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Serological Studies of *Clostridium botulinum* Type E and Related Organisms

R. K. LYNT, JR., H. M. SOLOMON, D. A. KAUTTER, AND T. LILLY, JR.
Division of Microbiology, Food and Drug Administration, Washington, D.C.

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ABSTRACT

Clostridium botulinum type E antigens prepared from washed cells by either Formalin treatment or heating at 100 C were used for immunizing rabbits. Agglutination tests showed that high levels of antibody were produced by both types of preparations. Flagellar antigens were highly strain-specific, whereas the somatic antigens were sufficiently similar to produce complete cross-agglutination. One toxigenic strain produced toxigenic and nontoxigenic progeny which were physiologically and antigenically identical in all other respects. Other nontoxigenic strains whose growth, physiological, and morphological characters were identical to type E and strains which had some physiological differences completely cross-agglutinated with type E strains via the somatic antigen. Neither type of antiserum agglutinated other clostridia against which they were tested except for *C. acetobutylicum*. This reaction seems to be due to a nonspecific anamnestic response and does not appear to be related to the immunizing strains. The nontoxigenic strains studied seem to have no greater antigenic differences from type E strains than the type E strains have from each other.

Members of the genus *Clostridium* are usually classified on the basis of their morphological, cultural, and physiological characteristics, and, wherever applicable, by the identification of their specific toxins.

The existence of nontoxigenic strains that resemble toxin producers, and the variations in morphology and physiology among some toxigenic strains, may cause difficulty in classification.

Hoogerheide (5), for example, found it impossible to distinguish between *C. sporogenes* and a variant of *C. histolyticum* on the basis of morphology and fermentation patterns. Conceivably, if he had characterized the two strains by using additional criteria he might have separated them, but his inability to do so illustrates the shortcomings of classification based on morphology and fermentation patterns. Although identification of the specific toxin is a useful method of classification, Shattock (20), reviewing the serological classification of bacteria, suggested that toxin production is not a sufficiently stable character for taxonomy.

Mandia (11) based his classification of the proteolytic clostridia on antigenic formulas. He stated that the primary groupings should be based on the heat-stable antigens. *C. sporogenes* and *C. tetani* were found to share common heat-stable antigens with other proteolytic clostridia, but had additional antigens of their own. Moussa (12, 13), working with strains of *C. chauvoei* and *C. septicum*, found insufficient antigenic differences to warrant separation into two distinct species. He recommended that the classification of the genus *Clostridium* be based on antigenic formulas including flagellar, somatic, and spore antigens.

As early as 1938, McCoy and McClung (9), reviewing the literature, found that strains belonging to the same toxigenic type could be divided into smaller groups on the basis of common antigens. When a distinction was made between flagellar and somatic antigens, the flagellar antigens showed a narrow range of specificity and the somatic antigens were often common to all members of the species or type. However, since toxins can be identified readily, agglutination tests generally have not been used.

Nontoxigenic strains agglutinated by antisera produced against toxigenic clostridia have presented a problem of classification. Schoenholz and Meyer (19) considered such strains to be *C. botulinum*. On the other hand, Starin and Dack (22) found a strain which, though it was fully toxic, was not antigenic and was not agglutinated by antisera to their toxigenic strains.

McGaughey (10) selected two variants of *C. welchii* which differed in toxicity and antigenic structure from the parent strain. Although they cross-agglutinated with each other and neither cross-agglutinated with the parent strain, one was virtually nontoxigenic and the other was three to six times as toxigenic as the parent strain.

Smith (21) has found that *C. histolyticum* and *C. sporogenes* have common somatic antigens; *C. bifermentans* and *C. sordellii*, although they share common flagellar and somatic antigens as shown by Huang et al. (6), have been separated by Walker (23) on the basis of spore antigens.

Investigations dealing with the serological classification of strains of *C. botulinum* type E have been limited. Gunnison et al. (3) found two cultures of type E from Russia to be identical by agglutination and agglutinin absorption tests. On the other hand, Hazen (4) found strains from two different sources to be antigenically dissimilar. Nakamura et al. (14) used slide agglutination tests to classify strains isolated in the vicinity of Hokkaido, Japan, into five serological groups. Ono (16, 17), studying strains from the same source, found that the heat-labile antigens had a high degree of group specificity, whereas the heat-stable antigens were common to all; by cross-absorption experiments he identified antigenic components of some strains. He was also able to select variants which differed antigenically from the parent strain.

