To my Parents

IMMUNOFLUORESCENCE STUDIES OF SPORES OF *CLOSTRIDIUM BOTULINUM* TYPE E

by

Augustine C. Emeruwa, B.Sc.

The University of Manitoba

Canada

A Thesis

submitted to

The Faculty of Graduate Studies and Research

The University of Manitoba

In partial fulfilment

of the requirements for the degree

of

Master of Science

June, 1970

ACKNOWLEDGEMENTS

The author wishes to express his sincere gratitude to Dr. R. Z. Hawirko of the Department of Microbiology, University of Manitoba, for her assistance and advice throughout the course of this investigation and during the preparation of the manuscript. Grateful thanks is extended also to Dr. I. E. Erdman of Food and Drug Directorate, Ottawa to Dr. D. A. Kautter of the Department of Health, Education and Welfare, Food and Drug Administration, Washington, D.C.; for their supply of the strains of Cl. botulinum type E and to Miss Eileen Mason of the Laboratory of Hygiene, Ottawa, for the supply of Cl. botulinum types A, B, C, F and other Clostridium spp. Sincere appreciation is also extended to Mr. A. W. Lantz for the supply of the frozen white smoked fish, Mr. Michael Bryan and Mr. John Giardino for their helpful suggestions on the photographic procedures.

ABSTRACT

Immunoglobulins of hyperimmune spore antisera which were prepared by the immunization of rabbits with heat killed, formol-treated spores of D8 and PM-15 strains were conjugated to fluorescein isothiocyanate. The conjugates were absorbed with the spores of Cl. bifermentans and diluted to eliminate non-specific staining and cross-reactivity with related serotypes.

The fluorescein-conjugates of the spore antisera globulins stained spores as well as vegetative cells of 19/20 E strains from diverse geographical areas and the moderate fluorescence of the cells was removed by absorption of the conjugates with young cells of the homologous strain. Since the fluorescein-conjugates, which were absorbed with young cells, stained the spores of E strains without any reduction in the intensity of fluorescence, it appears evident that the spore antigens confer the common determinant groups of type E specificity. Some of the cross-reacting type E strains showed the same degree of fluorescence as the homologous strain, suggesting that more than one antigen is shared by these strains. Enzyme treatment of spores prior to staining

and the washing of the stained spores in bicarbonate buffer, pH 9.0, enhanced the sensitivity of the F.A. test. The fluorescein-conjugates of PM-15, a non-toxigenic strain stained toxigenic as well as non-toxigenic strains indicating that toxigenicity is not associated with type E specificity. Photomicrographs of spores of the PM-15 strain showed a single tubular projection which fluoresced to the same degree as the spore body. Fluorescent spores were observed in smoked white fish samples experimentally inoculated with type E spores even after storage at 4C and at -20C. Trypsin digestion of the fish homogenates prior to F.A. staining reduced to a minimum the non-specific fluorescence of the tissues. Some spore germination occurred during the early stages of storage at 4C but cells were not observed in samples stored at -20C. These findings are indicative of the effectiveness and reliability of the immunofluorescence technique described for the identification of strains of Cl. botulinum type E in cultures and suspected food materials but field application of the test remains to be validated.

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INTRODUCTION

The reported increase of outbreaks of botulism caused by strains of Cl. botulinum type E, (1,2) and their transmission by commercially and home processed marine food products has re-emphasized the need for the development of more efficient methods for the detection of the strains present in suspected materials from diverse geographical areas.

Since the development of the fluorescent antibody test (FAT) by Coons et al. (18) and the study by Kalitina in 1960 (53) on the serotypes of Cl. botulinum, continuing research by workers such as Walker and Batty (96), Boothroyd and Georgala (5) has resulted in the evaluation of FAT for the detection of type E strains (4, 36, 53). Aside from the technical difficulties, which vary from laboratory to laboratory, the adoption of the test has been restricted by the need for fluorescein-conjugates with a broad enough specificity to encompass all strains of type E isolates.

Georgala and Boothroyd (36), Walker and Batty (96) have reported that conjugates prepared with spore antiserum showed a broader type E specificity than conjugates of vegetative cell antiserum and suggested that spore antigens are more widely distributed than vegetative cell antigens among E strains.

The present study was undertaken to develop a reliable F.A. method for the identification of type E strains isolated from different geographical regions and for the screening of suspected food materials. The work also included the evaluation of the spore specific fluorescein-conjugates of globulins of the high titer antispore serum and some factors known to enhance the intensity and specificity of immunofluorescence.

HISTORICAL REVIEW

During the last decade, reports of botulism attributed to type E *Clostridium botulinum* which have been isolated from commercially or home processed foods, have prompted renewed impetus in the study and the control of the anaerobic spore formers.

Toxigenic strains of type E were first isolated from sturgeon by Russian workers in 1937, identified and named as such by Gunnison *et al.* (42). Since then, isolates have been found in soil (60); marine foods such as smoked and home-canned salmon (28,43), pickled and smoked herring (29,31), salmon eggs (30), smoked eel (1), home-made izushi, a Japanese raw fish food (55), and various consumer-convenience food products (51). In addition, lethal toxins which were specifically neutralized by type E antitoxins have been identified in environmental materials. Spores of these strains are highly resistant to adverse conditions such as heat, chemicals, enzymes, radiation and freezing which accounts for the wide distribution of the spores in environmental materials throughout the world.

Botulism caused by type E strains has been reported primarily in Japan, Russia, United States, Canada, Sweden

and Denmark. Seventy-five outbreaks have been documented (31) for the Northern Hemisphere with a total of 374 cases and 112 deaths. Of these, 44 occurred in Japan (26 in Hokkaido and 18 in Northern Honshu); 13 in the United States (including seven in Alaska and six in other states); 11 in Canada (eight in British Columbia and three in Labrador); three in Sweden and two each in Denmark and the Soviet Union. In terms of total population, the highest case fatality rate occurred in Canada with 63% as compared with 39% for the United States, 26% for Japan and 30% overall.

In Canada, three classes of food stuffs, each with a defined consumer group have been the source of outbreaks of botulism. These have included uncooked fish consumed by continental Europeans in British Columbia, putrescent salmon eggs by North-west Pacific Coast Indians and raw seal products by Eskimoes in Labrador.

Uncooked fish was the vehicle in four episodes in the more populated southern parts of British Columbia as follows: home-canned salmon in 1944 (28,31), pickled and smoked herring in 1949 (29,31) and pickled trout in 1954 (31). The fourth occurred inland at Penticton in 1960.

Putrescent salmon eggs alone accounted for the botulism of 1940 in Yukon near Whitehorse and eight other outbreaks have been reported.

Raw seal products, a major component of the diet of Canadian Eskimoes, were the source of botulism which occurred in 1956 at Labrador. Twelve such episodes have occurred with a case fatality rate of 66% (31).

In the United States, commercially processed imported foods have been implicated in four out of five outbreaks in urban areas involving persons of Caucasian origin with a 40% case fatality rate. Vacuum packed smoked ciscoes from Lake Superior accounted for the Minneapolis episode in 1960. Raw salmon eggs accounted for four other incidences among the Pacific Coast Indians between 1959 to 1962. Muktuk, a piece of Beluga flipper was the carrier in five outbreaks and raw whale meat in the sixth among the Eskimoes of the Northern Coast of Alaska.

In Japan, botulism caused by type E strains have been recognized and isolated from herring izushi at Hakkaido near Iwanai and later at Honshu. Herring izushi was the source of the majority of the 44 outbreaks in this country but other vehicles have been documented including trout eggs, commercially canned mackerel and herring (Kirikomi) which was the carrier in the most widely spread outbreak occurring in Hakkaido in 1962 (31).

In Sweden, botulism has been transmitted by pickled herring in 1960 and three other outbreaks have involved six persons with one fatality. In Norway, type E strains were isolated from ranch mink in 1960 but human botulism has not been reported.

