IDENTIFICATION OF *CORYNEBACTERIUM DIPHTHERIAE* WITH FLUORESCENT ANTIBACTERIAL REAGENTS

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Received for publication 5 April 1963

ABSTRACT

MOODY, MAX D. (Communicable Disease Center, Atlanta, Ga.) AND WALLIS L. JONES. Identification of *Corynebacterium diphtheriae* with fluorescent antibacterial reagents. J. Bacteriol. **86**:285–293. 1963.—Conditions are described whereby fluorescent-antibody reagents could be prepared and used to identify *Corynebacterium diphtheriae* in pure and mixed cultures and in clinical materials. The use of O and OK antigens for immunization of rabbits to prepare the antibody was compared. The most satisfactory reagents were those made from serum of rabbits injected with live (OK) suspensions of *C. diphtheriae*. Such fluorescent reagents were used successfully in direct and indirect fluorescent-antibody tests to identify both toxinogenic and atoxinogenic *C. diphtheriae* but not to differentiate the two kinds of organisms.

Observation by conventional procedures of stained bacteria characteristic of *Corynebacterium diphtheriae* in smears made from throat swabs provides only suggestive evidence for the presence of *C. diphtheriae* and must be confirmed by more time-consuming bacteriological and serological tests. Rapid laboratory tests that would provide reliable information for the diagnosis of diphtheria would be an obvious advantage to the physician in choosing appropriate therapy in the early stages of the disease.

Fluorescent-antibody techniques make it possible to detect group A streptococci in the presence of other oral bacteria and extraneous material (Moody, Ellis, and Updyke, 1958; Moody, Baker, and Pittman, 1962) from throat swabs within a few hours after collecting the swab. Successful preparation of suitable fluorescent-antibody reagents is dependent upon the antigen(s) to be identified, its location in the cell, reactivity in an antigen-antibody test, and the ease with which homologous antibody can be produced and labeled. *C. diphtheriae* and occasional strains of *C. pseudodiphtheriticum* and *C. pseudotuberculosis* possess a common O antigen (O antigen 1), and most mitis strains possess a second O antigen (O antigen 2; Lautrop, 1950). Numerous K antigens, responsible for type-specific reactions of strains of *C. diphtheriae*, are also present. The O antigens are somatic antigens that withstand heating for 2 hr at 127 C, whereas the K antigens are heat-labile somatic surface antigens, the presence of which causes O inagglutinability in most strains.

The present investigation was designed to develop a fluorescent-antibody test for identifying *C. diphtheriae* from throat swabs. A preliminary report indicated that this would be feasible. (Moody and Jones, 1960). Inasmuch as both O and K antigens may play an important role in fluorescent-antibody detection of *C. diphtheriae* in clinical material, a study of the use of these antigens for producing antisera and fluorescein-labeled globulins for identifying *C. diphtheriae* in pure cultures and in clinical materials was conducted and is described.

MATERIALS AND METHODS

Strains. Six strains of *C. diphtheriae* obtained from Hans Lautrop, Statens Serum Institute, Copenhagen, Denmark, were used for producing O and OK antisera. Their characteristics were tabulated by Lautrop (1950). In the present communication, these strains will be referred to as strains 4, 5, 6, 7, 8, and 9. In addition, 21 strains isolated in the Communicable Disease Center Diagnostic Bacteriology Unit were included for testing. All were characterized by conventional biochemical and morphological tests as *C. diphtheriae* and typed as gravis, mitis, or minimus. In vivo and in vitro tests indicated that 21 of the strains were toxinogenic and 7 (strains 5, 6, 8, 9, 17, 18, and 28) were atoxinogenic; however, strains 17 and 18 were slightly toxinogenic in some tests. ["Toxinogenic" and "atoxinogenic"

as used by Lwoff (1953) and Barksdale, Garmise, and Rivera (1961) are preferred over "toxigenic" and "atoxigenic" to describe strains of *C. diphtheriae*, which do and do not liberate toxin.] Heterologous organisms examined included representative strains of beta-hemolytic streptococcal groups A, C, and G, alpha-hemolytic streptococci, *Staphylococcus aureus*, *C. ulcerans*, *C. pseudotuberculosis*, *C. pseudodiphtheriticum*, *C. renale*, *C. xerosis*, and *Listeria monocytogenes*.