Walker and Batty (24) found that both somatic agglutination tests and a fluorescent-antibody technique were equally specific and could be used to distinguish type E from other types of *C. botulinum*; however, nontoxigenic strains were not included in their studies. Previously, they had shown that the closely related *C. septicum* and *C. chauvoei* could be separated by these procedures (2).

Naturally occurring nontoxigenic anaerobes which are morphologically, culturally, and biochemically identical to type E and produce a bacteriocin active against *C. botulinum* type E have been reported by this laboratory (7). Numerous other organisms resembling type E have been found in environmental samples. When these organisms are present in the same materials with type E, the detection of the toxin and the isolation of type E is complicated. It would be desirable to know whether these nontoxigenic organisms are variants of type E or organisms of a closely related group.

The purpose of this investigation was to determine whether serological methods, apart from toxin neutralization tests, might be useful for identifying *C. botulinum* type E and distinghishing it from organisms which resemble it.

MATERIALS AND METHODS

Strains. The strains studied are identified in Table 1. Strain 066BNT is a nontoxigenic variant of 066B which was previously described (7). Strain 066B was chosen for study because it produces nontoxigenic variants. The nontoxigenic strain used in these studies was derived from a toxigenic single colony isolate whose progeny consisted of both toxigenic and nontoxigenic clones. Alternate platings and broth subcultures of single colonies of the toxigenic strain continually gave rise to both types of colonies, and separate toxigenic (066B Tox) and nontoxigenic colonies were selected from a single plate.

Strains S5, 28-2, and 38-1 have morphological, cultural, and biochemical characteristics identical with those of type E, except that they are nontoxigenic. Two of these strains (S5 and 28-2) produce a bacteriocin (7). Strains S9 and PM15, in addition to being nontoxigenic, differ in their fermentation patterns and hemolytic ability, but closely resemble type E in colonial morphology. The nontoxigenicity of these strains was determined by intraperitoneal injection of 0.5 ml of undiluted culture filtrate into mice. The injection of these filtrates did not produce botulism symptoms or death of the mice.

All strains used for preparation of immunizing or agglutinating antigens were checked for purity. They were plated on Liver Veal Agar (Difco) containing 4% sterile egg yolk, and single colonies were isolated and propagated in the Trypticase (BBL)-peptone-glucose broth (TPG) of Schmidt et al. (18). Primary cultures grown in TPG were tested for toxicity in mice. To minimize the possibility of antigenic variation resulting from selection by repeated subculture, stocks were subcultured in TPG about every 3 months. From these, a new working culture was prepared whenever needed.

Immunizing antigens. TPG cultures (16-hr) of the desired strains were grown at 28 C. Microscopic examination of 16-hr cultures showed only vegetative cells. The cells were separated by centrifugation, were washed three times, and were resuspended in physiological saline at one-twelfth the original volume. On the basis of tube dilution titrations, these suspensions contained about 10^8 to 10^9 vegetative cells per milliliter. Whole-cell (H-O) antigens were prepared by adding Formalin to the suspension to give a 0.4% final concentration. The antigen was incubated at 37 C for 3 to 4 days, and was tested for toxicity in mice and for sterility. The heat-stable (somatic or O) antigens were grown, washed, and resuspended in the same manner as for whole-cell antigens, and then were heated at 100 C for 1 hr. When the suspension was cool, Formalin was added as a preservative to give a 0.3% final concentration. Antigens for immunization were stored under refrigeration until needed.

Preparation of antisera. Hyperimmune antisera for agglutination tests were produced in New Zealand white rabbits. The rabbits were given 1.0 ml of the appropriate antigen intravenously every 3 to 4 days for a total of six injections. After 1 week, they were given another 1.0-ml injection, and were bled by

TABLE 1. *Identification of strains of Clostridium*[a]

