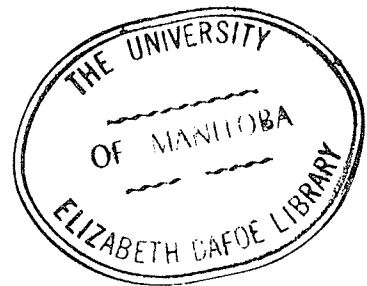


DETECTION OF CLOSTRIDIUM BOTULINUM
TYPE E BY DIRECT FLUORESCENT ANTIBODY TECHNIQUE

by
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To my Parents

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ABSTRACT

The detection of ten strains of Clostridium botulinum type E by direct staining with fluorescein isothiocyanate labelled type E antisera was investigated. Conjugates, prepared with antisera against three type E toxigenic strains 070, D8 and Beluga, stained their homologous strains with intense fluorescence. The conjugates were absorbed with Cl. bifermentans and then diluted to eliminate staining of Cl. botulinum types A, B, C and F and eight heterologous Clostridium spp. A significant degree of fluorescence was observed with 7/8 toxigenic type E strains and the 070 conjugate, 8/8 strains and the D8 conjugate and 3/9 strains and the Beluga conjugate. A very low degree of fluorescence was observed with two nontoxigenic type E strains.

Photomicrographs show that, in most cases, it is the spore body and not the vegetative cells of the cross-reacting strains which were stained by the 070 and D8 conjugates, suggesting that the antigen shared by the toxigenic strains is a component of the spores.

Fluorescent antibody technique applied to homogenized

suspensions of whitefish containing 10^8 cells per ml
allowed the detection of the type E cells with a minimal
degree of nonspecific staining of the fish tissue.

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INTRODUCTION

INTRODUCTION

In the last decade it has been shown that the transmission of botulism due to type E toxin formation is primarily caused by the distribution of type E strains of Clostridium botulinum in commercially processed fish and fish products.

The control of botulism both from the public health and food and drug aspects is dependent on reliable methods for the rapid detection of Cl. botulinum and the screening of large numbers of food samples.

The fluorescent antibody test appears to offer the most promise but is beset with the problems of nonspecific staining, cross-reactivity of related sero types and the preparation of conjugates which give type specific reactivity with all strains.

Immunofluorescent techniques were investigated in this study for the purpose of developing a fluorescent antibody test with specific reactivity for type E strains of Cl. botulinum.

HISTORICAL

HISTORICAL

Recent reports of Clostridium botulinum type E outbreaks throughout the world have renewed interest in the study of this organism.

Type E strains were first isolated by Russian workers in 1937 from sturgeon implicated in an outbreak of botulism. Since then strains have been isolated from smoked and home-canned salmon (24, 33), pickled and smoked herring (22, 23), salmon eggs (25) and home-made izushi (42, 59), a Japanese raw fish food.

Botulism caused by type E strains has occurred primarily in Japan, Russia, United States, Canada, Sweden and Denmark. A total of 75 outbreaks have been reported affecting 374 people with 112 deaths. Of these, 44 occurred in Japan, 13 in United States, 11 in Canada, three in Sweden and two each in Denmark and the Soviet Union. The highest fatality rate of 63% has occurred in Canada compared to 39% in the United States and 26% in Japan.

In Canada type E strains have been isolated from three categories of foodstuffs. These included the various forms of uncooked fish consumed by British Columbians from

continental Europe, putrescent salmon eggs by Indians and raw seal products by Labrador Eskimoes. Four outbreaks from raw fish occurred in the more populated southern parts of British Columbia, affecting seven persons with five deaths. Three of the four occurred at Nanaimo in 1944, Vancouver in 1949 and Natal in 1952, with home-canned salmon (24), pickled herring (22) and pickled trout (21) as the respective vehicles. The fourth outbreak occurred well inland at Penticton in 1960.

The first reported case from putrescent salmon eggs occurred near Whitehorse, in the Yukon in 1940. Eight type E strains have since been isolated from salmon eggs (25).

Consumption of seal meat resulted in two deaths at Paulatuk, N.W.T. in 1961, and seven deaths at Markham Bay, N.W.T. in 1965. A type E toxigenic strain was isolated from a left-over seal flipper at Labrador in 1956.

Due to the prevalence of botulism caused by C1. botulinum type E workers began to study the ecology of these microorganisms. Before the prototype E strains were isolated, Russian workers had shown that contamination of fish by type A or B spores was attributed to either environmental or fish intestinal sources (21). Once it became apparent that type

E strains had a predilection for fish the question arose whether type E spores were primarily telluric or marine in origin (22).

An important pioneer in these studies was Dolman (23, 24) who suggested that type E spores were terrestrial in origin, found thinly scattered, except in heavily seeded areas of the northern hemisphere. He proposed that spores were brought by rain and river erosion to the offshore seabottom and from these sources bottom feeding fish and their predators added the spores to their intestinal flora.

Such studies have now been supplemented from nearly all parts of the world. In Russia and Sweden workers found type E spores in samples of water and soil (45, 38). In Japan 5.9% of coastal and inland soil samples yielded toxigenic type E cultures (41). Type E strains have also been isolated from mud samples taken from the U.S. Pacific Coast (26), marine deposits off the coast of Scandinavia (7) and the Gulf of Maine (6). Ward and Carroll (75) demonstrated production of type E toxin from mud samples inoculated into cooked meat medium.

Several workers have isolated toxigenic type E strains from fish, including Cayuga Lake fish (9), whitefish chubs

(63), salmon (15), and steelhead and bottom fish (16). Hobbs et al. (35) isolated nontoxigenic strains identical in morphology and biochemical reactions to toxigenic strains from fish and marine deposits. Bott et al. (3, 4) reported that type E strains were present in the intestinal contents of fish from all four of the Great Lakes. The fish of the Green Bay of Lake Michigan proved to be a rich source of type E, occurring in 57% of the 728 samples tested (5). Eklund and Polsky (26) isolated a type E strain from crab.