Preparation of OK and O antigens for immunization and agglutination tests. Methods used were modifications of those reported by Lautrop (1955). *C. diphtheriae* strains were grown overnight at 37 C in Heart Infusion Broth containing 20% rabbit serum. The broth culture was spread evenly over the surface of Heart Infusion Agar containing 20% serum, and was incubated overnight at 37 C. The growth was collected in the appropriate suspending fluid as follows.

OK antigen for immunization. Cells were suspended in 0.85% NaCl and adjusted to contain approximately 5×10^8 cells per ml (as determined by plate counts). Fresh suspensions were prepared immediately prior to each inoculation; no antiseptic was used in the suspension.

OK antigen for agglutination tests. To prepare stable, homogeneous suspensions, gravis and intermedius strains were suspended in 2% NaCl containing 0.005 N sodium hydroxide, and the mitis strain was suspended in 0.85% NaCl. Formalin (final concentration, 0.25%) was added to each antigen, and the turbidity was adjusted to contain about 10^9 cells per ml.

O antigens for immunization. Cells were suspended in 0.005 N sodium hydroxide, and the turbidity was adjusted to contain approximately 50×10^9 cells per ml. The suspension was autoclaved for 2 hr at 127 C to remove traces of K antigen. From this point, the pH was kept between 8.8 and 9.6. Slight adjustments in density and pH were made when suspensions were unstable. For immunizing rabbits, the suspensions were diluted in physiological saline to contain approximately 10^9 cells per ml.

O antigens for agglutination tests. The concentrated cell suspension, after heating, was preserved by adding formalin (final concentration, 0.2%) which had been buffered with an equal amount of phosphate buffer (pH 7.5), and afterwards was brought up to pH 7.5 with sodium hydroxide and stored in a refrigerator. For use, a saline suspension containing approximately 10^9 cells per ml was used.

Production of OK and O antisera. At least three rabbits per antigen were injected in any given series. OK antisera were produced by injecting 0.5, 1.0, 2.0, and 2.0 ml subcutaneously at weekly intervals, followed by three injections of 2.0 ml each given intraperitoneally at weekly intervals. O antisera were produced by injecting 0.5, 1.0, 2.0, and 2.0 ml intravenously at 5-day intervals. All rabbits were test-bled on the fifth or sixth day after the last injection. Those which showed a significant antibody titer were bled out, and the serum was collected aseptically and preserved with Merthiolate in a concentration of 1:10,000. Sera with similar antibody levels in response to the same antigen were pooled and used for preparing globulin. It was noted that using live toxinogenic *C. diphtheriae* for producing OK antisera was fatal for occasional rabbits; however, this apparently is rare inasmuch as several different groups of rabbits immunized according to the same procedure have survived. Possibly the ability of the rabbits to survive was due to the gradual formation of protective antibody while the single weekly injections were administered.

Agglutination tests. Serum dilutions in 0.2-ml quantities were prepared in physiological saline, and 0.2 ml of antigen was added to each. The tubes were shaken by hand and incubated in a water bath at 50 to 52 C for 4 hr. Readings were made at 4 hr and after continued incubation overnight at 0 to 5 C. Antigen controls were included. Antigens used for agglutination tests were pipetted through a small piece of glass wool to eliminate coarse particles occasionally present in the antigen suspensions.

Preparation of fluorescent globulins. Globulins were obtained by precipitating the sera with ammonium sulfate at half saturation and were labeled with either fluorescein isocyanate (Coons and Kaplan, 1950) or fluorescein isothiocyanate (modification of Riggs et al., 1958).

Fluorescent-antibody tests. Fixed bacterial smears were stained 15 to 30 min with labeled globulin, rinsed in 0.01 M phosphate-buffered 0.85% saline (pH 7.2) for a total of 10 min, and then blotted gently. Except where the dilution of conjugate is specified, it was necessary to use low dilutions, usually not greater than 1:5 or 1:10. Indirect tests (Weller and Coons, 1954) were

performed by treating smears for 15 min with unlabeled antiserum or normal rabbit serum (control), rinsing for 10 min in buffered saline, staining for 30 min with labeled antirabbit globulin which had been prepared in goats (Moody, Biegeleisen, and Taylor, 1961); the smears were finally rinsed in buffered saline for 10 min and were blotted dry. All smears were mounted with buffered glycerol-saline and a cover slip. Examination of smears was accomplished with a fluorescence assembly using an Osram HBO-200 mercury vapor lamp and Schott BG_{12} (3 mm) and OG_1 (2 mm) filters. Intensity of fluorescence reactions was graded by plus values from − to 4+. A one-step inhibition test (Moody, Goldman, and Thomason, 1956) was used for establishing specificity of reactions.