Strain designation	Source of isolation	Geographical area	Supplied by
Toxigenic			
066B Tox, 070, 0421 0422, 517, 1553, 5191, 5192, 5194, 8074, 8077, 42013, 42015	Smoked whitefish chubs outbreak of 1963	Great Lakes	FDA[b]
Kalamazoo	Smoked whitefish outbreak of 1963	Great Lakes	FDA
D8	Canned tuna fish outbreak of 1963	Exact source undetermined	FDA
Minnesota	Smoked ciscoes outbreak of 1960	Great Lakes	FDA
Su1, Su3	Viscera of Green Bay suckers	Great Lakes	FDA
4A25	Green Bay mud	Great Lakes	FDA
VH	Pickled herring	Vancouver, B.C.	C. F. Schmidt
Beluga	"Muktuk"	Alaska	Continental Can Co., Chicago, Ill.
Alaska	Unknown to authors	Alaska	
8E	Intestinal tract of sturgeon	Russia	
Iwanai, Memanbetsu	"Izushi"	Hokkaido, Japan	Hiroo Iida
211, 715, 1304	Lake Abashiri mud		Hokkaido Institute of Public Health, Sapporo, Japan
4203, 4213	Unknown to authors	Sweden	Geoffrey Hobbs, Torry Research Station, Aberdeen, Scotland
Nontoxigenic			
066BNT	Derived from 066B Tox	See above	FDA
S5, S9	Viscera of Lake Erie smelt	Great Lakes	FDA
28-2, 38-1	Lake Huron mud	Great Lakes	FDA
PM15	Potomac River mud	Washington, D.C.	FDA

[a] *C. botulinum* types A, B, and C, *C. perfringens*, *C. bifermentans*, *C. sporogenes*, and *C. acetobutylicum* were from the Food and Drug Administration culture collection. *C. botulinum* type F was supplied by the Communicable Disease Center. *C. perfringens* and *C. bifermentans* were identified in this laboratory. These strains were used to obtain the results presented in all the subsequent tables.

[b] Food and Drug Administration.

cardiac puncture 10 days after this booster. Antiserum was stored frozen without a preservative.

Agglutination tests. H agglutination tests were standard tube tests by use of 0.5-ml portions of antigen and antiserum in Wassermann tubes. Antigens for these tests were either the Formalin-treated antigens used for immunization or untreated 16-hr TPG cultures. The Formalin-treated antigens were diluted to an optical density (OD) between 0.45 and 0.55 at a wavelength of 600 mμ, as determined with a Bausch & Lomb Spectronic-20 colorimeter. The OD of 16-hr broth cultures ranged from 0.33 to 0.68; the majority of readings fell within the same range as the diluted Formalin-treated antigens. However, it was found that, as long as turbidity could be detected by eye, they made satisfactory antigens. The antigen-antibody mixtures were lightly shaken, incubated in a water bath at 50 C for 2 hr, and were read immediately.

The somatic agglutination test procedure was a variation of a method first described by Noble (15), modified by Lamanna (8) for study of the genus *Bacillus*, and used by Batty and Walker (2, 23) for clostridia. Quantities (0.1-ml) of antiserum and antigen, diluted to the same optical density as in the H agglutination tests, were incubated at 50 C for 2 hr; 0.5 ml of saline was added at the end of the incubation time to facilitate reading, and the solutions were read immediately. This procedure gave higher titers than shaking for 2 min at room temperature as originally described. Titers were based on the volume of antiserum in the end point dilution.

Antigens for the somatic agglutination tests were prepared from 16-hr TPG cultures grown at 28 C. The cultures were heated at 100 C for 1 hr; the cells were separated by centrifugation and were resuspended to about one-tenth the original volume with physiological saline. Phenol was added to the suspension to give a 0.2% final concentration. Somatic antigens of the other clostridia, which tended to auto-

agglutinate, were stabilized by the addition of Tween 80 to give a 0.5% final concentration. Stock antigens prepared in this manner could be kept at 4 C for an indefinite time without altering their ability to agglutinate. The stock antigens were diluted with physiological saline containing 0.2% phenol or 0.2% phenol plus 0.5% Tween 80 to an OD of 0.45 to 0.55 for agglutination tests.

At the end of the incubation period, both the H and the O agglutination tests were read by gently flicking the tubes to resuspend the sedimented cells. The presence or absence of agglutination was determined by visual examination with a bright light against a black background. To facilitate reading end points, a 3.5 × hand lens was used. Reading by use of sedimentation patterns was unsatisfactory, since some control antigens tended to form a pattern but after resuspension were indistinguishable from those settling in a button. Likewise, clumps that readily dispersed on shaking were not counted as agglutination. Titers were recorded as the highest dilution giving clearly discernible agglutination.

Agglutinin absorption. Cells for the absorption of antibody were grown for 16 hr in TPG at 28 C, and were packed by centrifugation. When absorbing somatic antibody, cells were heated for 1 hr at 100 C before packing.