The 1960 and 1963 outbreaks of type E botulism from consumption of smoked whitefish (1, 43, 62) reemphasized the need of adequate controls and more reliable methods for the detection of type E strains.

The fluorescent antibody technique introduced by Coons (13) would appear to offer a highly sensitive test for screening of environmental materials and implicated samples for the presence of type E organisms. The method of labelling antibodies with fluorescein isocyanate was originally developed for detecting pneumococcal antigen in tissue (14). Riggs et al. (69) replaced the isocyanate of fluorescein by the more stable isothiocyanate. Fluorescein

isothiocyanate is the most widely used fluorochrome as it emits a brilliant yellow green fluorescence easily differentiated from the white autofluorescence of cells and tissues. Rhodamine B dyes are less commonly encountered and have been used as a second label for staining mixed cultures (37).

Improvements have been introduced to prepare a more specific conjugate. Preliminary purification of gamma-globulin by precipitation with ammonium sulphate is now standard procedure (57). However this method precipitates impurities such as alpha and beta globulins and albumin, which combine with fluorescent dye more readily than gamma-globulins and may cause nonspecific staining (6, 46, 48).

Various attempts have been made to eliminate nonspecific staining. Ion exchange methods have been used to purify gamma-globulins from other serum components (19, 47, 65). As suggested by Nairn (57) gradual labelling of the antiserum provides a more suitable conjugate than rapid addition of fluorescent dye to the globulins (52). Clark and Shepard (11) labelled gamma-globulins by dialysis and found that this method reduced but did not completely remove the nonspecific staining by conjugates. This was confirmed

by Dedmond et al. (19) and Hebert et al. (34).

Nonspecific staining has been considerably reduced by removal of unreacted fluorescent dye by column chromatography. Goldstein et al. (30) used Sephadex G-50 while other workers (19, 31, 66) used Sephadex G-25. Riggs et al. (68) used DEAE-cellulose to remove unreacted dye and at the same time to separate from the conjugate a component which gave specific staining. This was subsequently employed by several workers (17, 30, 53).

The presence of negatively-charged ions in the conjugate may also cause nonspecific staining. Absorption of these ions and unreacted dye with liver powder (8, 39), charcoal (27) and ethyl acetate (20) have been advocated. Dedmond et al. (19) removed negative ions by passing labelled globulins through DEAE-Sephadex A-50.

One of the important factors related to nonspecific staining is the fluorescein to protein (F/P) ratio of conjugates. Hebert et al. (34) showed that nonspecific staining increased linearly to the (F/P) ratio. These workers also showed that purifying the dye reduced the nonspecific staining. This was confirmed by Pittman (67). Methods have been published on the measurement of the F/P

ratio (44, 48, 57, 79).

The direct staining method, in which fluorescein-labelled antiserum is applied directly to smears containing the antigen, is the simplest and most widely used. The indirect method, in which the antigen is first treated with unconjugated specific antiserum and then made visible with a fluorescent-labelled antiserum specific for the unlabelled immunoglobulins is considered to be more sensitive (78).

Although there have been widespread studies of immunofluorescence for the detection of bacteria as a diagnostic aid (2, 57), the direct fluorescent antibody test has been restricted to the identification of group A streptococci in clinical specimens (12, 32, 77) and of Shigella sonnei (71) and Escherichia coli (42, 60) in faeces. The indirect method has been used most frequently for the detection of treponemal antibody (81).

One of the more important factors in obtaining a specific reactant for immunofluorescence has been the use of absorption to remove nonspecific staining and cross-reacting antibody of related bacteria. Moody et al. (56) showed that a conjugate of group A streptococcus absorbed with a group C strain was specific for group A strains. Deacon et al. (18)

found that conjugates prepared from antisera to gonococci could be freed from cross-reactions with Neisseria meningitidis. Thomason et al. (72) purified Vi antigen by absorption with O antigen in immunofluorescent studies on Salmonella spp.

Immunofluorescent studies on Cl. botulinum by Walker and Batty in 1964 differentiated among three groups; proteolytic types A, B and F, non-proteolytic types C and D and non-proteolytic type E (73).

The problem of preparing a conjugate which would react specifically with all type E strains has not been resolved. Georgala and Boothroyd (29) prepared conjugates which stained the vegetative cells of 22/41 type E strains. Two conjugates of Baltic type E isolates stained 20/27 strains from the Baltic area, but only 2/14 strains from North America.

Few studies have been carried out with conjugates prepared from type E spores. Walker and Batty (74) found that a type E spore conjugate stained 19/19 type E strains. There were no cross-reactions with spores of Cl. botulinum types A or B of other Clostridium spp. Georgala and Boothroyd (29) used a type E conjugate, made specific for

spores by absorption with a young culture of vegetative cells, and stained 41/51 toxigenic type E strains from various parts of the world.

The fluorescent antibody test has only recently been used to detect type E organisms in environmental materials or in experimentally inoculated foods. Ward et al. (76) compared fluorescence with mouse toxigenicity for the detection of Cl. botulinum type E in estuarine waters of the U.S. Gulf Coast. Of 37 samples which were positive by mouse protection tests only six showed significant fluorescence. Midura et al. (54, 55) used the fluorescent antibody test on spore inoculated food products and detected fluorescence of vegetative cells for a period of fourteen days.

The evidence which has accumulated to date indicates that the fluorescent antibody test shows considerable promise as a reliable method for the detection of Cl. botulinum but the production of specific conjugates which will detect all type E strains and the standardization of the fluorescent antibody test must be attained before results from various laboratories can be compared.

