing 2 g of gelatin, 1 mg of streptomycin, and 10^6 U of penicillin per liter. After rotary shaking at 250 rpm for 2 h the fluid was treated with trypsin as described above. The amount of toxin present was determined by injecting two mice with twofold dilutions. The type of toxin present was determined as described for culture fluids.

Serum neutralization test. The serum neutralization test was carried out as described by Ohishi et al. (10). Type B-L toxin, purified by the methods of Kozaki et al. (6), was diluted with 0.05 M phosphate buffer (pH 6.0) containing 0.2% gelatin. The toxin was activated by trypsin as described above. The reaction was terminated by adding soybean trypsin inhibitor (Sigma) at a final concentration of 0.4 mg/ml. In the tests 1 ml of the toxin dilution containing 20 intraperitoneal mouse 50% lethal doses (LD_{50}) of toxin was mixed with 1 ml of serum. After incubation at 37°C for 30 min, 0.5 ml of the mixture was injected intraperitoneally into two mice. The mice were observed for up to 5 days.

RESULTS

pH and a_w of grass silages and the presence of *C. botulinum* and botulinum toxin in grass silages. All grass silages fed to the cows since October 1978 were investigated. In the silage prepared in 1977 before the outbreak of botulism, *C. botulinum* and its toxin were not detected (Table 1). In the three silages prepared in 1978 (no. 2, 3, and 4) *C. botulinum* type B was present; however, toxin was not found in these silages. In the silages prepared in 1979 *C. botulinum* was detected in 6 of the 8 samples investigated. Toxin type B was demonstrated in 4 of the 8 samples. In two samples (edge samples no. 11 and 15) *C. botulinum* was present in 0.0001 g. In sample no. 16 *C. botulinum* could not be detected, although 20 intraperitoneal mouse LD_{50} of toxin type B was present.

The a_w of the silage prepared in 1979 were higher than those of the silages prepared in 1978. Edge samples showed the highest a_w values.

Presence of *C. botulinum* in other fodder. A total of 30 samples of maize silage were investigated for the presence of *C. botulinum*. *C. botulinum* was not detected in these samples. Also, in 25 samples of concentrates *C. botulinum* was not detected.

Excretion of *C. botulinum* by cows: clinical aspects. From the results it could be concluded that *C. botulinum* was accurately detectable only if the inoculated fortified egg meat media were heat shocked before incubation. Therefore, the most probable numbers were determined from heated samples (2). The average most probable numbers per gram of feces of the 10 cows investigated are presented in Fig. 1. In this figure the identities of the silages fed to the cows are also indicated. There was a correlation between the level of contamination of the feces and the level of contamination of the grass silages. When silage fodder not contaminated with *C. botulinum* (silage no. 1) was fed to the cows,

TABLE 1. *pH and a_w of grass silages and occurrence of C. botulinum and botulinum toxin*

Grass silage no.	Yr of preparation	Place of sampling	Sample no.	pH	a_w	*C. botulinum* (type) in:				Type (and amount[a]) of toxin
						0.1 g	0.01 g	0.001 g	0.0001 g	
1[b]	1977	Vertical stab	1	NI[c]	NI	–[d]	–	–	–	–
		Vertical stab	2	NI	NI	–	–	–	–	–
2	1978	Vertical stab	3	5.77	0.93	+ (B)	–	–	–	–
		Center	4	6.35	0.94	+ (B)	+ (B)	–	–	–
3	1978	Vertical stab	5	6.26	0.94	–	–	–	–	–
		Edge	6	5.40	0.98	+ (B)	+ (B)	–	–	–
4	1978	Vertical stab	7	5.30	0.96	+ (B)	–	–	–	–
		Vertical stab	8	NI	NI	+ (B)	+ (B)	–	–	–
5	1979	Vertical stab	9	6.58	0.98	–	–	–	–	–
		Vertical stab	10	6.33	0.97	+ (B)	–	–	–	–
		Edge	11	5.95	0.98	+ (B)	+ (B)	+ (B)	+ (B)	B (17)
		Edge	12	6.02	0.98	+ (B)	–	–	–	–
6	1979	Vertical stab	13	5.82	0.97	+ (B)	+ (B)	–	–	B (2)
		Vertical stab	14	5.85	0.97	–	–	–	–	–
		Edge	15	5.60	0.99	+ (B)	+ (B)	+ (B)	+ (B)	B (15)
		Edge	16	6.00	0.99	–	–	–	–	B (20)

[a] Amount of toxin expressed as intraperitoneal mouse LD_{50} per gram.
[b] Grass silage no. 1 was prepared before the outbreak of botulism.
[c] NI, Not investigated.
[d] –, *C. botulinum* or toxin not detected.

