Use of Immunofluorescence to Identify *Clostridium botulinum* Types A, B, and E

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THE DETECTION of *Clostridium botulinum* in food or cultures usually requires the demonstration of toxin by mouse inoculation and the identification of toxic type by mouse protection tests using the specific antitoxins. Since cultural and animal tests are very time consuming, there is a need for specific, more rapid methods to detect *C. botulinum* in food. The fluorescent antibody (FA) technique has been useful in the rapid identification of pathogens (*1*). If a specific FA method could be developed for detecting *C. botulinum* organisms, it would greatly facilitate examination of food and cultures.

A search of the relevant literature showed interesting and varied findings. Kalitina (*2*), using a fluorescein-labeled antiserum prepared against a formalinized culture of *C. botulinum* type A, found that all types of *C. botulinum* organisms were stained but none of the other species tested were stained. Bulatova and Kabanova (*3*) found that only types A and B were stained by fluorescein-labeled antiserum prepared against type A organisms, but 3 of 17 strains of *C. sporogenes* were stained. Using FA techniques, Walker and Batty (*4*) found it possible to differentiate among three important groups: proteolytic types A, B, and F; nonproteolytic types C and D; and nonproteolytic type E. No cross-reactions with *C. sporogenes* were observed by Walker and Batty. All three groups of investigators agreed that the FA technique holds great promise.

This study was undertaken to obtain data on the application of a direct FA technique for the detection and identification of types A, B, and E *C. botulinum* organisms in food and in cultures.

Methods and Materials

Antiserum production. The cultures used in the preparation of the antiserum were obtained from the Communicable Disease Center, Atlanta, Ga., and were designated as *C. botulinum* type A (Hall), *C. botulinum* type B (KA 40), and *C. botulinum* type E (KA 2).

The organisms were cultured in tubes containing freshly steamed and cooled cooked-meat medium (Difco) for 24–48 hours at 37° C. Subcultures were made in NIH thioglycollate broth without agar (Difco). A gram stain was prepared on each culture after 18 hours' incubation at 37° C. to determine the purity as well as to make a morphological study for the presence of

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vegetative cells and spores. Transfers of cultures were made in NIH thioglycollate broth at intervals of 9 to 16 hours to keep the presence of spores at a minimum and the presence of the vegetative cells at a maximum.

After 4 to 6 transfers had been made, the cultures showed only the presence of vegetative cells. The cultures were centrifuged at 2,000 x g. for 40 minutes. Vegetative cells were then washed twice in sterile distilled water and twice in sterile 0.85 percent saline. The final deposit of organisms was resuspended in formalinized saline (0.85 percent sodium chloride with 0.4 percent by volume of formalin). The antigens were stored at 4° C. The preparations were checked for sterility by inoculation into thioglycollate broth.

Antigen suspensions were adjusted to a No. 3 density on the MacFarland Scale. Two rabbits were given seven intravenous inoculations each, with an initial dose of 0.5 ml., followed by inoculations of 1-, 2-, 3-, 4-, and 5-ml. at 3- to 4-day intervals. Blood samples were drawn from each rabbit 1 week after the 5-ml. dose. A booster dose of 5 ml. of the antigen was given, and blood samples were drawn again 1 week later.

Tube agglutination tests were performed using 0.5 ml. of the immunizing agent (adjusted to No. 2 MacFarland) and 0.5 ml. of serial dilutions of the antiserums. The tubes were incubated at 37° C. for 30 minutes and spun at 2,000 x g. for 7 minutes in a No. 1 International centrifuge. This determined the titer and the specificity of the antiserums. Titers were recorded as the reciprocal of the highest serum dilution showing clumps that did not break up when the base of the tube was tapped sharply.

Preparation and testing of conjugates. The serums immune to the vegetative cells of *C. botulinum* toxigenic types A, B, and E were fractionated to globulin with cold 50 percent ammonium sulfate solution and dialyzed with 0.85 percent saline to remove excess sulfate ions. The crude globulin was conjugated with fluorescein isothiocyanate (BBL) at a ratio of 1: 50 of the dye to protein by the method of Riggs, Loh, and Eveland (*5*).