MATERIALS AND METHODS

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Cultures

Clostridium botulinum type E cultures used in this study were toxigenic strains 070, D8, VH, 5191, 5192, 715 and nontoxigenic 066BNT and PM-15 received from Food and Drug Administration, Washington, D.C.; the toxigenic strain ATCC 9564 was obtained from the American Type Culture Collection and the Beluga strain from the Laboratory of Hygiene, Ottawa.

Cultures of Cl. botulinum types A, B, C and F, Clostridium sporogenes G221, Clostridium bifermentans G301, Clostridium tertium G186, Clostridium chauvoei G306, Clostridium difficile G311, Clostridium histolyticum G141, and Clostridium septicum G326 were supplied by the Laboratory of Hygiene, Ottawa.

Clostridium sporogenes ATCC 19404 and Clostridium novyi 27 were obtained from the culture collection of the Department of Microbiology, University of Manitoba.

The toxigenicity of the type E strains was verified by mouse protection tests using three groups of four mice.

The test group of mice received untreated culture filtrates and died within four hours. The second group which received untreated culture filtrates combined with type E antitoxic serum survived. The third group inoculated with culture filtrates heated to 100° C for 10 minutes also survived.

Antisera

Vaccines were prepared from type E strains grown on blood agar at 28° for 24 hours under anaerobic conditions. The surface growth was harvested in saline and washed four times. The cell mass was diluted in 0.4% formalized saline to a turbidity equivalent to a McFarland tube No. 2, kept at 37° C for 3 to 4 days and tested for toxicity and sterility.

Albino adult rabbits were immunized with type E vaccines 070, D8 and Beluga by an intensive intravenous schedule of three injections per week for five to six weeks. Rabbits which showed serum agglutin titers 640 were bled by cardiac puncture. The serum was collected, preserved with merthiolate and stored at -20° C.

Agglutination Tests

H antigens were prepared by the method used for

vaccines except that the cells were grown in trypticase-peptone-glucose broth (TPG), (70) and diluted with formalized saline to an optical density (O.D.) of 0.45 to 0.50 on a Beckman Model C colorimeter. Serial dilutions of type E antisera with equal volumes of antigen were incubated at 50° C for 2 hours and macroscopic agglutination recorded.

Somatic O antigens were prepared with cells grown in TPG, suspended in saline and heated at 100° C for one hour. The organisms were diluted in 0.2% phenol saline to an O.D. of 0.45 to 0.50. Agglutination tests were carried out according to the method of Lynt (51). Serial dilutions of antiserum and an equal volume of O antigen in a total volume of 0.2 ml were incubated at 50° C for 2 hours. Prior to reading 0.8 ml of saline was added to each tube.

Isolation of Gamma-Globulin

Gamma-globulins were separated from other serum components by three methods. The procedures were carried out at 4° C with type E antisera and with a pool of normal rabbit sera.

In the first method the serum was treated with an equal volume of 40% ammonium sulphate. The precipitated

globulins were dissolved in a minimal amount of saline and dialyzed against saline until the sulphate ions were removed. The protein content was measured by the Lowry method (50) and adjusted to 1% (10 mg/ml).

In the second method serum was applied to a 2.5 x 45 cm DEAE-cellulose column and eluted by means of a continuous gradient obtained with 0.02 M phosphate buffer, pH 7.8, joined by a siphon to an equal volume of 0.02 M phosphate buffer containing 0.2 M NaCl (pH 6.5). The fractions were collected and read for protein content at 280 m μ on a Beckman DU spectrophotometer. The fractions representing the first major peak were collected, concentrated by dialysis against 15% polyvinylpyrrolidone (PVP) in phosphate buffered saline (PBS), pH 7.4, and examined by cellulose acetate electrophoresis to confirm the presence of gamma-globulin. The protein content was adjusted to 1% by the addition of saline.

The third method was carried out using the batch technique described by Perper *et al.* (65). Sixty ml of serum was mixed with 24 g of DEAE-Sephadex A-50, stirred continuously for one hour and filtered through a Buchner funnel. The process was repeated four times. The separation

and homogeneity of the gamma-globulin was confirmed by cellulose acetate electrophoresis.

Conjugation of Gamma-Globulin

In the first method the gamma-globulins were precipitated with ammonium sulfate and conjugated to fluorescein isothiocyanate (FITC) by the method of Marshall et al. (52) using a dye-globulin ratio of 1:10. After labelling proceeded for 20 hours, the conjugate was clarified by centrifugation and passed through a Sephadex G-25 column with PBS (0.01 M, pH 7.4) to remove unconjugated fluorescein. The conjugate was concentrated by dialysis against PVP and stored at -20° C.

In the second method the gamma-globulin was separated by column chromatography and conjugated to FITC in a dialysis bag by the method of Clark and Shepard (11). Labelling proceeded for 20 hours and unconjugated fluorescein was removed by passing the conjugate through a Sephadex G-25 column. The conjugated globulins were concentrated and stored at -20° C.

Prior to use the conjugates were dialyzed against PBS for 48 hours.

Absorption of Conjugates

The conjugates were absorbed with an equal volume of a dense suspension of Cl. bifermentans for 2 hours at room temperature, clarified by centrifugation and stored at -20° C.

The Direct Fluorescent Antibody Test

Smears were prepared with cells of Clostridium spp. grown in TPG for 21 to 24 hours and heat fixed. Two drops of fluorescein conjugate were added to each smear. After staining for 30 minutes the smears were washed with 0.01 M phosphate buffer pH 7.2 for 10 minutes and rinsed in carbonate buffer pH 9.0.

Controls included cells of Clostridium spp. stained with fluorescein conjugates of normal globulins and cells pretreated with homologous unconjugated antiserum for 30 minutes prior to staining with fluorescein conjugates of antisera.