RESULTS

Agglutinin response in rabbits. Agglutinin titers of pooled sera representative of those obtained by immunizing rabbits with 12 different antigens are given in Table 1. None of the sera was absorbed before testing. OK antisera usually agglutinated OK antigens of homologous strains in higher dilutions than those of heterologous strains. Completely negative reactions of O antigens of homologous strains resulted; however, O antigens of several heterologous strains reacted in low dilutions of OK antisera. Reactions of O antisera with homologous strains of O antigens were of the same magnitude as those obtained with homologous strains of OK antigens. Many cross reactions occurred between O antisera and O antigens; presumably these were caused by common O antigens among the strains. Presence of K antigen failed to block most of the reactions of O antisera. Since the ultimate objective of the study was to develop fluorescent-antibody reagents that would be suitable for identifying *C. diphtheriae* regardless of their K serotype, absorption of the sera to obtain type specificity was not done.

Agglutination tests performed with globulin fractions of the sera indicated that a slight drop in titer often occurred during fractionation. The labeling procedure eliminated the titer.

Fluorescent staining reactions. Smears of formalized and autoclaved antigens of the six immunizing strains were treated with undiluted fluorescent antibody prepared against each of the antigens. Fluorescent OK globulins stained OK antigens of homologous strains brilliantly, and a few heterologous strains stained at least moderately well (Table 2). O antigens failed to react with OK globulins.

Using the indirect method of staining, both OK and O antigens stained brilliantly when homologous antiserum was used as the primary reagent (Table 2). Numerous cross reactions among heterologous antigens of diphtheria strains and sera resulted, regardless of the antigen tested and

TABLE 1. *Agglutination tests with Corynebacterium diphtheriae*

Immunizing strain and antiserum	Reciprocal agglutinin titers obtained with strain antigens*											
	OK antigens (formalinized)						O antigens (autoclaved)					
	4	5	6	7	8	9	4	5	6	7	8	9
4—OK	*320*	320	—	—	160	160	—	—	—	—	—	—
5—OK	80	*1,280*	—	40	80	—	80	—	—	—	—	—
6—OK	—	—	*320*	160	80	320	80	40	—	80	—	20
7—OK	—	—	320	*320*	40	160	20	—	40	—	80	20
8—OK	—	—	—	—	*320*	—	20	—	20	—	—	—
9—OK	—	—	80	160	—	*320*	80	20	40	20	—	—
4—O	320	320	80	160	160	—	*640*	20	20	—	160	20
5—O	160	320	—	—	—	—	40	*320*	20	—	40	80
6—O	—	320	160	160	20	—	160	40	*80*	20	40	20
7—O	—	—	80	320	20	—	160	20	320	*320*	160	160
8—O	—	80	40	160	80	—	40	—	40	—	*640*	—
9—O	—	—	80	80	40	320	80	40	20	80	—	*320*

* Strictly homologous antigen-antibody reactions are italicized.

TABLE 2. *Direct and indirect staining reactions of Corynebacterium diphtheriae**

Immunizing strain and antiserum	Direct tests												Indirect tests											
	OK antigens (formalinized)						O antigens (autoclaved)						OK antigens (formalinized)						O antigens (autoclaved)					
	4	5	6	7	8	9	4	5	6	7	8	9	4	5	6	7	8	9	4	5	6	7	8	9
4—OK	*3–4*	2	3-4	1	2	—	2	—	—	—	3–4	1	*4*	1–2	—	1	3	—	—	—	—	—	—	—
5—OK	2–3	*4*	3–4	2–3	3–4	—	4	1–2	—	—	—	—	2	*3–4*	—	—	3–4	—	1	—	—	—	—	—
6—OK	1–2	1	*4*	4	3–4	2	3	—	3–4	3	4	—	—	—	*4*	4	2–3	—	—	—	—	—	—	—
7—OK	—	—	4	*4*	3–4	1	2–3	—	2	2	4	—	—	—	3	*4*	3	—	1	—	—	—	1	—
8—OK	2	—	3–4	2	*4*	—	—	—	—	2–3	1–2	—	—	—	—	2	*4*	—	—	—	—	—	1	—
9—OK	—	—	4	3–4	2	*3*	—	1	—	—	3	—	—	—	—	1	—	*4*	—	—	—	—	—	—
4—O	3	2	—	1	3	3	*4*	—	2–3	3	4	2	2	—	—	±	1	—	*1–2*	—	2	2	—	—
5—O	2	1–2	2	2	1–2	1	2–3	*4*	—	2	—	—	1	1–2	—	±	±	1	—	*1–2*	2	1	2–3	1
6—O	—	—	4	3–4	2	—	1–2	—	*3*	2–3	3–4	—	1	1	1–2	1	2–3	—	—	—	*1–2*	1–2	—	—
7—O	—	—	4	4	2	1–2	2	—	2	*4*	3	—	1	1	1	2	2–3	—	—	2	—	*1*	3	2
8—O	2–3	—	4	1–2	2	1	2–3	3–4	3–4	1–2	*4*	—	—	—	—	1	1–2	—	—	—	—	—	—	—
9—O	—	—	3	2	1–2	1	2	1	1	2	3	*3–4*	—	—	—	—	2	1	—	—	—	—	—	*1*