In order to determine the primary origin and the environmental distribution of type E strains, extensive surveys of soil and water samples from various geographical areas have been under investigation. On the basis of data from North America, Japan, Sweden, and Russia, Dr. Dolman, one of the pioneer workers in this field, concluded that although type E strains have a predilection for fish as a vehicle, the microorganisms are of terristerial origin and show a definite affinity for marine waters. He suggested that bottom feeding fishes and their predators added the spores to their intestinal flora from such habitats (37).

Confirmatory data on the ecology and origin of type E strains has shown that the spores were isolated from water and soil of Russia, Sweden, and Japan (60, 32, 52, 54); the mud of the United States Pacific Coast (33, 24, 25), marine deposits of the Scandinavian Coast (11) and the Gulf of Maine (79). And also E strains were isolated from fishes as follows: white fish chubs from Milwaukee area (80); salmon steelhead,

a few crabs and bottom fish from the Pacific Coast of the U.S.A. (33, 24, 25); and a variety of species from the Great Lakes (7, 6) and the Green Bay area of Lake Michigan (8), including Cayuga Lake fish from New York State (14) and fresh water perch from a river near Paris, France (84).

Non-toxigenic strains of type E have shown the same ecological distribution as the toxigenic strains and have been isolated from fish and marine deposits (45).

In the standard procedures for the cultivation and identification of the type E strains in environmental materials, much emphasis has been placed on toxigenicity tests with mice. These techniques require elaborate laboratory facilities, are time consuming, and are not suitable for routine screening of large numbers of samples. The fluorescent antibody test has been recommended as an appropriate procedure (5, 53, 95).

In 1941, the fluorescent antibody test made its first debut in a publication by Coons et al. (18) and in the following year, the same authors published a detailed description of the preparation of conjugates using the fluorescein isocyanate dye which emitted a yellow-green fluorescence, for the detection of pneumoccal antigen in infected mice (19).

After World War II, significant practical and theoretical contributions ensued. The high specificity of the technique was demonstrated by localizing a native protein in normal tissue, by Marshall et al. in 1951 (70). In 1957, the original conjugation procedure was simplified and the denaturing effect of organic solvents eliminated by combining the buffered globulins directly to the fluorescein dye without the addition of acetone and dioxone (37, 69). In 1958, Riggs et al. (87), replaced the fluorescein isocyanate with fluorescein isothiocyanate (FITC) which is easier to synthesize, less toxic and more stable. Other fluorochromes have included Rhodamine B, a red fluorescent dye often utilized as a counterstain because it provided excellent contrast to the yellow-green of fluorescein and the blue autofluorescence of tissues; acriflavin, thioflavin, primulin and dimethylamine naphthaline-5-sulfonyl chloride (DMANSC), The fluorescein isothiocyanate dye has proved to be most acceptable because it emits a brilliant fluorescence.

Gamma globulins are usually obtained for conjugation by precipitation with ammonium sulphate or cold ethanol (76), but nonspecific fluorescence due to impurities has been reported (10, 64). The ion exchange method has been used (27, 63, 82) but has offered no advantage.

Conjugation procedures have been modified by many workers in order to achieve a more specific and sensitive reagent. Nairn (76) has shown that a gradual addition of the dye to the globulins yielded a more effective conjugate. Conjugation by dialysis (69) has been tried but showed no improvement over the simpler procedures. More recently, critical studies have shown that the labelling reaction occurs more rapidly at room temperature without loss of the antibody activity (58, 71, 90) and was significantly improved at pH 9.5. The concentration of globulins for conjugation could be varied between 5 to 50 mg. with little effect on the final degree of labelling achieved (38). Globulins conjugated to multiple fluorescent dyes have had limited application (77, 75).

The removal of unreacted dye from the fluorescein-conjugates to reduce nonspecific fluorescence by simple dialysis against repeated changes of water and of buffered saline has proved to be time consuming. Critical analysis has also revealed that some unreacted dyes remained even after prolonged dialysis (13, 69). Several workers have used separation according to molecular sizes by gel filtration on Sephadex, a cross-linked dextran (100, 39, 35, 41, 57, 65). This is now the method of choice because of the technical simplicity,

efficiency, and high yield of undenatured conjugates unequalled by other procedures. Extraction of unreacted dye with charcoal has also been tried by Fothergill et al. (34) with little success due to the great loss of protein involved.

The conjugates eluted from the Sephadex columns are easily concentrated by dialysis against water-soluble high molecular weight compounds such as dextran, polyethelene glycol and polyvinyl pyrrolidone without altering the ionic strength (65, 59).

Objectionable fluorescence due to nonspecific staining, cross-reactivity and autofluorescence has been a problem in fluorescence staining. Fluorescence which was not produced by the interaction of antigen with antibody has been shown to be caused by a charge effect resulting from the union of some basic proteins of the cells with the acidic proteins from the conjugates (39, 69, 87, 70, 26, 78). Methods of controlling nonspecific fluorescence have included simple dilution of the conjugates beyond the range of nonspecific staining without altering the specificity. Conjugates purified on DEAE-cellulose columns have yielded fractions with a high degree of specificity and a minimum of nonspecific fluorescene (39, 87, 26, 70).

Curtain showed that the protein dye ratio played an

important role in nonspecific staining 1961 (25) and Wells et al. (99) and other workers have published the procedures of calculating the ratio of dye to protein (76, 64, 58, 56). The ratios reflect the average amount of fluorescein per protein molecule (F/P) and the F/P ratio of 1 to 2 is considered optimal for fluorescein isothiocyanate. Nonspecific fluorescence is minimal at this range of ratios and increases with a deviation from the range (49).

Absorption of conjugates with tissue powders have been shown to reduce nonspecific fluorescence of tissue cells by Coons and Kaplan (20). Monospecific conjugates have been prepared by absorption with the cross-reacting antigen (74, 92, 85) or by the one-step inhibition technique in which a small amount of the unconjugated antiserum was added to the cross-reacting antigen prior to the absorption procedure.

Smears stained with fluorescein-conjugates and rinsed with a buffer, pH 9.0, have shown a minimum nonspecific fluorescence (69) and an enhanced degree of fluorescence (83). Autofluorescence is also diminished by the selection of appropriate lens system, filters, and staining techniques.

The fluorescent antibody tests in general use are the direct staining method developed by Coons et al.(18) and the

indirect method described by Weller and Coons (98). The direct method consists of the application of a few drops of appropriately diluted fluorescein-conjugate to an antigen and has the advantage that only a single reagent is needed. In this method, immunological specificity of the fluorescence is established by control tests including staining with conjugates of normal serum, absorption of the conjugate with homologous antigen and inhibition tests with globulins prior to staining with fluorescein-conjugates.

The indirect method, also known as anti-gamma globulin test, consists of pretreatment of the antigen with unlabelled specific globulins followed by staining with fluorescein-conjugates of antiglobulin serum. This method is based on the principle that antibody molecules, in addition to reacting with antigens, are capable of serving as antigens which are made visible by staining with conjugates of antiglobulin sera. The technique also shows an increased sensitivity.

Other methods of fluorescent antibody staining have included complement fixation with fluorescein conjugate of anticomplement serum. The sensitivity of this method has been reported to be higher than the antiglobulin method (40, 23, 46). Also direct staining with a second conjugate of

contrasting dye has been used to provide information on multiple antigens present in the same preparation or even within the same cells (9, 26, 6, 47, 72, 88).

The identification of a diverse range of microorganisms by the fluorescent antibody test (FAT) has prompted workers such as Kalitina to investigate the feasibility of adapting the FAT for the detection and identification of Cl. botulinum type E. In 1960, Kalitina (53) demonstrated that fluorescein-conjugates of antiserum to Cl. botulinum stained the other serotypes. For four years Walker and Batty studied Clostridium spp by FATand in 1964 showed that the test could be used as a rapid method for differentiating the serotypes of Cl. botulinum into two main groups, the proteolytic types A, B, and F; and the non-proteolytic types C, D, and E (95). Independently, in the same year, Boothroyd and Georgala (5) confirmed this finding as well as showed that the vegetative cells of type E strains have a restricted range of strain specificity. Fluorescein-conjugates of vegetative cell antiserum have been shown to stain the spore bodies rather than the cells of most of the cross-reacting strains (3).