Whitefish sections were macerated in a Waring blender, homogenized in a stainless steel tissue grinder and suspended in phosphate buffer pH 7.2. Fish homogenates were prepared to contain 10^8 Clostridium spp. per ml. Smears were fixed

in absolute methanol and stained with the fluorescein conjugates as previously described. A counterstain of Evans blue or Rhodamine B dye in carbonate buffer pH 9.0 was applied for 1 minute.

Fluorescein stained smears were mounted in a glycerol-saline mixture (9:1), pH 8.2. A Zeiss photomicroscope with a 200 watt mercury arc pressure lamp as the ultraviolet source, an achromatic-aplantic condenser and a planapochromatic objective N.A. 1.3 was used to read fluorescence. The ocular barrier system consisted of an ultraviolet excluding filter No. 47. Exciter filters BG 38/2.5, BG 12/4, BG 3/4 and UG 5/3 were used in combination to obtain the maximal fluorescence.

Intensity of fluorescence was recorded as 4+, very intense; 3+, intense; 2+, moderate; +, doubtful; and -, negative. Intensities of 2-4+ were considered to be positive.

Photomicrographs were taken on Tri-X film (ASA 400) using an overhead Zeiss Icarex 35 mm camera with exposure times ranging from 4 to 5 minutes. Negatives were developed in Autofine developer for 8 minutes.

RESULTS

RESULTS

Agglutination tests with type E 070, D8 and Beluga antisera are shown in Table 1. Each antiserum showed an anti-O titer of 800 and an anti-H titer of 640 with the homologous strains. Anti-O cross-agglutination occurred with 8/9 type E botulinum strains and the 070 antiserum, 7/9 strains and the D8 antiserum and 3/9 strains and the Beluga antiserum. Anti-H cross-agglutination occurred between the 070 and D8 strains.

The globulins from the 070 and D8 antisera prepared by ammonium sulphate precipitation and conjugated by the method of Marshall (52) gave more intense staining than that observed with the globulins isolated by column chromatography and conjugated through a dialysis bag.

Undiluted and unabsorbed 070, D8 and Beluga conjugates, which were prepared from ammonium sulphate precipitated globulins, stained their homologous strains with an intense (3-4+) degree of fluorescence. The same conjugates stained Cl. bifermentans, Cl. sporogenes and Cl. botulinum types A and B with moderate fluorescence. Serial dilutions of the

conjugates showed that a dilution of 1:20 eliminated fluorescence of the heterologous Clostridium spp. but also failed to stain the type E D8 strain.

The effect of absorption of the conjugates with packed cells of Cl. bifermentans is shown in Table 2. Fluorescence of the D8 and other type E strains was not significantly reduced. Fluorescence of Cl. bifermentans and Cl. sporogenes was eliminated by a 1:4 dilution of the 070 conjugate and a 1:2 dilution of the D8 and Beluga conjugates.

The intensity of fluorescence of ten type E strains stained with conjugates which were absorbed and diluted is shown in Table 3. A significant degree of fluorescence was observed with 7/8 toxigenic type E strains and the 070 conjugate, 8/8 strains and the D8 conjugate and 3/8 strains and the Beluga conjugate. A very low degree of fluorescence was observed with the two nontoxigenic type E strains. Cl. botulinum types A, B, C and F and six unrelated Clostridium spp. did not fluoresce with the conjugates (Table 3).

Photomicrographs of direct stains of 24 hour cultures observed by fluorescence microscopy are shown in Figs. 1-21. Homologous strains showed an intense degree of fluorescence

(Figs. 1, 2, 3). The cross-reactions of the type strains are shown in Figs. 4-21. In a number of cases the 070 and D8 conjugates gave fluorescence of the spore bodies (Figs. 7-10, 13-17) and not the vegetative cells. Very limited fluorescence was observed with the nontoxigenic strains (Figs. 18 and 19).

Control smears of cells stained with absorbed fluorescein-labelled normal globulins did not fluoresce. Smears pretreated with homologous unconjugated antisera prior to staining with absorbed fluorescein-conjugated antisera showed partial inhibition of staining.

Homologous type E cells suspended in whitefish homogenates showed fluorescence from 2-4+. A minimal degree of nonspecific staining of the fish tissue was observed.

TABLE 1
Agglutination Titers

Antigen	<u>Cl. botulinum</u> Type E Antisera					
	070		D8		Beluga	
	O	H	O	H	O	H
<u>Cl. botulinum</u>						
Type E strains						
070	6400*	10,240	800	1280	-	-
D8	1200	160	6400	1280	400	-
Beluga	200	-	400	-	1600	640
VH	200	-	6400	-	-	-
5191	1600	-	400	-	-	-
5192	1600	-	1600	-	-	-
715	1600	-	1600	-	-	-
9564	200	-	1600	-	-	-
PM-15	200	-	-	-	600	-
066BNT	-	-	-	-	-	-
<u>Cl. sporogenes</u>	-	-	-	-	-	-
<u>Cl. bifermentans</u>	-	-	-	-	-	-

* Reciprocal of highest dilution showing visible clumping.

TABLE 2

The effect of dilution on nonspecific immunofluorescence of Clostridium spp.