Fluorescent antibody staining was performed by the direct method. A 0.03-ml. quantity of the antigens was heat-fixed on alcohol-rinsed, ringed glass slides. A 0.03-ml. quantity of the appropriate dilution of conjugate was pipetted on the antigen, and the slides were placed in a moist chamber at 37° C. for 30 minutes. The preparations were washed twice in phosphate buffered saline (pH 7.2) during a 10-minute interval and mounted. Mounting fluid was prepared by mixing nine parts of neutral glycerin and one part of pH 9.0 carbonate buffer (*6*). The tests were read on a Leitz fluorescence microscope, using a BG–12 primary filter with blue absorbing (Leitz) secondary filter.

Examination of food and cultures. Food samples were either purchased randomly in local supermarkets or submitted for botulinum toxin examination to the Microbial Diseases Laboratory, California State Department of Public Health. The cultivation and isolation of clostridial organisms were performed by procedures discussed by Lewis and Angelotti (*7*). Clostridial cultures were obtained from the culture collections in the Division of Laboratories, California State Department of Public Health, or were new isolates submitted for identification.

Initially, the following method was used to examine food samples and cooked-meat enrichment medium subcultures of these food samples and of pure clostridial cultures.

1. Place approximately 0.03- to 0.05-ml. aliquots of the sample on ringed glass slides (alcohol rinsed) and allow to air dry.
2. Fix with absolute methanol.
3. Pipette 0.03- to 0.05-ml. of the appropriate dilution of the conjugate on the dry smears and place the slides in a moist chamber at 37° C. for 30 minutes.
4. Wash for 5 minutes in cold phosphate buffer pH 7.2.

Table 1. Tube agglutination titers of toxigenic types A, B, and E, *C. botulinum*

Vegetative antigen	*C. botulinum* antiserums		
	Type A	Type B	Type E
Type A	1:5120	1+ cross to 1:160.	± cross to 1:160.
Type B	---	1:5120	---
Type E	---	± cross to 1:160.	1:5120.

Table 2. Staining intensity of *C. botulinum* with fluorescein-labeled vegetative cell conjugates

Antigen	Dilutions of conjugates															
	Type A					Type B					Type E					NRSC [1] control
	10	20	40	80	160	10	20	40	70	160	10	20	40	80	160	
Type A	4+	4+	4+	3+	-----	±	±	-----	-----	-----	±	-----	-----	-----	-----	--------
Type B	±	-----	-----	-----	-----	4+	4+	3+	2+	2+	±	-----	-----	-----	-----	--------
Type E	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	4+	4+	4+	3+	2+	--------

[1] Normal rabbit serum conjugate.

5. Rinse in distilled water, blot lightly, and air dry.

6. Mount and examine as previously described.

Because of a problem with nonspecific background staining material, this method was modified by the use of a counterstain procedure described by White and Kellog (*8*). Following step 4, the smears were immersed in a 1 percent aqueous solution of Evans' blue for 10 minutes at room temperature. A final rinse was made for 1 minute in two changes of pH 9.0 carbonate buffer. The smears were blotted dry and mounted and examined as previously described.

Table 3. Staining intensity of clostridial species with fluorescein-labeled *C. botulinum* vegetative cell conjugates.

Clostridial species	Number of strains	Intensity of fluorescence with *C. botulinum* conjugates at 1 : 20 dilution		
		Type A	Type B	Type E
botulinum type A	4	4+	------	------
botulinum type B	2	------	4+	------
botulinum type B	1	[1] 4+	4+	------
botulinum type C	3	------	------	------
botulinum type D	2	------	------	------
botulinum type E	3	------	------	4+
botulinum type F	1	------	------	------
novyi type A	1	------	------	------
sporogenes	5	------	------	------
bifermentans	5	------	------	------
capitovale	1	------	------	------
difficile	1	------	------	------
haemolyticum	1	------	------	------
histolyticum	1	------	------	------
septicum	3	------	------	------
tertium	4	------	------	------
tetani	1	------	------	------
perfringens	18	------	------	------

[1] This strain gave a 4+ intensity, but the mouse neutralization test reconfirmed it as a type B.