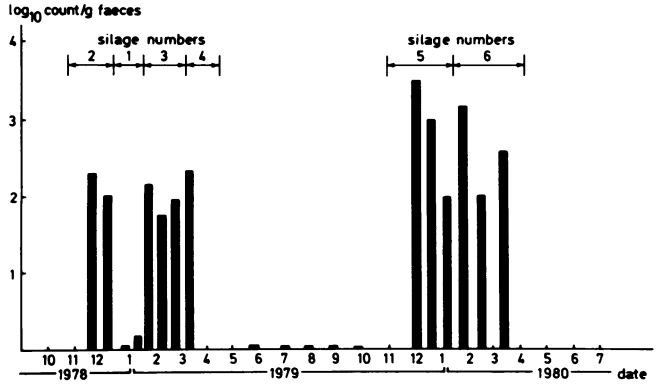

FIG. 1. *Excretion of C. botulinum type B by cows on a cattle farm affected by botulism in June 1977.*

C. botulinum organisms were present in the feces only in low numbers. Also, in summertime, when the cows were not fed any silage foder, the contamination of the feces was low.

The average number of *C. botulinum* organisms present in the feces during the housing period of the winter of 1978 to 1979 was about 100/g. In the housing period of 1979 to 1980 the contamination was higher and amounted to about 600/g.

No clinical symptoms of botulism were observed among the cows during the investigation. Also, no reduction of the normal milk yield was observed. In the sera of the cows no antibodies against botulinum toxin type B were detected.

Feces samples obtained from cows of four other farms affected by botulism in 1977 also contained *C. botulinum*. From each farm 20 samples were investigated; *C. botulinum* type B was demonstrated in 60 to 100% of the 0.5-g samples.

Presence of *C. botulinum* in the grass pastures and in feces of birds. The numbers of *C. botulinum* type B organisms present in the soil samples increased significantly after the outbreak of botulism (Table 2). Immediately after the onset of the disease *C. botulinum* type B was not detected. In 1980, 2.5 years later, more than 100 cells of *C. botulinum* were present per g of soil.

Feces of fowl foraging in these pastures were also contaminated with *C. botulinum* type B (Table 3). In 19 of 25 samples this bacterium could be demonstrated. Besides *C. botulinum* type B, type C was detected six times, and type D was detected once.

TABLE 2. *Numbers of C. botulinum type B organisms present in soil samples obtained from pastures*

Date of sampling	Most probable number per g of soil	95% confidence limits
1 August 1977	<0.2	<0.1–1.0
15 March 1978	2.7	1.3–6.8
7 May 1979	17	7–48
1 March 1980	130	50–390

TABLE 3. *Presence of C. botulinum in feces of birds foraging on grass pastures*

Date of sampling	No. of samples	No. of positive samples	No. of *C. botulinum* type:		
			B	C	D
15 March 1978	5	4	4	2	
7 May 1979	10	9	7	1	1
1 March 1980	10	8	8	3	

DISCUSSION

On dairy farms involved in botulism outbreaks, cycles of *C. botulinum* type B have occurred. In Fig. 2 the links of the cycles are presented schematically; the outbreaks of botulism are initiated by supplementary feeding of brewers' grain contaminated with *C. botulinum* type B. From the results of Notermans et al. (8) it was clear that during digestion of this fodder no decrease in the number of bacteria occurred. As a consequence, feces became contaminated with *C. botulinum* type B. Spreading of manure increased the contamination of the pastures. Therefore, it is not surprising that *C. botulinum* type B was present in grass silages prepared with grass from these contaminated pastures. It was demonstrated that in such grass silages the number of *C. botulinum* type B organisms increased, and that botulinum toxin type B was produced. Grass silages therefore form a link in the cycle of *C. botulinum* in which multiplication occurs, thus increasing the level of contamination on the farm. Feeding cows with the contaminated silate fodder completes the cycle.

Growth of *C. botulinum* in grass is determined, among other factors, by pH and a_w (9). In grass silages prepared with wilted grass the lactic acid fermentation is delayed. Therefore, the pH decreases only slightly during storage. The a_w, which is the most important stabilizing factor, is not uniform throughout a silo. Especially at the edges of the silo, the a_w may increase due to transport of moisture caused by changes in temperature, thus allowing outgrowth of *C. botulinum*. Growth of *C. botulinum* in grass silages steadily increases the contamination of the farm.