<u>Clostridium</u> spp.	Fluorescein-conjugated antisera*											
	070				D8				Beluga			
	1:1	1:2	1:4	4+	1:1	1:2	1:4	2+	1:1	1:2	1:4	2+
type E 070	4+	4+	4+	4+	3+	2+	2+	2+	+	+	-	-
type E D8	3+	2+	2+	2+	4+	3+	2+	2+	+	+	+	+
type E Beluga	+	-	-	-	2+	2+	+	+	3+	3+	2+	2+
<u>Cl. sporogenes</u> (2 strains)	2+	+	-	-	-	-	-	-	2+	-	-	-
<u>Cl. bifermentans</u>	2+	2+	+	+	-	-	-	-	2+	-	-	-

* Absorbed with Cl. bifermentans.
4+ very intense, 3+ intense, 2+ moderate, + doubtful

TABLE 3

Direct staining of Clostridium spp. with three type E conjugates

<u>Cl. botulinum</u> type E strains	<u>Fluorescein-conjugated</u> <u>antisera</u>			<u>Clostridium</u> spp.	<u>Fluorescein-conjugated</u> <u>antisera</u>		
	070	D8	Beluga		070	D8	Beluga
070	4+	2+	+	<u>bifermentans</u>	+	+	+
D8	2+	3+	+	<u>sporogenes</u> (2 strains)	-	-	-
Beluga	+	2+	3+	<u>botulinum</u> type A	-	-	-
VH	2+	2+	3+				
5191	2+	2+	+	<u>botulinum</u> type B	+	-	+
5192	2+	2+	-	<u>botulinum</u> type C	-	-	-
715	2+	3+	-	<u>botulinum</u> type F	-	-	-
9654	2+	2+	2+	<u>tercium</u>	-	-	-
				<u>septicum</u>	-	-	-
PM-15	+	+	+	<u>novyi</u>	-	-	-
066BNT	-	+	+	<u>chauvoei</u>	-	-	-

Figs. 1-3. Fluorescent photomicrographs of homologous type E strains. (Magnification approximately 6250X).

Fig. 1. 070 strain stained with 070 conjugate.

Fig. 2. D8 strain stained with D8 conjugate.

Fig. 3. Beluga strain stained with Beluga conjugate.



FIGURE 1



FIGURE 2

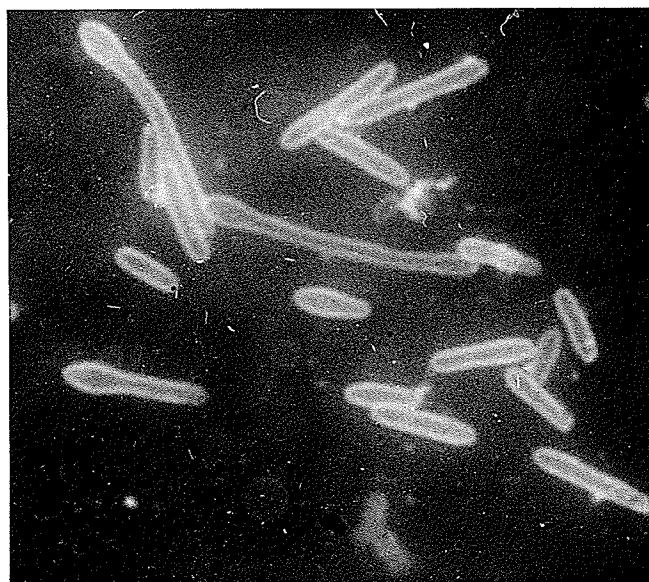


FIGURE 3

Figs. 4-10. Fluorescent photomicrographs of type E
strains stained with 070 conjugate.
(Magnification approximately 6250X).