The immunization of rabbits with vegetative cells yielded agglutinins for types A, B, and E *C. botulinum*. The immunizing antigens were adjusted to a MacFarland No. 2 standard and used as antigens for the tube agglutination tests. There seemed to be no variation in the ability of the three different types to stimulate agglutinin production, for each serum gave a titer of 1 : 5120 against its homologous antigen. Cross-agglutination tests were then conducted to the 1 : 160 dilution; the results are given in table 1.

Each antiserum type was titered for fluorescent staining, using the homologous and heterologous *C. botulinum* vegetative cell antigens. The results are shown in table 2.

The specificity of the *C. botulinum* conjugates, A, B, and E was evaluated by testing them against other clostridial organisms. Of all the *C. botulinum* strains tested, only one—a toxigenic type B—fluoresced strongly with the type A conjugate. The experimental results, summarized in table 3, show no other significant crossings and show the specificity of each fluorescein-labeled antiserum at a 1 : 20 dilution.

The specific staining obtained with the conjugates suggested that the immunofluorescence technique might be useful in detecting *C. botulinum* organisms in food under experimental and actual conditions.

Experimentally, six commercial food products were inoculated with type E spores, incubated anaerobically at 30° C. for 11 days, and examined by the FA method. Vegetative cells of *C. botulinum* type E were detected in all

food samples: barbecued salmon, luncheon meat, spiced ham, green pea puree, soybean cake, and turkey roll.

Seven food specimens and cultures, which were submitted to the Microbial Diseases Laboratory for botulinum toxin determination, also were examined by the FA technique. Smears were prepared from the material directly and from cooked-meat enrichment cultures incubated at 30° C. for 24 hours. Among the specimens were two cans of herring, a can of asparagus, and sliced ham, packaged in plastic. All gave negative results by FA and toxin testing. Rods giving a 3+ fluorescence with the type A conjugate were observed in a smear from canned peas; no toxin or viable bacteria could be found. On this basis, it was assumed that the fluorescing organisms were destroyed by thermal processing. Positive results by FA and toxin testing were obtained from a sample of home-canned albacore and from a culture made of the washings from a container of home-canned mixed vegetables.

Antiserums against the vegetative cells of *C. botulinum* types C, D, and F have been prepared to complete the production of immune serums for all known toxigenic types.

Discussion

The most significant findings of this investigation were (*a*) the production of stable, sensitive, and specific fluorescein-labeled antiserums to vegetative cells of types A, B, and E *C. botulinum*, (*b*) the ability to differentiate *C. botulinum* types by FA tests, and (*c*) the absence of cross reactions with other clostridial species.

Immunofluorescence appears to have practical value in the detection and identification of *C. botulinum* vegetative cells in food and cultures. Some fundamental possibilities and problems were presented by the results. *C. botulinum* type E vegetative cells were successfully detected in six toxic foods experimentally inoculated with spores. Two actual food specimens in which type A cells were indicated by FA tests were confirmed as containing type A toxin by mouse protection tests.

With smears made from actual specimens submitted for botulinal toxin examination, problems were presented by nonspecific background staining and often in a lack of sufficient vegetative cells. The first problem was overcome by using a counterstain method in which the vegetative cells fluoresced in a red background. The latter problem was overcome by culturing an inoculum of food in cooked-meat medium (Difco) for 24 hours at 30° C. and then preparing smears for FA examination.

Since this FA test indicated only the presence of vegetative cells of *C. botulinum*, the determination of toxicity and toxin type still had to be performed by animal protection tests using specific antitoxins. The problem of atoxigenic strains was not considered in this study since the major criterion for the identification of *C. botulinum* is toxin production. However, detection of botulinum organisms in food or cultures by FA procedures could serve for presumptive diagnosis and could then be confirmed by toxin determination and cultural procedures.

For example, a sample of unshucked oysters indicated the presence of a heat-labile toxin by animal tests. When *C. botulinum* toxin production by a subculture could not be clearly ascertained by mouse toxin-antitoxin tests, an immunofluorescence examination was performed. The examination indicated that organisms were fluorescing with types A and E *C. botulinum* conjugates. Subsequent cultural, biochemical, and animal tests confirmed the presence of *C. botulinum* type E and the presence of either *C. sporogenes* or an atoxigenic *C. botulinum* type A. The FA test helped to explain the results of the other tests.