Portions (2-ml) of the antiserum to be absorbed were treated with 1 to 2 ml of packed cells by suspending the cells in undiluted antiserum and incubating at 50 C for 1 hr with occasional shaking. After the cells were separated by centrifugation, the antiserum was tested for agglutination with the absorbing strain. If agglutinins to the absorbing strain remained, the absorption was repeated. To serve as a control, another portion of the same antiserum was incubated along with the one being absorbed.

RESULTS

High levels of antibody were produced in rabbits immunized with *C. botulinum* type E and nontoxigenic organisms resembling it. Titers of H and O antibody were about equal, but, whereas the former had a very narrow range of specificity, the latter cross-reacted completely.

H agglutination with H-O antisera. The results of agglutination tests with H-O antisera tested against either Formalin-killed antigens or overnight broth cultures are shown in Table 2. Results are given for tests on 11 hyperimmune rabbit antisera against 24 strains of type E and organisms resembling type E. The only complete

TABLE 2. *Agglutinin titers of H-O antisera against Formalin-treated antigens or overnight broth cultures*

Antigen[a]	Antiserum										
	070	5192	066B Tox	066BNT	VH	Beluga	1304	211	715	S5	28-2
Group 1											
070	5,120	—[b]	—	—	—	—	—	—	—	—	—
5192	—	5,120	—	—	—	—	—	—	—	—	—
066B Tox	—	—	5,120	5,120	40	—	—	40	—	—	—
066BNT	—	—	5,120	5,120	40	—	—	40	—	—	—
VH	—	—	—	—	2,560	2,560	—	—	—	160	20
Beluga	—	—	—	—	2,560	2,560	80	—	—	160	160
1304	—	—	—	—	40	—	1,280	—	—	—	—
211	—	—	160	160	80	—	40	1,280	—	160	80
715	—	—	—	—	—	—	—	—	20,480	—	—
S5	—	—	—	—	320	—	—	—	—	2,560	—
28-2	—	—	—	—	—	—	—	40	—	—	2,560
Group 2											
5191	—	—	320	320	2,560	—	80	80	—	—	—
4A25	—	—	—	—	20	40	—	—	—	—	80
D8	—	—	—	—	40	2,560	—	40	—	—	40
Minnesota	80	—	—	—	80	—	—	80	—	—	—
4203	—	—	—	—	—	—	—	—	80	—	—
4213	—	—	—	—	—	—	—	—	—	80	40
1553	—	—	—	—	—	—	—	—	—	—	20
SU1	—	—	—	—	—	—	—	—	—	—	—
SU3	—	—	—	—	—	—	—	—	—	—	—
Alaska	—	—	—	—	—	—	—	—	—	—	—
38-1	—	320	—	—	—	—	—	—	—	—	—
S9	—	—	20	—	—	160	—	—	—	—	80
PM15	—	—	—	80	—	—	—	160	—	—	40

[a] Group 1 consisted of Formalin-treated antigens or overnight broth cultures, and group 2 consisted of overnight broth cultures.

[b] Symbols: —, no agglutination at 1:20.

cross-reactions are between the toxigenic and nontoxigenic 066B strains and between VH and Beluga. The VH antiserum also reacted to full titer with 5191, and the Beluga antiserum with D8. However, VH antiserum did not react with D8; nor did Beluga antiserum react with 5191.

The remaining cross-reactions were all of low titer and generally could be removed by absorbing the antiserum with its homologous O antigen, suggesting that these crosses occur by way of the somatic antibody. For example, all of the low-titer cross-reactions with the VH antiserum, except that with S5, were removed by absorbing it with VH somatic antigen. All H-O antisera were also tested against *C. botulinum* types A, B, and F, *C. perfringens*, *C. bifermantans*, *C. sporogenes*, and *C. acetobutylicum*. None of them was agglutinated. Preimmunization sera were tested against the strains with which the rabbits were immunized. None of the normal rabbit sera showed evidence of antibody.

In addition to the cross-reactions shown in Table 2, antisera prepared against strains 070 and 5192, which were isolated in the same smoked whitefish chub outbreak (1), reacted with a number of other strains. The results of agglutination and agglutinin absorption tests with 070 antiserum are given in Table 3. Five strains in addition to the homologous antigen were agglutinated to full titer by this antiserum. In addition, strain Minnesota was agglutinated slightly. Absorption with unheated cells of strains 8E, Kalamazoo, or 42013 removed all antibody, as did the homologous strain. If, however, this antiserum was absorbed with the Minnesota strain or with 070 somatic antigen, the antibody reacting with the Minnesota strain was removed, but the reactions with the other strains remained unaltered. Thus, the agglutination with Minnesota seems to occur by way of the O antigen, whereas H antigens are responsible for high titers against the other strains. Therefore, these strains, with the exception of Minnesota, appear to be antigenically identical with respect to their H antigens. Similar results with strain 5192 are shown in Table 4.