Fluorescein-conjugates prepared from spore antisera by Walker and Batty (96) as well as Boothroyd and Georgala (5) stained spores of 41/51 toxigenic strains of type E isolated

from different parts of the world. Vegetative cells showed only a slight fluorescence and no cross-reactivity with related spp. More recently, agglutination (91) and Agar diffusion tests (62) have shown that spore antigens are widely distributed and suggested that spores carry common as well as specific antigenic determinant groups.

The fluorescent antibody test also appears promising as a screening method for the detection of type E spores in suspected materials such as raw or processed foods for control purposes. Type E cells have been reported to show specific fluorescence in 6/37 samples from the estuarine waters of the United States Gulf Coast (97) and in experimentally inoculated food during the entire incubation period (73).

The fluorescent antibody test offers considerable promise as a rapid and reliable procedure which would permit the detection of type E strains in commercially processed foods and suspected materials. However, there is the need for a monospecific antiserum for the preparation of fluorescein-conjugates with a broad enough specificity to encompass all type E strains and to allow the standardization of conjugates for the investigation into many facets of the control of botulism.

MATERIALS AND METHODS

Cultures

Clostridium botulinum type E toxigenic strains D8, 070, 5191, VH and non-toxigenic PM-15 and 066BNT were received from the Food and Drug Administration, Washington, D.C.; toxigenic strains Beluga OT, VHOT, Minnesota, Russian, Hazen-salmon, F-106 and non-toxigenic strains Beluga 0 and VHO, from the Food and Drug Directorate, Ottawa; the Beluga strain, *Cl. botulinum* types A, B, C and F and *Cl. bifermentans* were supplied by the Laboratory of Hygiene; *Cl. sporogenes* ATCC 19404, ATCC 9564, 9565, and 17786 from the American Type Culture Collection. The toxigenicity of type E strains, D8, 070, VH, 715, 5192, 9564, Beluga, 066BNT and PM-15 was verified by the mouse protection tests (3).

The type E strains showing geographical areas, and original source of isolation are listed in Table I.

Preparation of antisera

Suspensions of spores were prepared from the type E strains D8, and PM-15. The cultures were grown in Trypticase (BBL) Peptone (Difco) glucose (TPG) broth (88) for 48 hr. at 30 C and transferred repeatedly at intervals of 48 hr. until 80% sporulation was achieved as estimated by phase contrast.

microscopy. The spores were collected by centrifugation at 3000 g. for 30 min. at 4 C, treated with 1 mg/ml trypsin (Mann Research Lab.) and 2 mg/ml lysozyme (Mann Research Lab.) for 2 hours at 37 C and washed three times in 0.01 M phosphate buffered saline (PBS), pH 7.2.

The washed spores were heated for one hour at 100 C, suspended in 0.4% formol saline, and stored for one week at 4 C. The suspension was tested for sterility and adjusted to a density corresponding to McFarland tube No. 5 prior to inoculation.

Albino rabbits were immunized by a series of intensive intravenous inoculations three times a week for four weeks.

Agglutination Tests

One week following immunization, the titers of the antisera were estimated using the tube agglutination method of Lamanna (89). The spore antiserum was set up in series of halving dilutions ranging from 1:10 to 1:20480 in 0.5 ml. volumes. An equal volume of standardised spore suspension, McFarland no. 2, was added to each dilution and to an antigen control tube containing 0.5 ml. saline. The test was incubated for 45 min. at 30 C with constant shaking, followed by overnight refrigeration at 4 C prior to reading. The titer was recorded

as the highest dilution of the antiserum showing visible agglutination. The immunization series were repeated after an interval of one month and antisera showing agglutinin titers > 1280 were stored at -20 C.

Conjugation

Gamma globulins were precipitated by the addition of an equal vol. of saturated ammonium sulfate to the spore antiserum. After four hr. at 4 C, the globulins were collected by centrifugation and redissolved in an equal vol. of phosphate buffered saline PBS, 0.01 M, pH 7.2, (84 ml. of 0.2 M Na_2HPO_4, (28.4 g/L), 16 ml. of 0.2 M $NaH_2PO_4 \cdot 2H_2O$ (31.1 g/L), 1,900 ml. distilled water, 17,53 NaCl adjusted to pH 7.2). The procedure was repeated twice and the globulins were dialyzed against distilled water and PBS until the sulfate ions were removed. The protein content of the globulins was determined by the Lowry method (66) and adjusted to 2% (20 mg/ml) with saline.

Conjugation of the gamma globulins was carried out by the method of Nairn (77) with fluorescein isothiocyanate (BBL), FITC, 12.5 µg per mg globulin, in 15 ml. aliquots pH 9.5, for 2 hr. at 25 C. The unreacted FITC dye was removed by gel filtration on Sephadex G-25 (Pharmacia) packed in a 125 ml. column and equilibrated with PBS at 4 C. The labelled globulins

were concentrated by dialysis against 15% polyvinyl pyrrolidone (Mann Research Lab.) for 20 hr. at 4 C and stored at -20 C. Preimmune serum was also fractionated and conjugated with FITC.

Absorption and Dilution of Conjugates

Fluorescein-conjugates were absorbed with spore suspension of Cl. bifermentans for two hr. at room temp. using 1:2 vol. spores to conjugate and the procedure was repeated twice. Similarly spore specific conjugates were prepared by absorption with vegetative cells from 18 hr. TPG cultures of the homologous strain. Fluorescein-conjugates were diluted in series ranging from 1:2 to 1:16 and the optimal dilution showing significant specific fluorescent staining was determined.

Preparation of Spores.

Sporulating cultures from 20 type E strains, serotypes A, B, C and F and two other Clostridium spp. were propagated in 10 ml. vol. of TPG for 48 hr. at 30 C. The spores were collected by centrifugation at 3000 g. for 30 min. at 4 C, washed three times and suspended in 10 ml. PBS.

Spores from 10 ml. TPG cultures were suspended in 5 ml. of 0.05% trypsin and 0.05% ribonuclease and digested for three hr. at 30 C. and then washed in PBS (17).

For control tests, vegetative cells were collected from 18 hr. TPG cultures at 30 C, washed and suspended in 10 ml. PBS.

Frozen smoked white fish, which were received from the Fisheries Research Board, University of Manitoba, were thawed and inoculated asceptically through the sealed plastic bags. Three designated areas of each fish were injected with one ml. of 10^6 spores suspended in PBS. Ten fish samples were used in preliminary trials to determine the size of the spore inoculum, the concentration of the trypsin and time required for digestion of the fish tissues. The test samples included six inoculated fish stored at 4 C and six at -20 C. Control samples included one inoculated and one uninoculated fish tested without storage. Also, uninoculated fish were stored for 42 days at 4 C and at -20 C. At weekly intervals for six weeks, one fish stored at 4 C and one at -20 C were thawed at room temp. Approximately, one gram sample of each inoculated area was homogenized in a tissue homogenizer (Bellco) and digested in 5 ml. of 1% trypsin for 6 hr. at 30 C. The deposits were collected by centrifugation at 3000 g. for 30 min. at 4 C, washed and suspended in PBS.

Fluorescent Antibody Staining

A loopful of spore suspension was spread on a greasefree slide, air dried and gently heat fixed. The smears were flooded with fluorescein-conjugated globulins and left for 30 min. at 37 C in a moist chamber. The smears were then rinsed gently with 0.5 M bicarbonate buffer pH 9.0 (0.5 M $NaHCO_3$ (42.0 gm/L) and 0.5 M Na_2CO_3 (53.0 gm/L), pH 9.0) for one min. and placed in a Coplin jar filled with bicarbonate buffer for 10 mins. (83). The slides were allowed to air dry in a slantwise position.

Duplicate smears of the fish homogenates were fixed in acetone for 15 mins. at room temp. and stained with a fluorescein-conjugate.