There are a number of other possible applications of the FA method in the detection of *C. botulinum* organisms in food. In epidemiologic investigations of botulism outbreaks where the source or mode of contamination is important, a more thorough study could be conducted because a larger number of samples could be handled for presumptive testing by immunofluorescence. The presumptive results could then be confirmed by the use of animal and cultural procedures, thus reducing the total number of samples tested by the more laborious methods. However, it must be remembered that *C. botulinum* organisms may not be found even though toxin may be present.

Also, in connection with control programs in commercial establishments, FA procedures may be feasible while animal tests may not be practical.

Conclusions

The described immunofluorescence method is reliable and rapid when vegetative cells of *C. botulinum* types A, B, and E are present. Specific staining of organisms in food specimens suspected of containing botulinum toxin might serve for presumptive diagnosis followed by toxin tests and cultural procedures to confirm the diagnosis. Additional research and evaluation is necessary to determine the feasibility and value of such a procedure.

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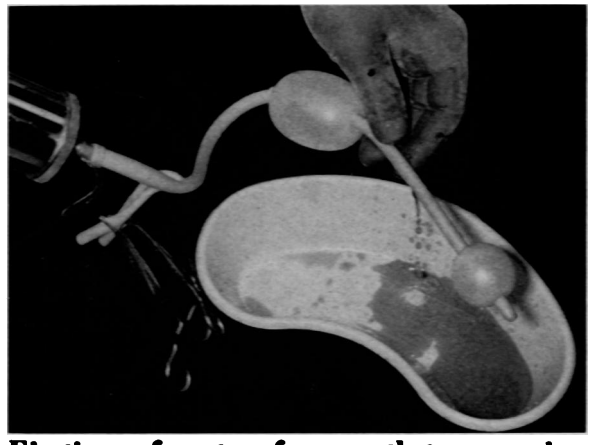
Catheter for Suture Line Testing

The catheter for suture line testing is made of rubber, inflatable, and modeled after the ordinary Foley type urethral catheter. Attached are two inflatable bags, one at the distal end and the other about 7 cm. proximal to the distal bag.

Water can be injected into each bag through separate channels, and the bags are inflated. Through a third channel water can be injected through the catheter, and this stream of water ejects from a hole in the catheter between the two inflated bags.

The purpose of the catheter is to isolate a portion of the pharynx and esophagus, after laryngectomy, by having the distal bag distended in the esophagus and the upper bag distended in the pharynx. Once the pharynx is closed by suturing, injection of water through the third hole, in the catheter itself, distends the pharynx and tests the tightness of the line of sutures.



Ejection of water from catheter opening between inflated bags

After the test the catheter, which was inserted into the esophagus through the mouth, is deflated and withdrawn.—WILLIAM H. SAUNDERS, M.D., *professor and chairman, department of otolaryngology, Ohio State University.* *This invention was developed under Public Health Service grant No. T1-NB-5397.*

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SYNOPSES

BUTLER, JOSEPH MILES, Jr. (Public Health Service), FERGUSON, FREDERICK F., and PALMER, JUAN R.: *New field device for quantitative recovery of* Schistosoma mansoni *cercariae. Public Health Reports, Vol. 82, March 1967, pp. 250–252.*

A new device based on the continuous-flow centrifuge technique has been used successfully to recover *Schistosoma mansoni* cercariae from both clear and turbid natural waters. A 5-gallon sample of water can be put through the device in 20 to 30 minutes. While the head of the centrifuge is still spinning, addition of 10 percent formalin with picric acid kills, preserves, and stains the cercariae for subsequent counting.

MAY, ROBERT P. (University of Texas Southwestern Medical School), BARNETT, JACK A., and SANFORD, JAY P.: *Characterization of the antibody response to acetone-killed typhoid vaccine. Public Health Reports, Vol. 82, March 1967, pp. 257–260.*

Clinical and experimental observations suggested that gamma G antibodies might provide more protection against infective organisms than gamma M antibodies. Acetone-killed typhoid vaccine has been shown to produce better protection than the heat-killed phenolized type. In an attempt to relate these two observations, 12 volunteers were immunized with acetone-killed typhoid vaccine to determine if gamma G anti-O antibodies would be formed rather than the gamma M anti-O antibodies that develop in response to heat-killed phenolized vaccine. Differences in the antibodies formed might account, it was believed, for differences in the protective capacity of the two types of vaccine. The antibody responses to injection of acetone-killed vaccine, however, were quantitatively and qualitatively similar to those observed after immunization with heat-killed phenolized vaccine.