* Strictly homologous antigen-antibody reactions are italicized.

TABLE 3. *Staining viable Corynebacterium diphtheriae by direct and indirect fluorescent-antibody tests*

Immunizing strain and serun	No. of strains stained by	
	Direct test (24 strains tested)	Indirect test (25 strains tested)
4—OK	23	24
5—OK	24	25
6—OK	20	23
7—OK	23	24
8—OK	21	25
9—OK	20	24
4—O	18	23
5—O	23	24
6—O	18	22
7—O	22	20
8—O	20	23
9—O	20	20

the staining method used. From these tests, it appeared that the indirect method of staining gave better results than the direct method, in that both OK and O antigens were stained brilliantly after treatment with homologous antibody.

The direct and indirect staining methods were tested with 24 strains of viable *C. diphtheriae* and representative strains of several diphtheroids. Smears for staining were prepared from saline suspensions of organisms grown overnight at 37 C in Heart Infusion Broth containing 0.05% dextrose. Smears were air-dried and fixed gently with heat prior to staining. Normal rabbit serum (control) and the 12 antisera corresponding with the fluorescent globulins described above were used in the indirect tests. More strains were stained more brilliantly when OK rather than O antisera or globulins were used (Table 3). Staining obtained by the indirect method was slightly better than that obtained by the direct method; however, from a practical standpoint the indirect method was more difficult to perform, and there are more inherent possibilities for cross reactions to occur. In this series, however, no staining reactions occurred when normal rabbit serum was substituted for antisera in the indirect tests. Also, some strains were stained by labeled goat anti-rabbit globulin tested directly without pretreatment of smears with antiserum. With the exception of occasional toxinogenic strains of *C. ulcerans* that gave weak to moderate reactions, strains of diphtheroids failed to react in either the direct or indirect fluorescent-antibody tests.

Additional proofs of specificity of direct and indirect staining methods for *C. diphtheriae* were established by the facts that (i) staining was not accomplished with antisera or labeled globulins absorbed with *C. diphtheriae*, (ii) direct staining reactions could be blocked by mixing diphtheria antiserum with the conjugate but not by using normal rabbit serum, and (iii) 13 additional

diphtheroid strains isolated from throats and 7 strains of *L. monocytogenes* failed to stain.

In the interest of using a single reagent for preparing a fluorescent globulin that would stain all tested strains of viable *C. diphtheriae*, the individual reactions tabulated in Table 3 were examined closely. Results indicated that using a mixture of OK antisera produced with strains 5 and 7 would serve this purpose. This was demonstrated to be the case; therefore, fluorescent globulins prepared from such a mixture were used in the phases of the study described below.

Comparison of effects of fixation methods and storage conditions upon staining intensity of C. diphtheriae. Smears of overnight broth cultures of two toxinogenic strains of *C. diphtheriae* were prepared and allowed to air-dry. Replicate smears were fixed by the following methods: (i) 95% ethanol for 2 min, (ii) 0.5 or 10% formalin for 5 min, and (iii) acetone for 5 min or 4 hr at −20, 0 to 5, and 25 C. Complete sets of smears fixed by each method were stored at 0 to 5 C and at room temperature up to 30 to 36 days. Representative smears of each set were stained by the direct method immediately after fixation and after storage for 1, 4, 6, and 30 to 36 days. All cover slips were sealed with nail polish to prevent evaporation during storage. Fluorescence reactions were read immediately after staining and after storage of stained preparations at 0 to 5 C and at room temperature for 1, 2, 4, 7, and 25 days.