The spores, which were pretreated with enzymes, were suspended in 2.5 ml. fluorescein-conjugates of globulins and left for 255 mins. at 30 C. The labeled spores were collected by centrifugation, washed and suspended in bicarbonate buffer. A loopful of the spore suspension was spread on a greasefree slide, air dried and gently heat fixed.

Control tests included spores of E strains stained with conjugates of preimmune serum as well as spores stained with conjugates of specific spore antiserum after absorption with spores of homologous strain. In addition, spores

were stained with a fluorescein-conjugate of spore antiserum which was incubated for one hr. at room temp. with an equal vol. of dilutions of unconjugated spore antiserum. Control tests included homogenates of inoculated fish samples stained before storage and uninoculated fish samples stained after storage at the low temperatures.

Fluorescence Microscopy

Smears were mounted in immersion oil nD 1.5 (nonfluoresent) and examined with a Zeiss fluorescence photomicroscope equipped with a bright field condenser, an OSRAM HBO-200W Mercury Vapour Pressure Lamp and filters consisting of Schott BG-38/2.5, BG 12/4, BG 3/4 and UG 5/3 exciter filters in combination with barrier filter No. 47.

The intensity of fluorescence was rated on a scale of 0 to 4+ and fluorescent staining reactions which gave 2+ or greater were considered positive.

Photomicrographs were taken on Pan X (ASA 100) with an overhead Zeiss Icarex 35mm. camera with exposure times of 35 to 45 seconds. The negatives were developed in autofine developer for six minutes.

RESULTS

Agglutination tests on antisera from rabbits immunized with spore suspensions of the D8 and of the PM-15 strains showed titers of 2560 with homologous strains and titers ranging from 320 to 2560 with heterologous type E strains (Table II). The antisera did not agglutinate spores of the 066BNT, non-toxigenic strain.

Spores of type E strains of Cl. botulinum stained with unabsorbed fluorescein-conjugates of spore antisera showed intense fluorescence. The moderate degree of fluorescence observed with spores of Cl. botulinum types A, B, C and F Cl. bifermentans and Cl. sporogenes was eliminated by absorption of the conjugates with spores of Cl. bifermentans followed by a dilution of 1/8.

Vegetative cells of E strains showed a moderate degree of fluorescence with the absorbed and diluted fluorescein-conjugates.

Fluorescein-conjugates of spore antisera of the D8 and PM-15 strains which were reabsorbed with young cells of homologous strain (Table III) stained 16/16 toxigenic and 3/4 non-toxigenic type E strains with significant fluorescence.

Spores of the E strains treated with enzymes prior to prolonged staining showed an increased degree of fluorescence (Table III).

Fluorescent spores were observed in samples of experimentally inoculated fish on an average of five spores per microscopic field before storage and two to five after storage at the low temps. (Table IV). Fluorescent vegetative cells were observed in test samples examined during the first 14 days of storage at 4 C but were not seen in fish stored at -20 C (Table IV). The non-specific fluorescence of the fish tissue was markedly reduced by trypsin digestion of the homogenates.

Control tests showed specific inhibition of the fluorescence of spores stained with a mixture of unconjugated and fluorescein-conjugated spore antisera. Also, spores stained with conjugates of preimmune serum did not fluoresce. The fluorescence of type E vegetative cells was completely eliminated by absorption of the fluorescein-conjugates of spore antiserum with vegetative cells of the homologous strain. Correspondingly, the fluorescence of the spore body was abolished by absorption of the conjugates with spores of the homologous strain. Furthermore, cells and spores were not observed in the uninoculated fish samples.

Photomicrographs of fluorescent type E cells and spores are shown in figs. 1-23. The spore body of the PM-15 strain showed a single tubular appendage as illustrated in fig. 24. Fluorescent spores detected in the experimentally inoculated smoked fish is shown in fig. 25.

Table I

Clostridium spp.	Geographical Area	Source of Isolation
Cl. botulinum		
Type E. Strains		
08	uncertain	Canned tuna fish, outbreak
070, 5191 and 5192	Great Lakes	Smoked whitefish chubs
Beluga, O, OT	Alaska	"Muktuk"
VH, VH-O, VH-OT	Vancouver, B.C.	Pickled herring
715	Hokkaido, Japan	Lake Abashiri mud
9564	Nova Scotia	Smoked salmon
9565	Nova Scotia	German-canned "sprat"
Minnesota	Great Lakes	Smoked ciscoes, outbreak
Russian	Russia	Sea of Azov
Hazen-salmon	Nova Scotia	smoked salmon
F106		
Type E, nontoxogenic:		
PM-15	Washington, D.C.	Potomac River mud
066BNT	Great Lakes	Smoked whitefish chubs
Cl. botulinum		
Type A		Cow liver
Type B		
Type C		
Type F	Pacific Coast	Marine sediment
Cl. bifermentans	Great Lakes	Sediments

TABLE II

Agglutinin Titers* of Spore Suspensions

of *Cl. Botulinum* Type E Strains.

Spore Suspensions	Antiserum to Spores of the D8 Strain
Type E Toxigenic Strains	
D8	2560
070	2560
Beluga	640
VH	640
715	2560
5191	640
5192	1280
9564	2560
9565	640
17786	320
Minnesota	640
Russian	640
Hazen-salmon	320
F-106	320

TABLE II Cont'd.

Spore Suspensions	Antiserum to Spores of the D8 Strain
Type E Non-toxigenic Strains	
PM-15	2560
066BNT	0
Cl. botulinum	
A	0
B	0
C	0
F	0

0, no visible agglutination.

* titer expressed as the reciprocal of the highest dilution showing visible agglutination.

Table III

Fluorescent Staining of Spores of *Clostridium spp.* with Conjugates of Spore Antiserum.

Cultures	Degree of Fluorescence		
Cl. botulinum,	Conjugates		
Type E Strains	PM-15	D8	D8
D8	3+	3+	4+*
070	3+	3+	4+
Beluga	2+	2+	3+
Beluga O (non-toxigenic)	2+	2+	3+
Beluga OT	2+	2+	3+
VH	2+	2+	3+
VHO (non-toxigenic)	2+	2+	2+
VHOT	2+	2+	2+
715	3+	3+	4+
5191	2+	2+	3+
5192	2+	3+	3+
9564	2+	3+	3+
9565	2+	2+	3+
17786	2+	2+	3+
Minnesota	2+	2+	3+
Russian	2+	2+	2+
Hazen-Salmon	2+	3+	3+
F-106	2+	2+	2+

Table III Cont'd.

Cultures	Degree of Fluorescence		
Non-toxigenic	Conjugates		
	PM-15	D8	D8
PM-15	3+	3+	4+
066BNT	-	-	-
Cl. botulinum			
Type A	-	-	±
Type B	=	-	-
Type C	-	-	+
Type F	-	-	+
Cl. sporogenes	-	-	+
Cl. bifermentans	-	-	-

*Trypsin treated spores.

TABLE IV

Fluorescent Antibody Test on Smoked White Fish

Experimentally Inoculated with Type E Spores

	Storage Temperature			
	4°		-20°	
Days	Cells/Field	Spores/Field	Cells/Field	Spores/Field
Zero	0	5	0	5
7	1	2	0	4
14	4	5	0	4
21	2	4	0	4
28	2	4	0	4
35	2	4	0	4
42	2	3	0	3

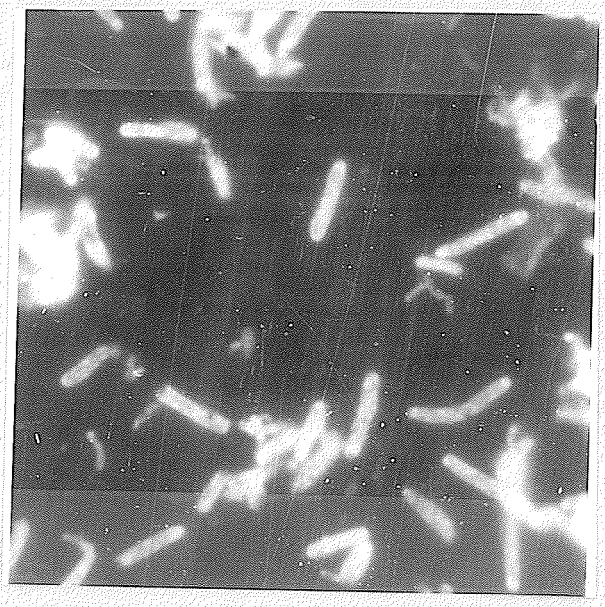

Fig. 1. Vegetative cells of 070 strain stained with a conjugate of D8 spore antiserum. (6000 X).

