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Fluorescent-Antibody Test for Detection of *Clostridium difficile* in Stool Specimens

KENNETH H. WILSON,[1,2]* JOSEPH SILVA,[1] AND F. ROBERT FEKETY[1]

Division of Infectious Diseases, University of Michigan,[1] and Section of Infectious Diseases, Ann Arbor Veterans Administration Medical Center,[2] Ann Arbor, Michigan 48105

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We evaluated a direct fluorescent-antibody test to detect *Clostridium difficile*, the most frequent cause of antibiotic-associated colitis. *C. difficile* organisms were injected into the ear veins of New Zealand White rabbits to induce antibodies, and the globulin fractions of their sera were conjugated to fluorescein isothiocyanate. The resulting conjugate strongly stained all 40 isolates of *C. difficile* tested. It also stained isolates of *C. sordellii*, *C. bifermentans*, *C. chauvoei*, and *C. sporogenes*, but not 20 other clostridial isolates or 10 isolates from other species. Results of testing fecal smears with the direct fluorescent-antibody method were compared with results of testing stools for *C. difficile* toxin and of culturing for *C. difficile* on a selective medium. A total of 158 fecal specimens from patients with antibiotic-associated diarrhea were tested. In these patients, the fluorescent-antibody test agreed with culture and toxin testing in 93% of the specimens. However, in normal adults, 62% of the fecal specimens from which *C. difficile* could not be cultured were positive by the fluorescent-antibody test. Absorption of the conjugate with *C. sordellii* led to a loss of reactivity to other clostridia as well as to 18 of 20 isolates of *C. difficile*.

Toxigenic *Clostridium difficile* is the etiology of most cases of antibiotic-associated colitis (AAC) (1). The treatment for *C. difficile*-associated colitis is vancomycin, a drug which is highly effective, but both costly and unpalatable (5, 11). Since vancomycin appears to be ineffective in the 80% of patients with antibiotic-associated diarrhea not caused by *C. difficile* or *Staphylococcus aureus* (5), it seems preferable to establish a diagnosis rather than to treat empirically.

A major obstacle to the diagnosis of AAC before initiating treatment is the difficulty in performing appropriate tests. Testing for *C. difficile* toxin in a tissue culture assay and culturing for *C. difficile* anaerobically on a selective medium require specialized equipment and personnel not available in many hospitals. Endoscopy of the colon may reveal pseudomembranes but may also reveal only nonspecific changes (6), in which case diagnosis requires colonic biopsy. Unless pseudomembranes are grossly apparent, all of these tests require 24 to 48 h to perform, a delay which may prove dangerous in the setting of *C. difficile* colitis.

A simple, rapid, and accurate method to aid in the diagnosis of *C. difficile* colitis is needed. Fluorescent-antibody (FA) tests for other disease states have shown these attributes. We therefore made an FA to *C. difficile*. The antibody was used to stain stool smears from patients with antibiotic-associated diarrhea, and these results were compared prospectively with testing for *C. difficile* toxin in tissue culture and with anaerobic culture on a selective medium.

MATERIALS AND METHODS

Animals. Six adult male New Zealand White rabbits (Langshaw, Augusta, Mich.) were used as a source of immune serum. The animals were caged separately and given Purina rabbit food pellets and water ad libitum.

Bacterial isolates. The immunizing strain of toxigenic *C. difficile* (49A) was isolated from a Syrian hamster with antibiotic-induced cecitis. The strain had been serially passed for several years in chopped meat broth and had consistently produced cytotoxin.

Isolates of *C. difficile* used to test the FA conjugate were obtained both from patients with AAC and from hamsters with cecitis. One isolate each of *C. bifermentans*, *C. paraputrificum*, *C. tertium*, *C. chauvoei*, and *C. histolyticum* was provided by the Centers for Disease Control, Atlanta, Ga. All of the other isolates were obtained from and identified by the Clinical Microbiology Laboratory at the University of Michigan Medical Center.