Strong fluorescence reactions were demonstrated on all smears, regardless of the fixation method used or the temperature at which unstained smears were stored, for at least 25 days. In general, the intense fluorescence of stained smears was retained during storage at either temperature for as long as 25 days. There were occasional slight decreases noted in smears stored at room temperature for 25 days but seldom after storage up to 6 days.

Effects of culture media upon fluorescence staining of C. diphtheriae. Frobisher and Updyke (1947) demonstrated, by growing organisms in fresh pork base medium, that antigenicity of avirulent *C. diphtheriae* was enhanced. Experiments were performed to determine the effect of this factor upon the staining qualities of *C. diphtheriae*. Three media were compared with 20 toxinogenic and 6 atoxinogenic strains of *C. diphtheriae* and 6 diphtheroid strains: Heart Infusion Broth (Difco) and meat infusion broth (plain) in which either fresh lean pork or beef infusion was incorporated (Carlquist, 1950). Tubes containing 10 ml of broth were inoculated and incubated for 4 hr at 37 C. Smears of each saline-washed broth sediment were air-dried, fixed with ethanol for 2 min, and then stained for 30 min with anti-OK diphtheria conjugate. Organisms in smears of all *C. diphtheriae* strains from all three media stained brilliantly with no detectable differences. None of the diphtheroid strains reacted, except for some toxinogenic strains of *C. ulcerans*.

In another series of experiments, several broth and semisolid media commonly used for transporting or isolating pathogens from the upper respiratory tract were tested. Broth media inoculated with *C. diphtheriae* and diphtheroids were Trypticase Soy (BBL), Heart Infusion Broth containing 0.05% dextrose, and Todd-Hewitt broth (Difco), each in 1- and 5-ml quantities. Media tested were Loeffler's, Pai's, and Trypticase Soy Agar (BBL) containing 5% rabbit blood. Broth cultures (1 ml) were incubated for 5 hr, and 5-ml cultures for approximately 20 hr, at 37 C. Cultures on slants of semisolid media were incubated at 37 C for 48 hr. Smears of broth cultures were prepared from a saline suspension of the centrifuged sediment. Smears of slant cultures were prepared from saline suspensions of organisms obtained after incubation for 24 and 48 hr. A total of 27 strains of *C. diphtheriae* and 6 diphtheroid strains grown in each medium were tested.

Of the broth media tested, Heart Infusion Broth containing dextrose was superior in that more strains stained at least 3+ in intensity and all strains (27) stained at least 2+ (Table 4). Similar results were obtained with cultures incubated for 4 and 20 hr. Todd-Hewitt broth was decidedly unsatisfactory.

Pai medium unquestionably was superior to Loeffler's medium, and was somewhat better than Trypticase Soy Agar when a 3+ staining intensity was desired. A few more strains were stained after incubation for 48 hr than after 24 hr. At the 2+ level, Pai and Trypticase Soy media gave similar results, except that one strain from the latter medium failed to react above the 1+ fluorescence level. None of the diphtheroid strains reacted above a ± to 1+ fluorescence level.

TABLE 4. *Staining Corynebacterium diphtheriae grown in various media (27 strains tested)*

Medium	Incubation period	No. of strains stained at various levels		
		≧1+	≧2+	≧3+
	hr			
Heart Infusion Broth with dextrose	4	27	27	23
	20	27	27	24
Trypticase Soy Broth	4	27	26	18
	20	27	27	21
Todd-Hewitt broth	4	27	22	8
	20	27	26	16
Pai slants	20	27	27	24
	48	27	27	27
Loeffler slants	20	23	15	6
	48	27	25	12
Trypticase Soy blood agar slants	20	27	26	10
	48	27	27	22

These data indicate that Heart Infusion Broth containing 0.05% dextrose and Pai medium are the media of choice for obtaining maximal fluorescence staining of the greatest number of strains of *C. diphtheriae*. The data also show that equivalent results can be obtained whether the meat infusion is commerically prepared or freshly prepared.