Table 5 presents the results of cross-agglutination tests between the toxigenic and nontoxigenic 066B strains. Strains 066B Tox and 066BNT mutually cross-agglutinate, and all antibody is removed by cross-absorption. These two antisera

TABLE 3. *Agglutinin titers of absorbed 070 H-O antiserum against related strains*

Absorbing strain	Antigens									
	Flagellar agglutination							Somatic agglutination		
	070	8E	Minnesota	Kalamazoo	42013	42015	0422	070	8E	Minnesota
None	5,120	5,120	80	5,120	5,120	5,120	5,120	2,560	1,280	1,280
Heated 070	2,560	2,560	—	2,560	2,560	2,560	2,560	—		
070	—	—	—	—	—	—	—	—	—	—
8E	—	—	—	—	—	—	—			
Kalamazoo	—	—	—	—	—	—	—	—	—	—
42013	—	—	—	—	—	—	—	—	—	—
Minnesota	2,560	2,560	—	2,560	2,560	2,560	2,560	80	80	—

TABLE 4. *Agglutinin titers of absorbed 5192 H-O antiserum against related strains*

Type of agglutination	Absorbing strain	Antigen								
		5192	0421	8074	8077	517	5194	Iwanai	Memanbetsu	38-1
Flagellar	None	5,120	5,120	5,120	5,120	5,120	5,120	2,560	5,120	
	Heated 5192	5,120	5,120	1,280	5,120	1,280	1,280	2,560	2,560	
	5192	—	—	—	—	—	—	—	—	
	0421	—	—	—	—	—	—	—	—	
	Iwanai	—	—	—	—	—	—	—	—	
Somatic	None	5,120	5,120	1,280	2,560	2,560	2,560	1,280	2,560	1,280
	Heated 5192	—	—	—	—	—	—	—	—	—
	5192	—	—	—	—	—	—	—	—	—
	0421	—	—	—	—	—	—	—	—	—
	Iwanai	1,280	640	320	1,280	640	1,280	—	—	

also react with strains 5191 and 211, but only to a fraction of the homologous titer. Absorption of the antiserum produced against 066B Tox with unheated cells of strain 5191 completely removed the component reacting with both 5191 and 211, but left a significant titer for both 066B strains. When the same antiserum was absorbed with 211 cells, the component reacting with 211 was removed but the antibody titers against the other strains were not affected. Thus, it appeared that the 066B strains are identical with respect to their heat-labile antigens, irrespective of whether or not they are toxigenic, and that strains 5191 and 211, while differing, share common antigens to some extent.

Somatic agglutination with H-O antisera. When the H-O antisera were tested against the O antigens, they all cross-reacted completely. Strains S5, 28-2, and 38-1, which are identical with type E in all respects except for their lack of toxigenicity, and strain 066BNT, which was derived from the typical toxigenic type E strain 066B, were indistinguishable from toxigenic type E strains in being agglutinated to full titer by these antisera. Strains S9 and PM15, which closely resemble type E in colonial morphology but are nontoxigenic and differ in their fermentation patterns and degree of hemolysis on blood-agar, were also agglutinated to full titer by these antisera. Since the agglutinin titers against the O antigens were all essentially the same, they are not presented. However, representative titers are shown in tables giving the results of absorption experiments. Somatic agglutination tests with H-O antisera absorbed either with homologous somatic antigens or unheated cells of strains whose H antigens agglutinated to full titer are also shown in Tables 3 through 5.

The somatic antibody titers of absorbed 070 H-O antiserum against strains 070, 8E, and Minnesota are presented in Table 3. The removal of all O antibody to the homologous strain by all absorbing strains indicates that these strains, except Minnesota, have complete antigenic identity. Although Minnesota appears to have the same O structure, it differs in its H antigens, as previously shown.