RENWICK, D. H. G. (British Columbia Department of Health Services and Hospital Insurance): *Estimating prevalence of certain chronic childhood conditions by use of a central registry. Public Health Reports, Vol. 82, March 1967, pp. 261–269.*

In using a voluntary registry of chronic childhood conditions, such as the British Columbia Handicapped Children's Registry, to estimate prevalence rates three major limitations must be recognized.

1. The voluntary nature of the registry restricts it to minimal estimates.

2. The frequent time lag between onset and registration results in relatively low rates in the youngest age groups. This is contrary to the true age-specific prevalence pattern of congenital conditions, which is a declining trend from birth due to mortality.

3. Rates of congenital conditions among children who are older than the registry itself tend to be understated, since treatment prior to its inception may have removed much of the incentive to register.

Because of the last two limitations, overall rates in British Columbia tend to be understated. Rates in age groups which are well represented on the registry often provide the most reliable estimates per 1,000 children in the general population.

Rates in selected age groups based on information accumulated since the beginning of registration in 1952 agree with other published rates. As the operation of the registry continues, estimated rates will be more reliable. Registry data indicate increasing prevalence trends of congenital heart defects and of mongolism in recent years due to improved survival.

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SYNOPSES

SELLS, CLIFFORD S. (University of Washington School of Medicine), LYMAN, EDWIN D., and KIRBY, CECIL D., Jr.: *A Community hospital immunization program. Public Health Reports, Vol. 82, March 1967, pp. 271–274.*

The first phase of a comprehensive hospital employee booster immunization program was carried out in 13 Omaha, Nebr., hospitals during the spring of 1965. Booster immunizations against diphtheria, tetanus, poliomyelitis, and smallpox were given to 4,447 persons, or 58.9 percent, of 7,554 hospital employees. Of the persons participating, 97.5 percent received boosters against diphtheria and tetanus, 81.3 percent against poliomyelitis, and 73.8 percent against smallpox. No serious reactions were recorded. The cost of the supplies averaged 60 cents per person or approximately $200 per hospital.

The second phase of the program, that of immunizing new employees at the time of employment, has not as yet been initiated by most of the hospitals.

MIDURA, THADDEUS F. (California State Department of Public Health), INOUYE, YOSHIHIKO, and BODILY, HOWARD L.: *Use of immunofluorescence to identify* Clostridium botulinum *types A, B, and E. Public Health Reports, Vol. 82, March 1967, pp. 275–279.*

The possible application of a direct immunofluorescence method for rapid identification of *Clostridium botulinum* organisms was investigated. Antiserums were prepared in rabbits against formalin-killed vegetative cells of *C. botulinum* types A, B, and E and conjugated with fluorescein isothiocyanate. Each of the conjugated antiserums was tested for titer and sensitivity against its homologous antigen. The specificity of the *C. botulinum* conjugates was evaluated by testing them against a total of 16 *C. botulinum* cultures and 42 other clostridial cultures.

The most significant findings were (*a*) the production of stable, sensitive, and specific fluorescein-labeled antiserums to vegetative cells of types A, B, and E *C. botulinum*, (*b*) the ability to differentiate *C. botulinum* types by FA tests, and (*c*) the absence of cross-reactions with other clostridial species.

The results suggested that the immunofluorescence technique might be useful in detecting *C. botulinum* organisms in food under experimental and actual conditions.

Six commercial food products were inoculated with type E spores, incubated anaerobically at 30° C. for 11 days, and examined by the FA method. Vegetative cells of *C. botulinum* type E were detected in all six food samples.

Seven food specimens and cultures, submitted for determination of botulinum toxin, also were examined by the FA technique. Four specimens gave negative results by FA and toxin tests; two gave positive results by both methods. A smear from canned peas showed rods fluorescing with the type A conjugate; no toxin or iable bacteria could be found. It was assumed that the fluorescing cells were organisms destroyed by thermal processing.

Immunofluorescence may prove to be a valuable tool for the presumptive determination of *C. botulinum* organisms in food and cultures, followed by toxin tests and cultural procedures to confirm the identification.