Staining heterologous bacteria commonly present in the throat. Other fluorescent-antibody studies (Moody et al., 1962) indicated the likelihood that fluorescent globulins prepared from serum of most nonimmunized rabbits will stain *S. aureus* and certain strains of groups C and G streptococci. As these organisms are sometimes present in throat cultures, and because diphtheria conjugates must be used in low dilutions, the extent to which diphtheria anti-OK fluorescent antibody would stain a variety of streptococci and staphylococci was investigated. Smears of four or five strains each of streptococcal groups A, B, C, D, F, and G, alpha streptococci, *S. aureus*, and *S. epidermidis* were treated with dilutions of conjugate up to 1:1,000.

It can be seen in Table 5 that some strains of group A streptococci reacted brilliantly with low dilutions of fluorescent globulin. Certain strains of groups C and G streptococci reacted with relatively high dilutions, and all strains of *S. aureus* reacted with a 1:100 dilution and most strains with a 1:500 dilution. None of the remaining organisms reacted.

These results suggest that such organisms, when present in clinical specimens, would be expected to stain. Unless they could be differentiated readily from *C. diphtheriae* on the basis of morphology, the reagent would need to be treated or used in some manner whereby reactions with heterologous bacteria could be eliminated.

Detection of C. diphtheriae in throat cultures. Heart Infusion Broth (with 0.05% dextrose) was inoculated with various combinations of six different strains of *C. diphtheriae*, group A streptococci, and other bacteria commonly found in the throat. After incubation overnight at 37 C, smears were prepared and stained with anti-OK conjugate. A second set of smears was stained with the same conjugate to which was added bovine serum labeled with rhodamine B (Smith, Marshall, and Eveland, 1959) to effect counterstaining of heterologous bacteria. *C. diphtheriae* stained with the characteristic brilliant yellow-green fluorescence in both series of smears. In the absence of counterstain, heterologous organisms failed to fluoresce, although some strains had reacted in some smears made from throat swabs tested in this laboratory. Heterologous bacteria stained red when counterstain was used in the conjugate. The use of counterstain did not affect the degree of brilliance exhibited by *C. diphtheriae* in these tests.

Smears of young broth cultures inoculated with throat swabs of seven diphtheria patients were stained with anti-OK diphtheria conjugate either mixed with or in the absence of equal parts of a mixture of rhodamine RB-200- (Chadwick, McEntegart, and Nairn, 1958) labeled anti-group A streptococcus globulin and anti-*S. aureus* globulin. Five of the specimens contained coccal forms as well as *C. diphtheriae*. In all specimens treated with rhodamine conjugate, *C. diphtheriae* as well as coccal forms fluoresced well when present; both kinds of organisms showed characteristic yellow-green fluorescence. When rhodamine conjugates were mixed with the diphtheria conjugate, *C. diphtheriae* fluoresced yellow-green and coccal forms red. In one specimen, however, the cocci fluoresced yellow-green, rather than red. The use of rhodamine conjugates did not decrease the intensity with which *C. diphtheriae* reacted.

TABLE 5. *Staining reactions of streptococci and staphylococci*

Strains tested	Fluorescence reactions with varying dilutions of anti-OK conjugate							
	Undiluted	1:5	1:10	1:25	1:50	1:100	1:500	1:1,000
Group A streptococci								
A-T1-1	4	3	3	1	—	—	—	—
A-T2-1	—	—	—	—	—	—	—	—
A-T6-1	3–4	2–3	—	—	—	—	—	—
A-T12-1	1–2	—	—	—	—	—	—	—
A-T22-1	1	1	—	—	—	—	—	—
Group C streptococci								
C-1	4	4	4	3–4	3	2–3	1	—
C-4	4	3	3	2–3	3	2	1–2	—
C-11	2–3	2	2	1–2	—	—	—	—
C-14	—	—	—	—	—	—	—	—
C-17	1–2	1	1	1	1	1	—	—
Group G streptococci								
G-1	3–4	3	2	2	1	1	—	—
G-4	2–3	1–2	1–2	1	1	—	—	—
G-9	4	3–4	3–4	3	3	1–2	1–2	1
G-11	2	1	1–2	1	1	1	—	—
G-13	3–4	3–4	3–4	2–3	2–3	2	1	1
Staphylococcus aureus								
857	4	4	4	4	4	4	4	2–3
55	4	4	4	4	4	4	4	2
214-8	4	4	4	4	4	4	3	1–2
PS-187	4	4	4	4	4	4	2–3	—
1503	4	4	4	4	4	4	1	—
Groups B, D, F, and alpha streptococci and *S. epidermidis* (5 strains each)	—	—	—	—	—	—	—	—

Control reagents for fluorescent anti-OK globulin. For routine use, experiments indicate that suitable negative control reagents for confirming specificity of the fluorescent anti-OK globulin may consist of at least one of the following: (i) fluorescent anti-OK globulin adequately absorbed with *C. diphtheriae* to remove homologous antibody, (ii) fluorescent anti-OK globulin mixed with anti-OK serum to inhibit reactions of *C. diphtheriae*, or (ii) fluorescent normal rabbit globulin that fails to stain *C. diphtheriae*. (Preferably the normal serum for this should be obtained from the same rabbits that are to be immunized with OK antigens.)