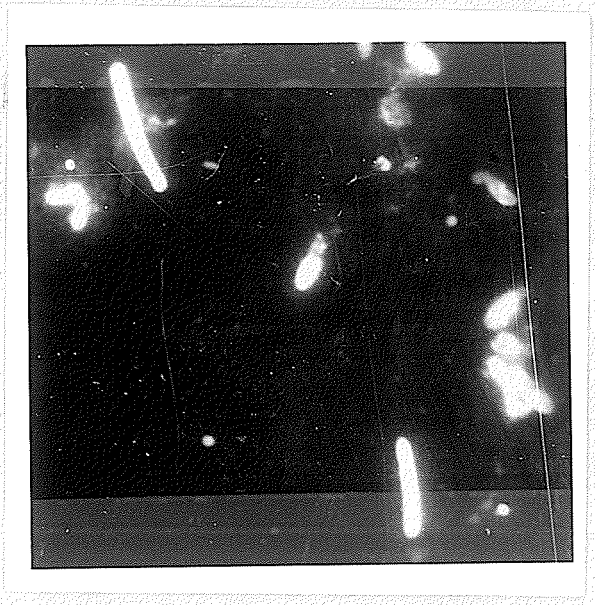

Fig. 2. Vegetative cells of 9564 strain stained with a conjugate of D8 spore antiserum. (6000 X).

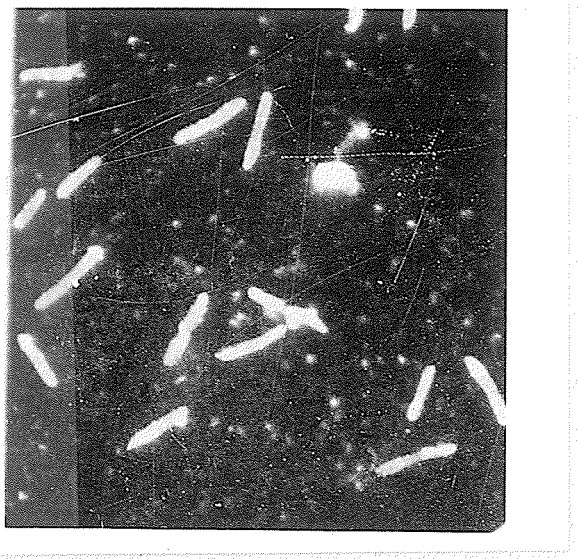

Fig. 3. Vegetative cells of PM-15 strain stained with a conjugate of D8 spore antiserum. (6000 X).

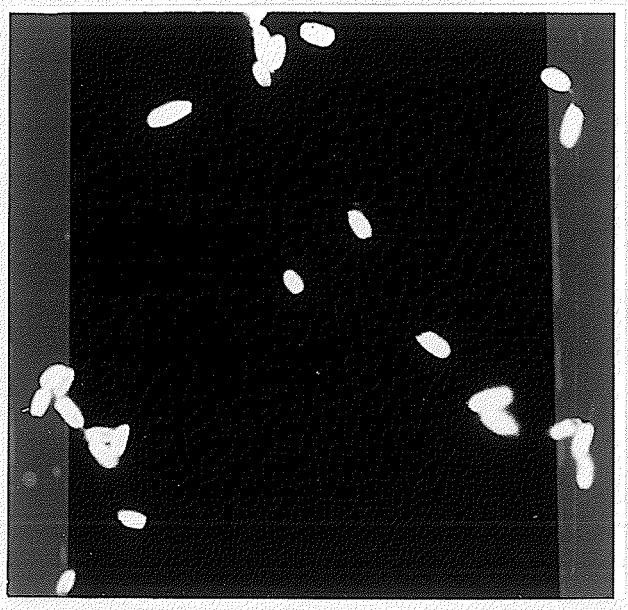

Fig. 4. Spores of D8 strain stained with a conjugate of D8 spore antiserum. (6000 X).

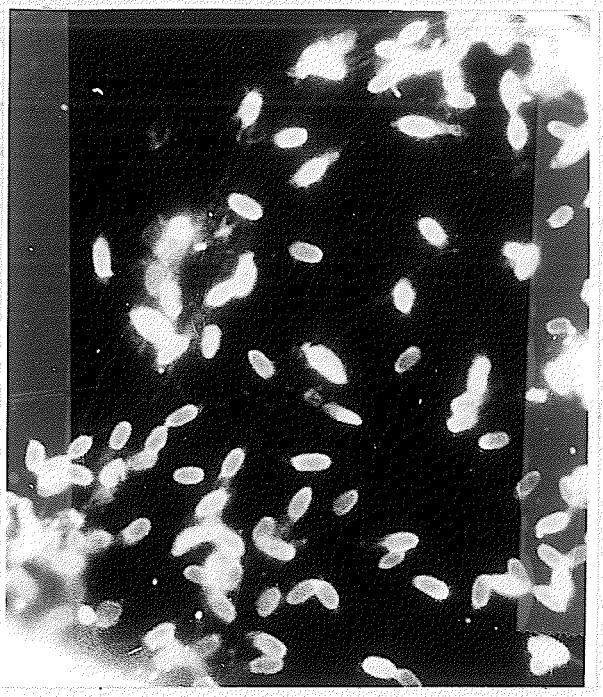

Fig. 5. Spores of 070 strain stained with a conjugate of D8 spore antiserum. (6000 X).

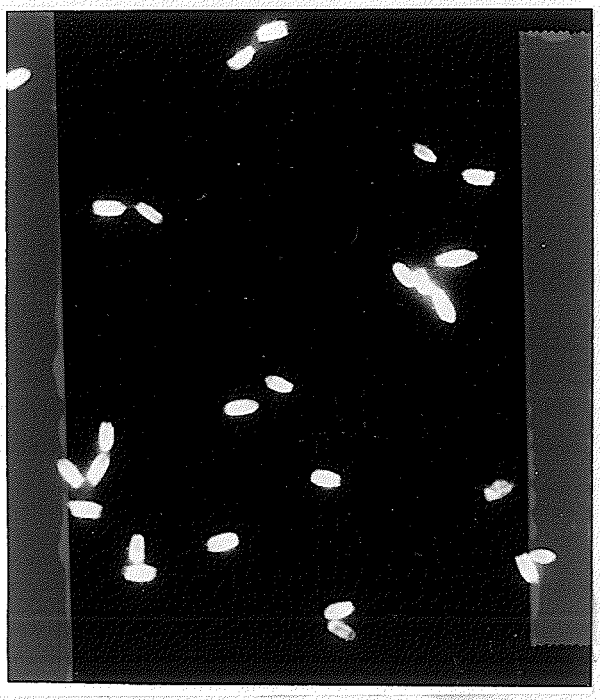

Fig. 6. Spores of 715 strain stained with a conjugate of D8 spore antiserum. (6000 X).

Fig. 7. Spores of PM-15 strain stained with a conjugate of D8 spore antiserum. (6000 X).