The results obtained with an absorbed 5192 H-O antiserum (Table 4) show that Iwanai differs somewhat in its somatic antigen from 5192 and is more like strain Memanbetsu, whereas strain 0421 is completely identical with 5192, since the H antigens also cross-agglutinate to full titer and are completely removed by absorption. When the antisera to both 066B strains were cross-absorbed with unheated cells, all somatic antibody was removed (Table 5). Absorption of antiserum to the toxigenic strain with unheated cells of 5191 also removed all somatic antibody. However, absorption with cells of strain 211 removed the components reacting with 211 and 5191 while leaving a residue which agglutinated with 066B strains.

Somatic agglutination with somatic antisera. Agglutination tests with somatic antigens against somatic antisera all showed high antibody titers and complete cross-agglutination, as they did against the H-O antisera. These somatic antisera were also tested against somatic antigens of *C. botulinum* types A, B, C, and F, and *C. perfringens*, *C. bifermentans*, *C. sporogenes*, and *C. acetobutylicum*. None of these antigens was agglutinated except *C. acetobutylicum*, which was agglutinated to high titer by all of the sera. The majority of preimmunization sera contained low levels of antibody to this strain, but none against the strain used for immunization. The rise in anti-

TABLE 5. *Agglutinin titers of absorbed 066B H-O antisera against related strains*

Antiserum	Absorbing strain	Antigens							
		Flagellar agglutination				Somatic agglutination			
		066B Tox	066BNT	5191	211	066B Tox	066BNT	5191	211
066B Tox	None	5,120	5,120	320	160	1,280	640	640	640
	066B Tox	—	—	—	—	—	—	—	—
	066BNT	—	—	—	—	—	—	—	—
	Heated 066BNT	2,560	2,560			20	20		
	5191	640	320	—	—	—	—	—	—
	211	5,120	2,560	320	—	160	160	—	—
066BNT	None	5,120	5,120	320	160	5,120	5,120	320	1,280
	066BNT	—	—	—	—	—	—		
	066B Tox	—	—	—	—	—	—		
	Heated 066B Tox	2,560	2,560			—	—		

body titer to *C. acetobutylicum* concurrent with the production of antibody to type E appears to be a non specific anamnestic response. Absorption with *C. acetobutylicum* removed only antibody to this organism without affecting the type E somatic antibody titer.

Agglutinin titers of type E somatic antisera absorbed with heterologous type E somatic antigens are shown in Table 6. The only significant residual antibody levels are found in the 5192 antiserum for the homologous strain. The low level of homologous antibody remaining in the 070 antiserum is probably of no significance, and cross-absorption tests with the Beluga and VH strains show the removal of all somatic antibody to both.

The results of somatic cross-absorption tests with type E strains and S5 are given in Tables 7 and 8. In general, where there is a significant residual antibody titer it is for the homologous or closely related strains. The exceptions to this are with the 070 antiserum, in which there is also residual antibody to Beluga and 1304, and the 28-2 antiserum, in which there is residual antibody for Beluga and VH. The S5 antiserum absorbed with strain 715 (Table 8) had a significant residual antibody titer for VH, Beluga, and 1304.

These results suggest that the O antigen complex is sufficiently alike among all strains of type E and those which closely resemble it to produce complete cross-agglutination. On the other hand, there may be minor differences in the antigenic components of some strains which give residual titers when absorbed with heterologous strains.

TABLE 6. *Agglutinin titers of type E somatic antisera absorbed with type E somatic antigens*

Antiserum	Absorbing strain	Antigen							
		5192	070	Iwanai	Memanbetsu	1304	Beluga	VH	S5
5192	None	5,120	5,120	2,560	2,560	2,560	10,240	10,240	2,560
	070	320	—	—	—	—	—	—	—
	Iwanai	320	—	—	—	—	—	—	—
	Memanbetsu	320	—	—	—	—	—	—	—
070	None	5,120	5,120	2,560	5,120	5,120	5,120	5,120	5,120
	5192	—	40	—	—	—	—	—	—
	1304	—	40	—	—	—	—	—	—
	Beluga	—	40	—	—	—	—	—	—
VH	None	5,120	5,120	5,120	5,120	2,560	5,120	5,120	2,560
	Beluga	—	—	—	—	—	—	—	—
Beluga	None	5,120	5,120	5,120	5,120	5,120	5,120	5,120	5,120
	VH	—	—	—	—	—	—	—	—