Each of the above controls was tested and found to be equally satisfactory, provided careful selection of sera and adequate testing was done to attain desired results. The careful selection of sera cannot be over-emphasized, inasmuch as some normal rabbit sera were found to posses diphtheria antibodies in low titer.

In practice, a positive control consisting of the demonstration of brilliant staining of known *C. diphtheriae* strains by the anti-OK conjugate should be included each time a series of unknowns is tested.

Discussion

This study demonstrates that fluorescent-antibody reagents can be prepared and used to identify *C. diphtheriae* accurately and rapidly. The organisms were detected in pure cultures, in mixed cultures, and in smears made from young cultures of throat swabs. The test described is designed to detect strains of *C. diphtheriae*, regardless of their type or their ability to produce

toxin. Therefore, it has potential value in diagnosis of acute cases of suspected diphtheria as well as in detection of carriers of both toxinogenic and atoxinogenic diphtheria bacilli. Toxinogenic *C. diphtheriae* cannot be differentiated from atoxinogenic strains, however, with the test described. Whitaker, Nelson, and Fink (1961) described a fluorescent-antitoxin test for staining toxinogenic *C. diphtheriae*. Other studies have indicated that such a reagent prepared with commercial antitoxin (Jones and Moody, 1960; Allen and Cluff, 1962) or that produced in rabbits (Jones and Moody, *unpublished data*) cannot be used to differentiate toxin-producing from toxin-nonproducing strains of *C. diphtheriae* with desirable accuracy.

Fluorescent O antibody appeared to be as specific as OK antibody, but fewer strains of viable *C. diphtheriae* were stained with O antibody than with OK antibody. Staining of O antigens may have been blocked by certain K antigens of viable cells. This phenomenon is similar to the O inagglutinability demonstrated with agglutination tests (Lautrop, 1950) in which absorbed antisera were used. A pool of fluorescent OK antibody prepared from sera of rabbits injected with two different strains of *C. diphtheriae* (5 and 7) stained all *C. diphtheriae* tested but none of the diphtheroid strains except for certain *C. ulcerans* strains which were toxinogenic. The latter reagent, therefore, was selected as the reagent of choice for detecting *C. diphtheriae* from clinical materials.

It has been our experience that fluorescent OK antibody for diphtheria loses its sensitivity for staining some strains if it is used in dilutions greater than 1:5. Fluorescent globulins used in this concentration often contain antibodies or substances that react with certain streptococci, *S. aureus*, and with substances present in clinical materials. Depending upon the material to be tested, these reactions may be a source of error if interpretation of the tests is not made with great care. Measures can be taken, however, to alleviate such problems by (i) sorbing the reagent with troublesome heterologous bacteria, ion-exchange resins, liver powder, diethylaminoethyl cellulose, etc., (ii) inhibition procedures, or (iii) counterstaining techniques.

Utilizing the information presented, evaluation studies in this laboratory with clinical material demonstrated that diphtheria fluorescent-antibody tests are as specific and sensitive as conventional methods (*unpublished data*). It was found, as with group A streptococci (Moody et al., 1962), that smears made directly from throat swabs of confirmed diphtheria cases were often unsatisfactory and that the swab must be incubated in broth approximately 3 to 6 hr prior to making smears for staining. In a few cases, diphtheria bacilli were observed in smears made directly from swabs, and the identification was confirmed by subsequent tests. Throat swabs that reach the diagnostic laboratory through the mails either as dry swabs or on Loeffler's slants often required incubation in broth for 18 to 20 hr before satisfactory smears could be made. Heart Infusion Broth containing 0.05% dextrose and Pai medium were the most satisfactory media for regular demonstration of intensely stained diphtheria organisms. The evaluation studies demonstrated the applicability of the diphtheria fluorescent-antibody tests as well as their limitations associated with staining clinical materials.

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