Immunizing schedule. A heavy 24-h growth of *C. difficile* (49A) was washed off a 10-cm-diameter blood agar plate into 15 ml of sterile phosphate-buffered saline (PBS), pH 7.2, and centrifuged at 5,000 × *g* for 5 min. The pellet was suspended in 20 ml of sterile PBS, and 1 ml of a 1:100 dilution of the suspension was

given initially into the marginal ear vein of rabbits. The initial dose then was doubled every other day until a dose of 1 ml of undiluted *C. difficile* suspension (10^8 to 10^9 organisms) was given. Each animal was injected with a full dose every 5 days for a total of six administrations. Eight weeks after the sixth injection, a second series of six injections was given. One week after the last injection, each animal was bled (50 ml) by cardiac puncture.

Selection of serum for direct conjugation. All of the rabbit sera were layered undiluted over ethanol-fixed smears of the immunizing strain of *C. difficile* for 1 h at 25°C and 100% relative humidity. The smears were then rinsed in PBS, and fluoresceinated sheep anti-rabbit globulin (GIBCO Laboratories, Grand Island, N.Y.) was layered over the smears for 1 h. Smears were then rinsed with PBS, dried, and examined for fluorescence under oil immersion at 1,000×, using a Zeiss fluorescent microscope with a mercury bulb filtered to emit several peaks at approximately 400 nm. The serum showing strongest fluorescence was chosen for conjugation with fluorescein.

Preparation of conjugate. The selected rabbit serum was conjugated by the method of Jones et al. (4). In brief, the globulin fraction was obtained by precipitation in 35% saturated ammonium sulfate, and the protein content of the resulting solution was assayed by using Biuret reagent. Fluorescein isothiocyanate (Eastman Organic Chemicals, Rochester, N.Y.) was added until the fluorescein/protein ratio was 30 mg/g. Conjugation was allowed to occur for 2.5 h at room temperature, and excess fluorescein isothiocyanate was removed by repetitive dialysis at 4°C in PBS (pH 9.0). Sodium azide (0.1%) was added as a preservative, and the conjugate was stored at 4°C and protected from light.

Staining of bacterial isolates. Bacterial isolates to be tested were grown for 24 to 48 h on blood agar plates, smeared on glass slides, and fixed for 5 min in absolute ethanol. Smears were dried and covered with a 1:20 dilution of fluorescent conjugate for 30 min at 25°C and 100% relative humidity. Slides were then rinsed for 10 min in PBS (pH 7.2), dried, and examined under a Zeiss fluorescent microscope as described above. Fluorescence of organisms was graded from 0 to 4+. A positive result was defined as fluorescence greater than 2+; a negative result was 2+ or less.

Staining of fecal smears. Each stool specimen was sampled thoroughly with a cotton swab, smeared on a glass slide, and then processed for FA testing as described for bacterial isolates. Fields (100 to 200) were examined for 5 min by an observer without knowledge of other test results. The criterion for a positive fecal FA test was the finding of two or more strongly fluorescent rods in 5 min.

***C. difficile* culture.** Fecal specimens were cultured for 24 to 48 h on prereduced cefoxitin-cycloserine fructose agar (2), a selective medium for *C. difficile*. All of the isolates were confirmed by our Clinical Microbiology Laboratory, using the API fermentation system and gas-liquid chromatography of volatile fatty acids (3).

Toxin testing. All of the fecal specimens were tested for a cytotoxin neutralized by *C. sordellii* antitoxin (Bureau of Biologics, Bethesda, Md.), using tissue cultures of human foreskin fibroblasts as previously described (9).

Absorption with *C. sordellii*. A solid lawn of *C. sordellii* was washed off two petri plates which had been incubated overnight. The organisms were suspended in 45 ml of PBS and centrifuged at 2,000 × *g* for 20 min. The pellet was suspended and recentrifuged. The supernatant was discarded, and the pellet was suspended in 12 ml of FA conjugate. The suspension was placed at 37°C for 1 h, kept overnight at 4°C, and then centrifuged at 39,000 × *g* for 20 min. The supernatant was retested for reactivity.

RESULTS

Staining of bacterial isolates. The reactivity of the FA conjugate at a 1:20 dilution is shown in Table 1. The FA conjugate stained strongly for all of the *C. difficile* isolates tested. It cross-reacted significantly with *C. sordellii*, *C. chauvoei*, and *C. bifermentans* as well as with *S. aureus*. The latter was easily distinguished from the clostridia morphologically, but all of the clostridia appeared to be similar to each other. All of the other isolates, including 20 clostridia and 10 nonclostridia, stained weakly or not at all. Dilution of the FA conjugate beyond 1:20 led to loss of staining of *C. difficile*.