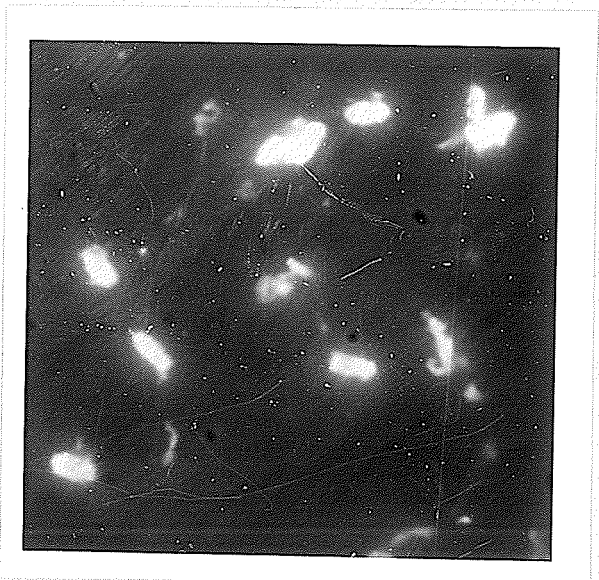

Fig. 8. Spores of Beluga strain stained with a conjugate of D8 spore antiserum. (6000 X).

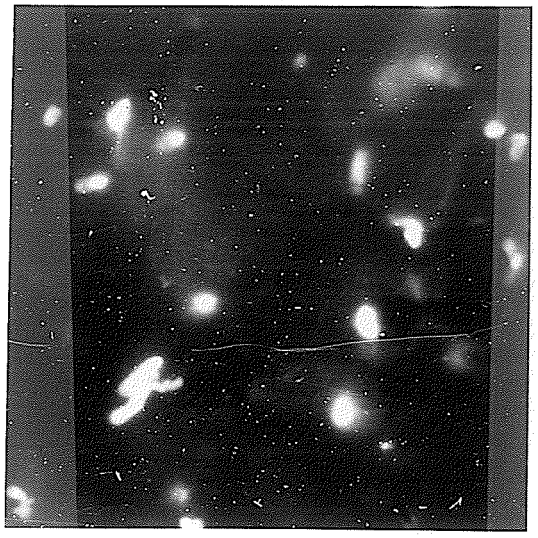

Fig. 9. Spores of Beluga O strain stained with a conjugate of D8 spore antiserum. (6000 X).

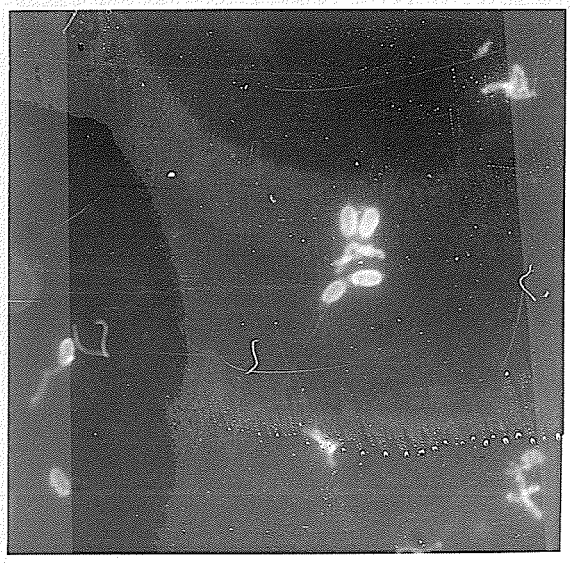

Fig. 10. Spores of Beluga OT strain stained with a conjugate of D8 spore antiserum. (6000 X).

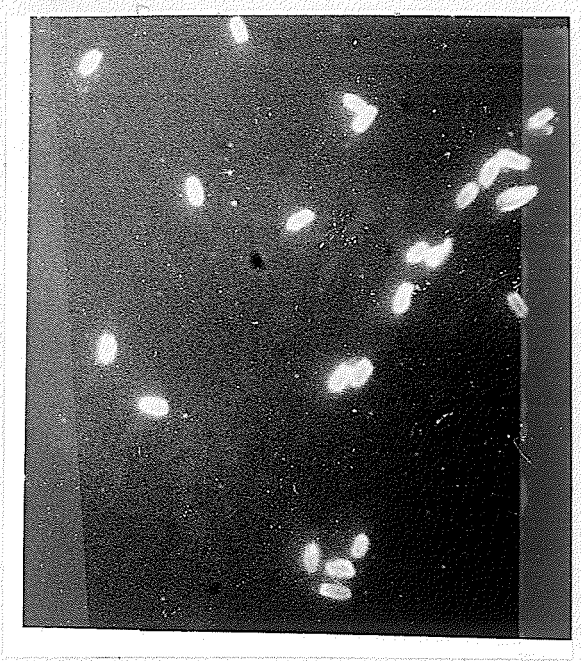

Fig. 11. Spores of VH strain stained with a conjugate of D8 spore antiserum. (6000 X).

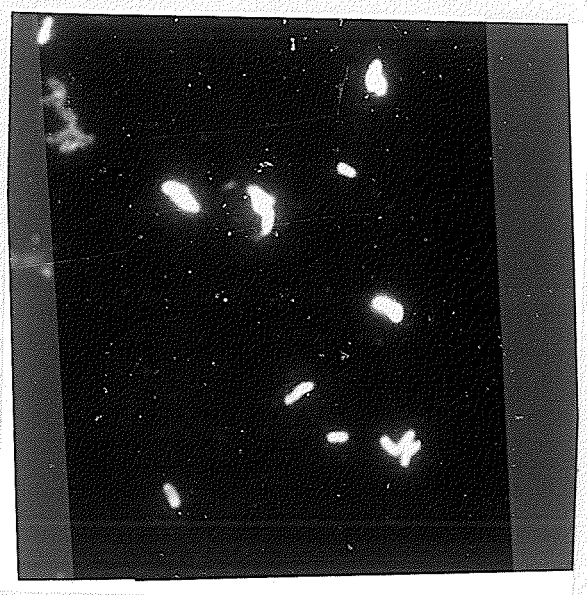

Fig. 12. Spores of VH-0 strain stained with a conjugate of D8 spore antiserum. (6000 X).

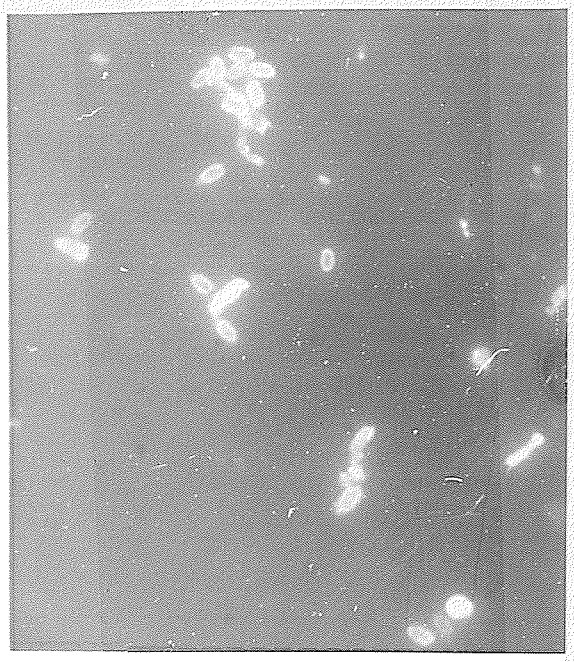

Fig. 13. Spores of VH-OT strain stained with a conjugate of D8 spore antiserum. (6000 X).

Fig. 14. Spores of 5191 strain stained with a conjugate of D8 spore antiserum. (6000 X).

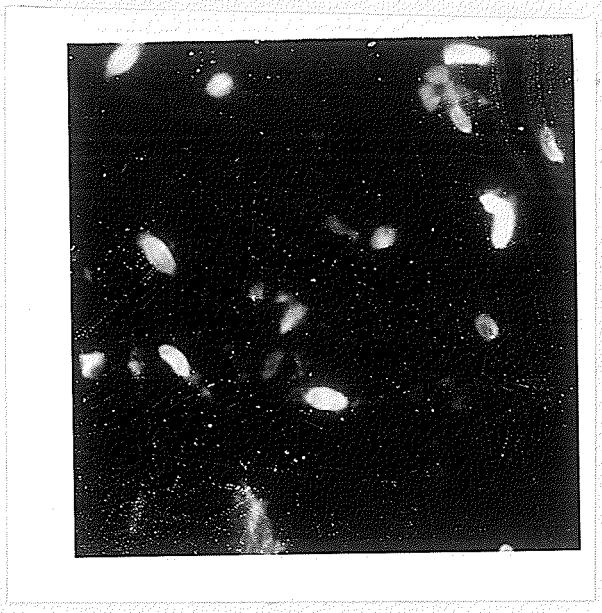

Fig. 15. Spores of 5192 strain stained with a conjugate of D8 spore antiserum. (6000 X).