TABLE 7. *Agglutinin titers of antisera absorbed with S5 somatic antigen*

Antiserum	Absorbing strain	Antigen							
		S5	Beluga	VH	1304	070	28-2	5192	715
S5	None	2,560	2,560	2,560	1,280	1,280	1,280	5,120	2,560
	S5	—	—	—	—	—	—	—	—
Beluga	None	5,120	5,120	5,120	5,120	10,240	5,120	5,120	5,120
	S5	—	640	640	—	—	—	—	—
VH	None	2,560	5,120	5,120	2,560	10,240	1,280	10,240	10,240
	S5	—	—	—	40	20	—	20	—
1304	None	5,120	5,120	5,120	5,120	5,120	2,560	10,240	5,120
	S5	—	20	—	640	—	—	—	20
070	None	5,120	10,240	10,240	10,240	10,240	5,120	10,240	10,240
	S5	—	160	—	160	160	—	—	—
28-2	None	2,560	10,240	5,120	5,120	5,120	5,120	10,240	10,240
	S5	—	160	160	20	80	320	—	—
5192	None	2,560	10,240	10,240	2,560	5,120	2,560	10,240	5,120
	S5	—	—	20	—	—	—	320	—
715	None	10,240	10,240	10,240	10,240	5,120	2,560	5,120	10,240
	S5	—	—	—	20	—	—	—	40

TABLE 8. *Agglutinin titers of somatic S5 antiserum absorbed with somatic antigens*

Absorbing strain	Antigen							
	S5	Beluga	VH	1304	070	28-2	38-1	715
None	2,560	2,560	2,560	1,280	1,280	1,280	1,280	2,560
S5	—	—	—	—	—	—	—	—
Beluga	—	—	—	—	—	—	—	—
VH	80	—	—	—	—	—	—	—
1304	80	—	—	—	—	—	—	—
070	40	—	—	—	—	—	—	—
28-2	80	—	—	—	—	—	—	—
38-1	80	—	—	—	—	—	—	—
715	320	320	160	160	40	—	—	—

DISCUSSION

The highly strain-specific H antigens of *C. botulinum* type E reveal both the diversity of antigenic groups found in the same outbreak and the number of similar strains isolated in widely separated geographical areas.

It is of interest that strain 070 from the 1963 Great Lakes smoked whitefish chub outbreak appears to be identical to strain 8E, the original Russian isolate. Similarly, strain 5192, from the same outbreak as 070 although differing somewhat in its somatic complex from the Japanese strains Iwanai and Memanbetsu, shares common H antigens with them.

Likewise, strain Beluga isolated from "muktuk," an Eskimo delicacy prepared from the skin and blubber of Beluga Whale flukes, shares common H antigens with strain VH isolated from Vancouver herring. In addition, strain VH shares H antigens with 5191 but not D8, whereas strain Beluga shares H antigens with D8 but not 5191. Strain 5191 came from smoked whitefish chubs originating in the Great Lakes; strain D8, from canned tuna fish of Pacific origin. Thus, a worldwide distribution of antigenic groups and components seems likely.

The fact that our toxigenic and nontoxigenic strains of 066B, derived from the same colony, are antigenically alike except for toxigenicity lends support to the use of cellular antigens as a supplementary aid in taxonomy, and shows clearly that nontoxigenic strains, otherwise the same as type E, may exist.

Other nontoxigenic strains studied have distinct H antigens from each other and from known strains of type E. However, since they have sufficiently similar O antigens to type E to completely cross-agglutinate with it, the lack of common H antigens does not, in itself, set them apart. It does suggest that, except for the lack of toxigenicity and in some cases the ability to produce a bacteriocin, they differ no more from type E than type E strains differ among themselves.

The multiplicity of H antigens among type E strains precludes the possibility of using H antigens as a means of identifying type E, but it may be useful in identifying or grouping strains. The utility of this procedure ultimately depends on how many different antigenic groups will be found.

Though it appears from this study that the somatic antigen of *C. botulinum* type E can be used to distinguish it from all other clostridia, a greater number of strains of type E and other clostridia will have to be studied before sufficient reliance can be placed on such a test.

Much remains to be done before the antigenic relationships among strains of *C. botulinum* type E can be fully understood. Further work is in progress in an attempt to group those strains which were not agglutinated by any of the sera used in this study, and to find other strains which may be agglutinated by sera which reacted only with the homologous antigen. Investigation of the antigenic relationships of additional nontoxigenic strains to type E is also under way. Since it has been suggested that spore antigens should be taken into account in the classification of the clostridia, this also is being done.

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