Antibiotic-associated diarrhea. Fecal specimens (158) from adult patients with antibiotic-associated diarrhea were tested with the FA conjugate when processed to culture for *C. difficile* and to analyze for *C. difficile* toxin (Table 2). All of the specimens had been sent from our own hospital or from other midwestern hospitals for *C. difficile* cytotoxin testing.

One hundred and twenty-six stools were culture and toxin negative; of these, five (4%) were positive by the FA test. One of the five was from a patient with severe antibiotic-associated diarrhea that subsequently responded promptly to oral vancomycin. A second specimen was obtained from a patient during metronidazole therapy for antibiotic-associated diarrhea; he had responded promptly to treatment. A third was from a patient who was subsequently retested and found to harbor *C. difficile* and toxin and whose diarrhea promptly responded to vancomycin. A fourth specimen was obtained during vancomycin therapy from a patient with documented pseudomembranous colitis. This patient was culture positive when vancomycin was stopped and relapsed when given penicillin orally. The fifth specimen was from an outpatient developing diarrhea after a course of ampicillin. The diarrhea resolved spontaneously, and the organisms seen by the FA test were atypical for *C. difficile* in that they exhibited central rather than subterminal sporulation. This latter specimen was the only one of the 158 tested in this group of patients that was clearly a false-positive.

Thirty-two stool specimens were positive for *C. difficile* or toxin or both, and 26 (81%) were

TABLE 1. Reactivity of FA conjugate with bacterial isolates

Isolate	No. of isolates[a]	Reactivity[b]		
		3^+–4^+	Trace–2^+	Absent
C. difficile	40	40	0	0
C. sordellii	4	3	1	0
C. chauvoei	4	2	1	1
C. bifermentans	1	1	0	0
S. aureus	2	1	1	0
C. butyricum, Escherichia coli	2	0	2	0
C. cadaveris, C. ramosum, Bacterioides thetaiotaomicron, Peptostreptococcus magnus, Propionibacterium acnes, Proteus mirabilis, Streptococcus faecalis	1	0	0	1
C. histolyticum, C. paraputrificum, C. septicum, C. tertium, Aeromonas hydrophila, Peptostreptococcus anaerobius, Peptococcus prevotii	1	0	1	0
C. innocuum	4	0	2	2
C. perfringens	3	0	1	2
C. sporogenes	2	0	1	1

[a] Number of isolates of each species in the group tested for fluorescence. Species that gave the same results are clustered in the same group.

[b] A positive result was defined as fluorescence greater than 2^+; a negative result was 2^+ or less.

also FA positive. Four specimens grew *C. difficile* but were toxin and FA negative. A repeat specimen from one of these patients was positive by all three tests, and the patient responded to oral vancomycin. Another specimen positive only by culture was from a patient with self-limited bloody diarrhea while receiving trimethoprim/sulfamethoxazole, but colonoscopy with biopsy did not indicate the presence of colitis. The other two were from oncology patients being treated with chemotherapy as well as antibiotics; one died but was not autopsied, and one had self-limited diarrhea. Specimens from two patients were toxin positive but were FA negative and grew no *C. difficile*. One patient was undergoing oral vancomycin therapy, and the other had self-limited diarrhea due to ampicillin therapy.

Colitis was histologically documented in seven instances. Fecal specimens from all seven of the patients with documented colitis were positive by the FA test. In all, *C. difficile* or toxin or both were present or patients responded to antibiotic treatment for *C. difficile* colitis 97% of the time when the FA test was positive.

Normal adults. Five fecal specimens (15%) from 39 normal adults grew *C. difficile*, and four of these five were positive by the FA test (Table 2). However, 21 of 34 specimens not growing *C. difficile* were also positive by the FA test. No specimen from a normal person was positive for *C. difficile* toxin.

Two specimens from normal controls that were positive only by the FA test were cultured repeatedly on cefoxitin-cycloserine fructose agar and also on the same medium without cefoxitin. These specimens were also heated to 80°C for 12 min with plating on cefoxitin-cycloserine fructose agar, cycloserine fructose agar, and a nonselective medium to isolate spore formers. All FA-positive isolates were identified and were clostridia. No *C. difficile* was isolated. One specimen yielded an FA-positive strain of *C. bifermentans*, and the other yielded an FA-positive strain of *C. sordellii*. Both yielded FA-positive strains of *C. sporogenes*, which is of interest in that two previously tested isolates of *C. sporogenes* had been FA negative.