Fig. 4. D8

Fig. 5. Beluga

Fig. 6. VH

Fig. 7. 5191

Fig. 8. 5192

Fig. 9. 715

Fig. 10. 9564



FIGURE 4



FIGURE 5

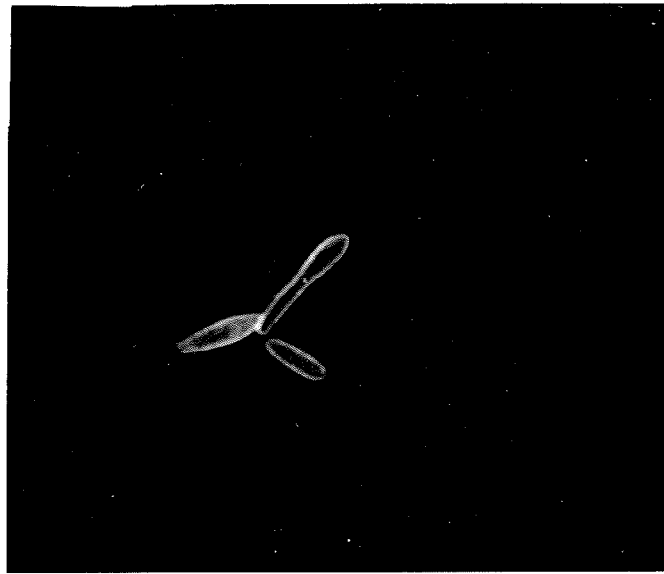


FIGURE 6

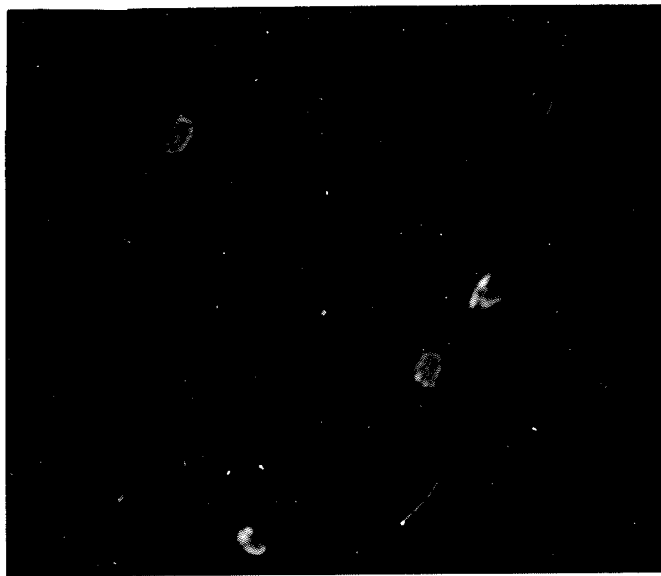


FIGURE 7



FIGURE 8

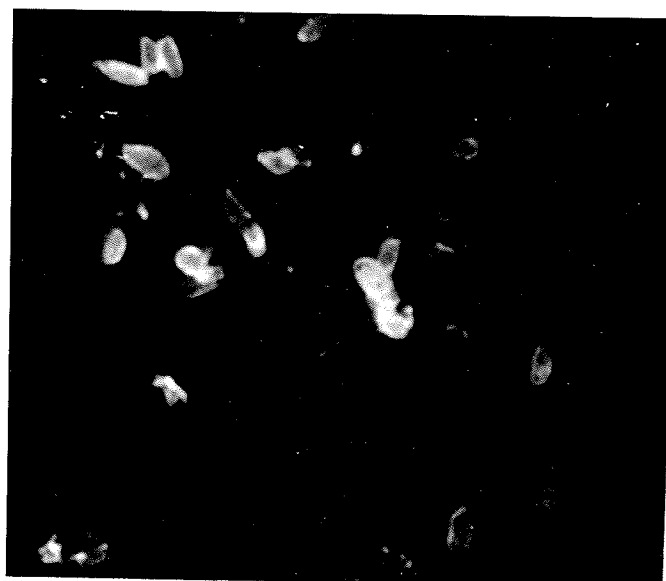


FIGURE 9

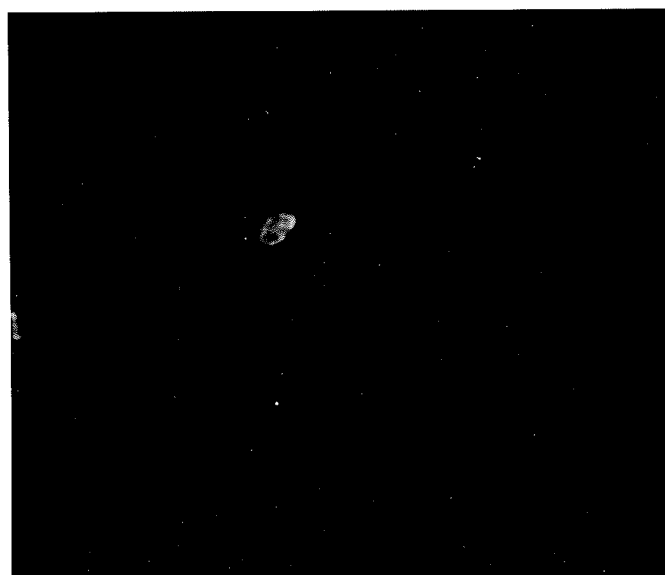


FIGURE 10

Figs. 11-19. Fluorescent photomicrographs of type E strains stained with D8 conjugate.

(Magnification approximately 6250X).