During this investigation no clinical symptoms of botulism were observed among the cows. The

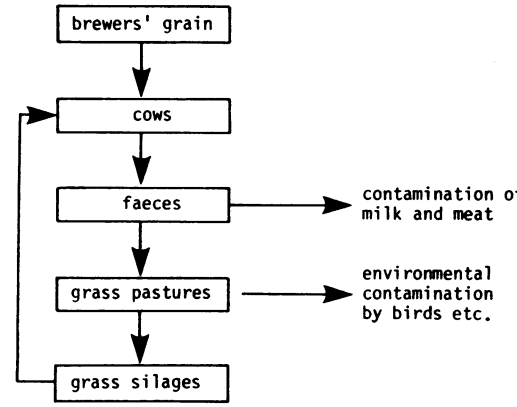

FIG. 2. *Schematic presentation of the cycle of C. botulinum type B on a cattle farm involved in a botulism outbreak caused by brewers' grain (malt).*

lethal oral dose for cows of crude toxin type B was estimated at 5.6×10^7 intraperitoneal mouse LD_{50}, whereas 6.4×10^6 intraperitoneal mouse LD_{50} sufficed to produce clinical signs of botulism (5). In this investigation such a dose would be obtained only if the cows were fed for a number of days with silage fodder from the edges of a silo. However, the laboratory experiments of Notermans et al. (9) showed that *C. botulinum* type B with grass as substrate may produce enough toxin to kill a cow that had consumed of 10 to 100 g of the contaminated grass. The fact that no circulating antibodies were detected in the serum of the cows suggests that the cows in these investigations were not exposed to large amounts of toxin. It also suggests that in all probability no intestinal production (in vivo production) of toxin had occurred.

The contamination of the feces of the cows is clearly caused by feeding contaminated silage fodder. In summertime when no silage fodder was fed the contamination of the feces was low. As a consequence of the contamination of the feces, milk produced by the animals may become contaminated with *C. botulinum*. If cows from such a farm are slaughtered it may also be expected that meat would be contaminated, since bacteria present on carcasses are mostly of fecal origin.

Besides contamination of human foodstuffs a contamination of the environment can also occur. Fowl, especially, are vectors capable of spreading *C. botulinum* over wide areas. In this way other grass pastures could become contaminated with *C. botulinum*, possibly resulting in cycles of *C. botulinum* on other farms without a previous history of botulism.

ACKNOWLEDGMENTS

We are grateful to L. DS. Smith and S. Kozaki for correction of the manuscript and critical review.

LITERATURE CITED

1. **Breukink, H. J., G. Wagenaar, T. Wensing, S. Notermans, and P. W. Poulos.** 1978. Food poisoning in cattle caused by ingestion of brewers' grains contaminated with *Clostridium botulinum* type B. Neth. J. Vet. Sci. **103**:303–311.
2. **De Man J. C.** 1975. The probability of most probable numbers. Eur. J. Appl. Microbiol. **1**:67–78.
3. **Gilyard, A. T., and R. Gilyard.** 1972. Grass silage: a hazardous horse feed. Cornell Vet. **32**:317–321.
4. **Haagsma, J., and E. A. Ter Laak.** 1978. Atypical cases of type B botulism in cattle, caused by supplementary feeding of brewers' grains. Neth. J. Vet. Sci. **103**:312–325.
5. **Haagsma, J., and E. A. Ter Laak.** 1978. Type B botulism in cattle caused by feeding grass silage. Report of a case. Neth. J. Vet. Sci. **103**:910–912.
6. **Kozaki, S., S. Sakaguchi, and G. Sakaguchi.** 1974. Purification and some properties of progenitor toxins of *Clostridium botulinum* type B. Infect. Immun. **10**:750–756.
7. **Northolt, M. D., C. A. H. Verhulsdonk, P. S. S. Soentoro, and W. E. Paulsch.** 1976. Effect of water activity and temperature on aflatoxin production by *Aspergillus parasiticus*. J. Milk Food Technol. **39**:170–174.
8. **Notermans, S., H. J. Breukink, T. Wensing, and G. Wagenaar.** 1978. Incidence of *Clostridium botulinum* in the rumen contents and faeces of cattle fed brewers' grains naturally contaminated with *Clostridium botulinum*. Neth. J. Vet. Sci. **103**:1327–1333.
9. **Notermans, S., S. Kozaki, and M. van Schothorst.** 1979. Toxin production by *Clostridium botulinum* in grass. Appl. Environm. Microbiol. **38**:767–771.
10. **Ohishi, I., G. Sakaguchi, H. Riemann, D. Behymer, and B. Hurvell.** 1979. Antibodies to *Clostridium botulinum* toxins in free-living birds and mammals. J. Wildl. Dis. **15**:3–9.
11. **Wieringa, G. W.** 1958. Effect of wilting on butyric acid fermentation in silages. Neth. J. Agric. Sci. **6**:204–210.