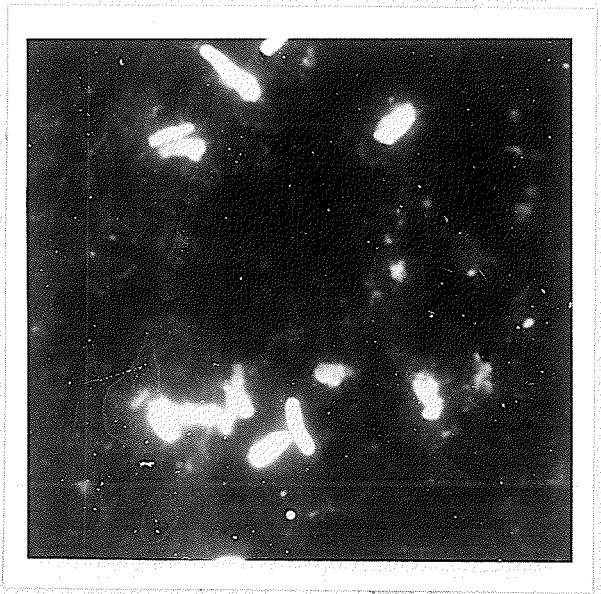

Fig. 16. Spores of 9564 strain stained with a conjugate of D8 spore antiserum. (6000 X).

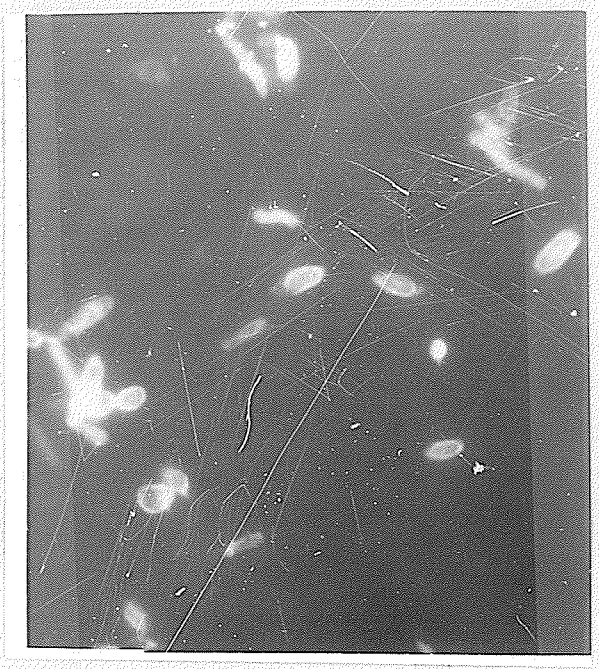

Fig. 17. Spores of 9565 strain stained with a conjugate of D8 spore antiserum. (6000 X).

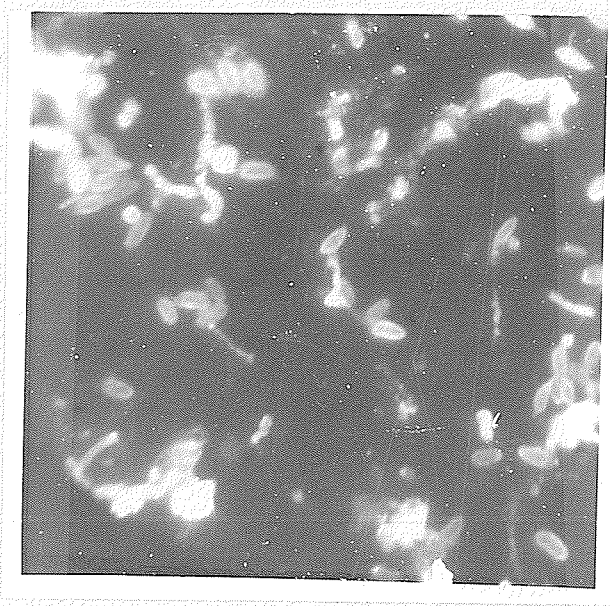

Fig. 18. Spores of 17786 strain stained with a conjugate of D8 spore antiserum. (6000 X).

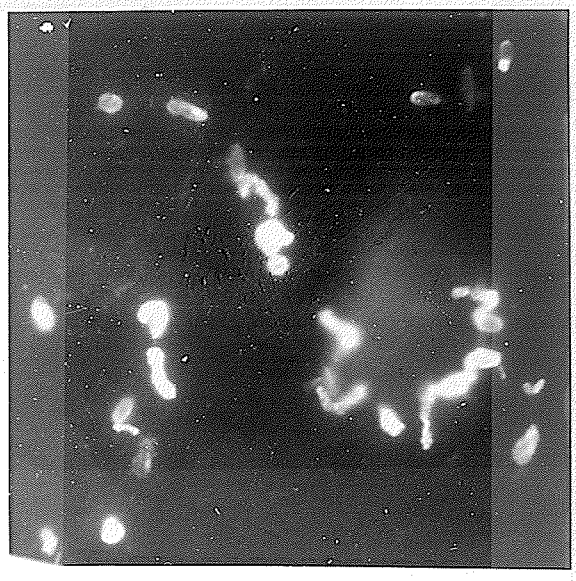

Fig. 19. Spores of Minnesota strain stained with a conjugate of D8 spore antiserum. (6000 X).

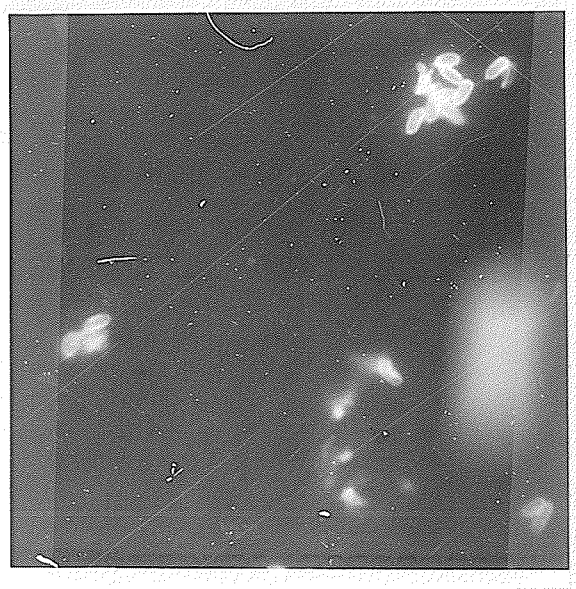

Fig. 20. Spores of Russian strain stained with a conjugate of D8 spore antiserum. (6000 X).

Fig. 21. Spores of Hazen-Salmon strain stained with a conjugate of D8 spore antiserum. (6000 X).

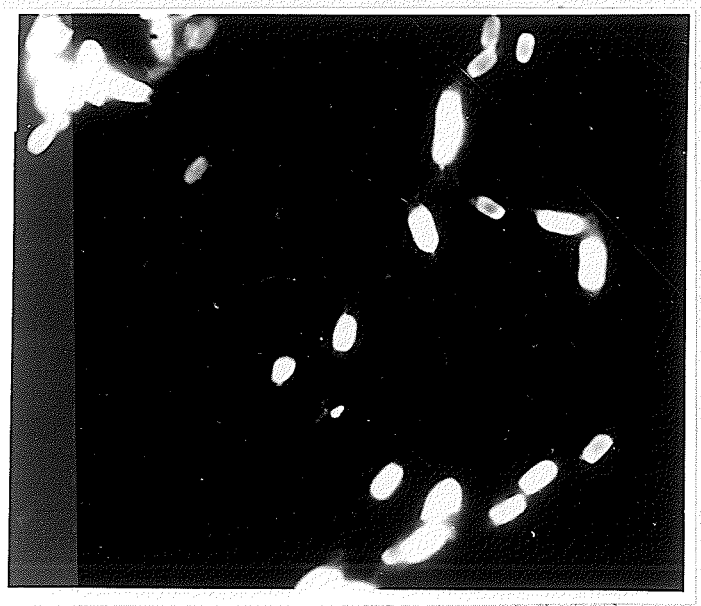

Fig. 22. Spores of PM-15 strain stained with a conjugate of PM-15 spore antiserum. (6000 X).