Fig. 11. 070

Fig. 12. Beluga

Fig. 13. VH

Fig. 14. 5191

Fig. 15. 5192

Fig. 16. 715

Fig. 17. 9564

Fig. 18. PM-15

Fig. 19. 066BNT

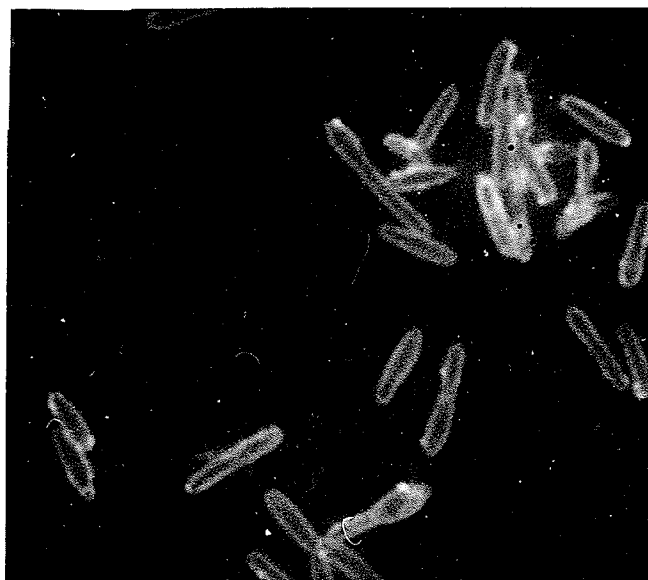


FIGURE 11

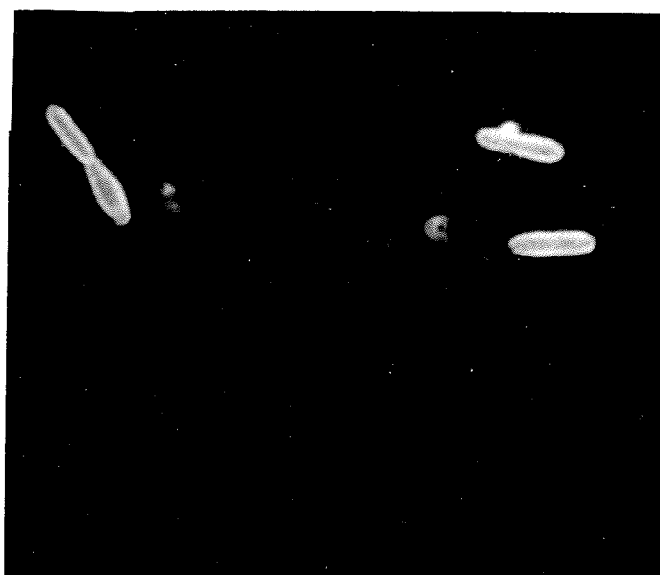


FIGURE 12

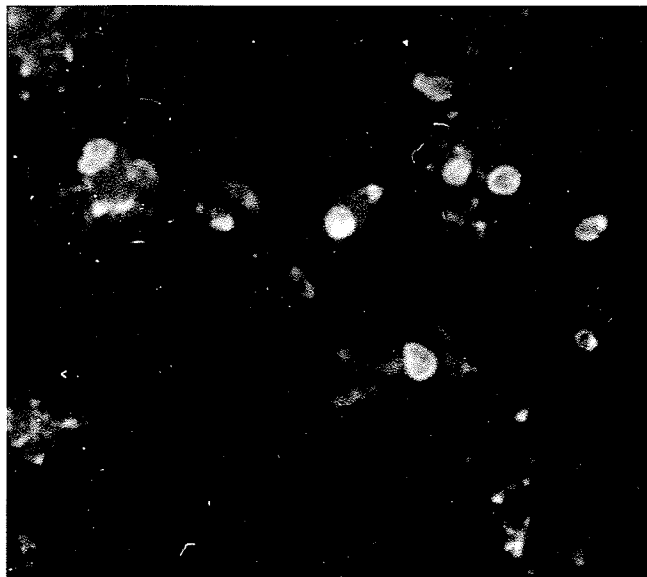


FIGURE 13

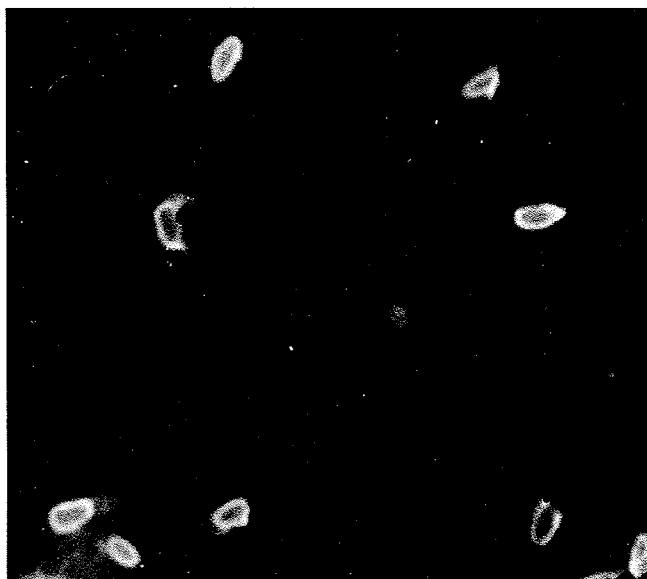


FIGURE 14

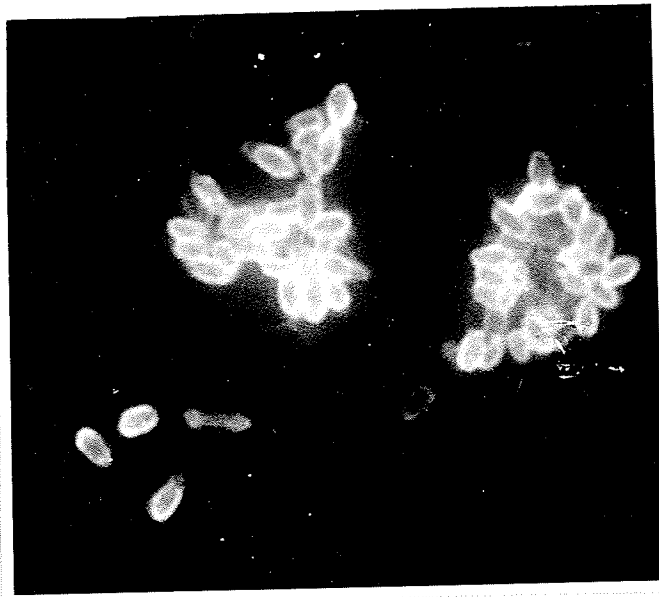


FIGURE 15



FIGURE 16

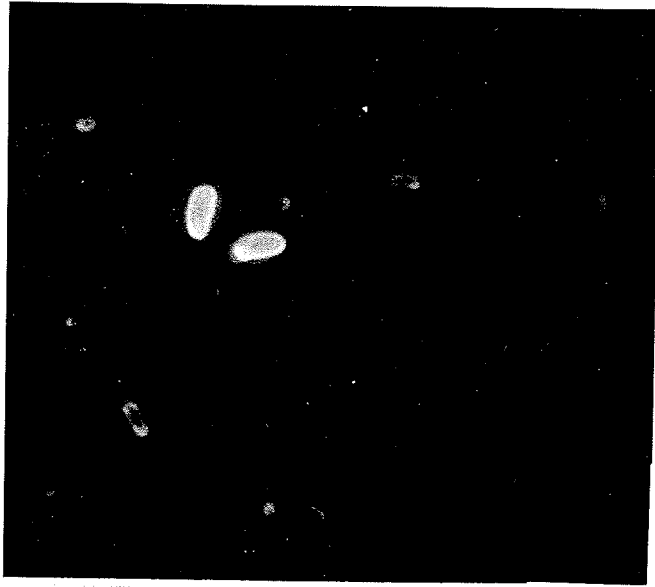


FIGURE 17



FIGURE 18

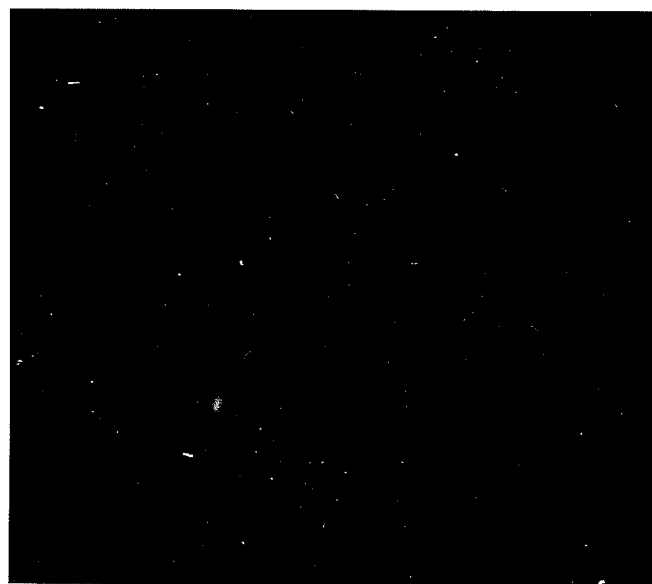


FIGURE 19

Figs. 20-21. Fluorescent photomicrographs of type E
Strains stained with Beluga conjugate.
(Magnification approximately 6250X).

Fig. 20. VH

Fig. 21. 9564

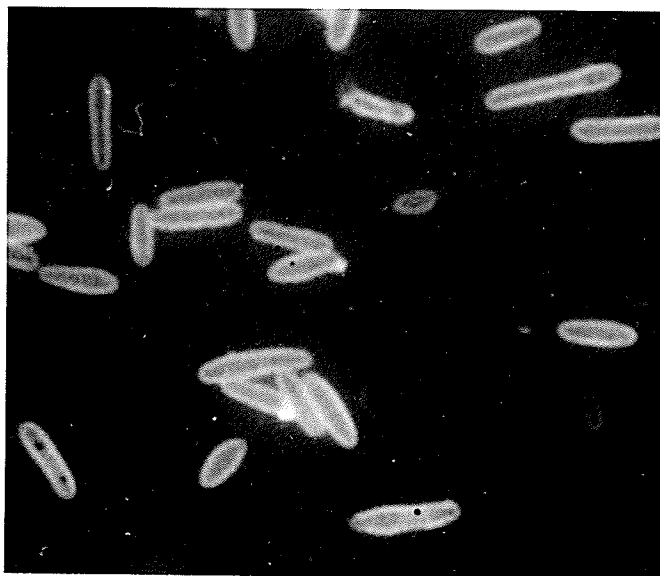


FIGURE 20



FIGURE 21

DISCUSSION

DISCUSSION

The nonspecific staining of bacteria and background materials observed in immunofluorescence has been a constant problem. The main source of reactivity is labelled contaminating serum proteins or labelled antibody globulins of heterogeneous net charge. Immunoglobulins isolated by ammonium sulphate precipitation gives a product of molecular heterogeneity so that ideally conjugates should be prepared from globulins fractionated by DEAE-cellulose column chromatography (58).

In this study the conjugates of globulins isolated by ammonium sulphate precipitation gave a more intense fluorescence than those isolated by column chromatography. Trypsin treatment of cells to enhance fluorescence (10) of globulins isolated by column chromatography failed to do so and therefore conjugates from ammonium sulphate precipitated globulins were used throughout the studies. Preparation of globulins in this manner also proved to be simpler, less expensive and time consuming.

Several workers (17, 30, 68) have recommended that heavily labelled globulins be removed after conjugation by

fractionation on DEAE-cellulose to obtain a more specific reactant. This procedure is useful where complete removal of nonspecific staining is required but reports have indicated that this technique yields an insufficient amount of specific labelled antibody for routine use in laboratories.

Although conjugates prepared from ammonium sulphate precipitated globulins gave intense staining, some nonspecific staining of unrelated bacteria and background material occurred. The problem of nonspecific staining with conjugates of Cl. botulinum types A and B has been resolved by absorbing the antisera or conjugates with heterologous species (29, 40). In this study cross-reactivity was eliminated by absorption of the type E conjugates with Cl. bifermentans provided that the conjugates were diluted 1:2-1:4. Intense fluorescence of the homologous strains was retained with the absorbed, diluted conjugates.

Several workers have used counterstains to reduce fluorescence of background materials (28, 36, 80). Nonspecific staining of whitefish tissue was not eliminated by counterstains of Rhodamine B or Evans blue although it was not especially troublesome. Lind (49) also reported that Evans blue or Flazo orange failed to diminish background

fluorescence in studies on the detection of N. gonorrhoeae in leucocytes.

The results of O agglutination tests with the 070 and D8 antisera agree with those of Lynt (5) who found that somatic antigens were sufficiently similar to produce a wide range of cross-agglutination of the type E strains. However, the Beluga antiserum appeared to be deficient in cross-reacting antibody both by agglutination and the fluorescent antibody test. The H agglutination tests also agree with those of Lynt in that the flagellar antibodies showed a narrow range of specificity. In cases when fluorescent staining of vegetative cells was clearly apparent (Figs. 1-4, 6, 11, 12, 20, 21) there was no indication of flagellar staining. This suggests that flagellar antibodies do not participate in fluorescent reactions.

Reports by Hobbs et al. (35) and Walker and Batty (74) have indicated that conjugates prepared from antiserum to toxigenic strains stained cells of nontoxigenic type E strains. However in this study the two nontoxigenic strains did not show a significant degree of fluorescence (Figs. 18 and 19). Further studies on a variety of nontoxigenic strains are required.

Photomicrographs show that in most cases the 070 and D8 conjugates stained the spore body and not the vegetative cells of cross-reacting type E strains (Figs. 7-10, 13-17). These results suggest that the spores of the toxigenic type E strains share at least one common antigen. Fluorescent antibody studies by Georgala and Boothroyd (29) also have indicated that spore antigens are more widely distributed than vegetative cell antigens among type E isolates from various geographical areas.

The detection of type E strains by the fluorescent antibody test requires a conjugate specific for a common antigen shared by all type E strains. Since a group antigen would appear to be a component of the spore body it could be isolated from purified spore suspensions by modern fractionation methods. A fluorescein-conjugate specific to the group antigen would then provide a reliable reagent for the detection of a diversity of type E strains and in addition could be used in studies on the distribution of spores in food and in natural environments.

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