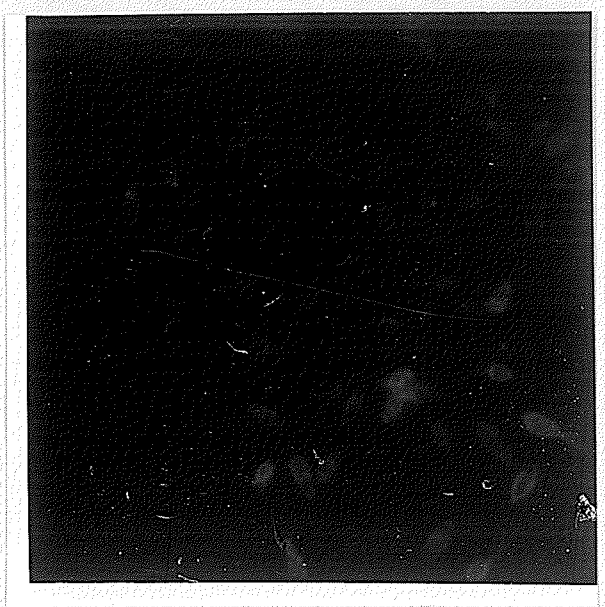

Fig. 23. Spores of 066BNT strain stained with a conjugate of D8 spore antiserum. (6000 X).

Fig. 24. Spores of PM-15 strain showing tubular projections.

Fig. 25. Spores of D8 strain in homogenates of experimentally inoculated fish. (6000 X).

DISCUSSION

In accord with the recent findings of Solomon et al. (91), the agglutination tests of spore suspensions, with the single exception of the 066BNT strain, showed that spore antigens possess a specificity restricted to type E strains of diverse origin. However, Solomon et al. reported identical agglutinin titers with heterologous E strains contrary to the quantitative differences of the titers observed with the cross-reacting stains in this study. The high titers of the spore antisera served as a suitable guide for the selection of antisera required for conjugation but are not necessarily indicators of immunofluorescence (45).

The fluorescent antibody test has been employed by some workers for the identification of Cl. botulinum type E in cultures and in environmental materials but its application on a wide scale has been hindered by the lack of a conjugate with a specificity inclusive of but limited to E strains. In this study, conjugates with a specificity restricted to E strains were prepared with spore antisera of high titers. Nonspecificity was eliminated, by gel filtration which removed the unreacted dye, by absorption with Cl. bifermentans which abolished the cross-reactivity of related serotypes and by dilution of the conjugates.

Since the fluorescein to protein ratios between land 2 areknown to yield · minimal nonspecific fluorescence (56), this may partly account for the high specificity and intense fluorescence exhibited by the fluorescein-conjugates of spore antisera which contained one per cent of the fluorescein carbomyl moiety. The enhanced sensitivity of conjugates maintained at pH 9.5 is considered to be due to the augmentation of the resonance of the enol-keto tautomerism involving the carboxyl group and one of the two phenolic groups (71). The degree of specific fluorescence was also enhanced by rinsing the stained smears with a bicarbonate buffer pH 9.0 (69,83). Furthermore, the brightest fluorescence, which was observed with spores treated with trypsin prior to staining (Table III), may be due to the partial separation of the protein coat (93) which reveals a greater area of the receptor sites for specific reactivity. Although the enzyme treatment increased the sensitivity of the FAT, it may also introduce some nonspecific fluorescence due to the prolonged staining.

The intense fluorescence of spores of 19/20 type E strains from diverse geographical origins which were stained with fluorescein-conjugates of spore antisera showed that antigenicity of type E spores is widely distributed among E strains as was suggested by Walker and Batty (96).

The moderate fluorescence of the vegetative cells of E strains, which was eliminated by absorption of the conjugates with young cells of the homologous strain without altering the fluorescence of the spores, confirms the non-identity of spore and cell antigens (91). And since significant fluorescence of spores of the heterologous E strains was observed with spore specific fluorescein-conjugates, it is evident that the spore antigens confer the determinant group of type E specificity. The findings of the FAT are in accord with the immunodiffusion analysis of spore extracts which showed that a spore antigen was common to E strains (62).

Some of the E strains including the 070, 715, 5192 and PM-15 showed the same degree of fluorescence as the homologous strain (Table III) suggesting that more than one spore antigen may be shared by these strains and the possibility of subgrouping closely related strains. Immunodiffusion test of spore extracts has also shown that a multiplicity of spore antigens is present among the strains (79). Because of the prevalence of variants among the cultures of type E strains (48), the E strains received from different laboratories including some identical strains such as VH and VH-OT, Beluga and Beluga-OT 9564 and Hazen-salmon were treated separately in order to show the stable character of the spore antigens detected by the FA test.

Type E specificity appears to be unrelated to toxigenicity since conjugates of the antiserum of the non-toxigenic PM-15 strain stained a wide variety of the toxigenic isolates (Table III). The antiserum of PM-15 strain also showed precipitin lines of identity with spore extracts of the toxigenic strains (unpublished observations). Only one strain, the non-toxigenic 066BNT failed to fluoresce with conjugates of spore antiserum. The 066BNT also did not agglutinate with type E spore antisera and there is some doubt as to the identity of some of the non-toxigenic variants which have been reported to show cross-reactivity with Cl. bifermentans, and Cl. sordelli antiserum (94).

FA test has been used by Midura et al. (73) for the detection of type E cells in experimentally inoculated food. In this study the test was applied to homogenates of experimentally inoculated smoked white fish stored for up to six weeks at low temperatures. From the preliminary trials a spore inoculum of 1 ml. of 10^6 spore suspension was selected based on the size and number of spores which showed 1 to 3 fluorescent spores per microscopic field. Similarly, the fish homogenates were fixed with alcohol, formalin and acetone but acetone fixation proved most satisfactory because it retained a majority of the organisms on the film. Non-specific fluorescence

of the fish tissues did not obscure the fluorescence of the spores, but it was difficult to obtain clear photomicrographs. This unwanted fluorescence was reduced by trypsin digestion of the fish homogenates and at the same time, the degree of fluorescence of the spores and vegetative cells was enhanced by the principle described earlier (93). Trypsin treatment of fish homogenates offers a twofold advantage for the screening of food materials because of its proteolytic activity and its ability to enhance the degree of fluorescence.

During the first 14 days of storage at 4 C the number of vegetative cells increased due to the germination of the spores, but during the subsequent four weeks, the number of the spores and vegetative cells remained constant. This observation appears to suggest that the problem of germination as well as toxin production occurs during the early periods of storage at 4 C. On the other hand, during the storage at -20 C, only spores were observed suggesting that type E spores present in frozen fish stored at -20 C do not germinate.

The photomicrograph of some of the spores of the PM-15 strain showed a single tubular appendage at one end of the spore body (fig.23) which is reminiscent of the multiple protrusions recently observed in electron photomicrographs of type E spores (49). The degree of fluorescence suggests that the antigenic group of the appendage is shared by the spore body.

These findings indicate that immunofluorescent staining technique described is a rapid, reliable and effective test for the detection of type E strains of Cl. botulinum in cultures and in samples of experimentally inoculated fish. The application of FA test for screening suspected materials appears promising in control programs. Since germination of spores does not occur in contaminated materials stored at -20 C it appears that more emphasis should be placed on the temperature of storage of raw food stuffs.

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