Nonantibiotic-associated diarrhea. Only one clearly false-positive result attributable to cross-reacting clostridia was seen in patients with antibiotic-associated diarrhea (Table 2). To see whether the false-positive results seen in normal controls could be eliminated by the washout effect of diarrhea itself, we tested 19 specimens

TABLE 2. Comparison of FA test with cytotoxin test and culture for *C. difficile*

Fecal specimens from:	Percentage of specimens that were FA positive	
	C. difficile positive[a]	*C. difficile* negative[b]
Antibiotic-associated diarrhea	81% (26/32)	4% (5/126)
Normal adults	80% (4/5)	62% (21/34)
Diarrhea not associated with antibiotics	75% (3/4)	47% (7/15)

[a] *C. difficile* or neutralizable cytotoxin or both were present.

[b] Neither *C. difficile* nor neutralizable cytotoxin was present.

from patients with nonantibiotic-associated diarrhea. These included one patient each with dumping syndrome, overdosage of stool softener, ulcerative colitis, and *Salmonella* and *Campylobacter* infection. The remainder of the patients had acute diarrhea of presumed viral etiologies. In general, diarrhea in these patients was of sudden onset and moderately severe. Four specimens grew *C. difficile* but were toxin negative; three of these were FA positive. Of the remaining 15 specimens which were culture and toxin negative for *C. difficile*, 7 were positive by the FA test; these were presumably false-positives. Again, a positive FA test did not accurately predict the presence of *C. difficile*.

Absorbed conjugate. After being absorbed by *C. sordellii*, the conjugate stained *C. sordellii* only at a 1:2 dilution (Table 3). Activity to *C. bifermentans* was also lost. The reactive strains of *C. chauvoei* and *C. sporogenes* were lost on subculture and could not be tested. The absorbed conjugate also lost activity to most strains of *C. difficile* other than the immunizing strain, to which it remained fully active.

At a 1:2 dilution, the conjugate stained 70% of the *C. difficile* strains tested, but did not stain *C. bifermentans*. Fifteen stool specimens from normal individuals previously positive by the FA test were treated again with the absorbed FA at a 1:2 dilution. Only 3 of 15 (20%) remained positive. Fourteen stool specimens from patients with antibiotic-associated diarrhea and which were positive for *C. difficile* and toxin were tested with the unabsorbed FA at a 1:2 dilution and the absorbed FA at a 1:2 dilution. With the unabsorbed conjugate, 13 (93%) were positive; with the absorbed conjugate, 11 (79%) were positive. With absorption, the conjugate appeared to gain specificity but lose sensitivity.

DISCUSSION

These initial results, using an FA conjugate to detect *C. difficile* in fecal specimens from patients with antibiotic-associated diarrhea, show the potential usefulness of an FA test in this setting. In our series of 158 specimens a positive test was 97% predictive of the presence of *C. difficile*, its toxin, or a response to therapy, and the test was at least 81% sensitive. These data are in keeping with those of Lowe et al. (B. R. Lowe, A. B. Onderdonk, and J. G. Bartlett, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, C231, p. 313), who reported an FA test for *C. difficile* to be 86% sensitive. Although colonic biopsy is the definitive diagnostic procedure for AAC, in practice this procedure is often not done. Seven patients in our series had AAC proven by colonic biopsy; all seven also had positive FA tests. In cases in which colonic biopsy was not performed, results of the FA test correlated well with those of other tests. In four of five instances in which the FA test was the only positive test, patients were either already on therapy or subsequently responded to therapy directed at *C. difficile*.

When the FA conjugate was applied to specimens from normal subjects or patients with other diarrheal illnesses, the incidence of false-positive results was very high. Two fecal specimens from normal adults contained other species of clostridia that reacted with the conjugate. We presume that false-positives were not a problem in the setting of antibiotic-associated diarrhea because antibiotics had eliminated the other FA-positive species of clostridia.

The lack of specificity of serological tests for *C. difficile* has been noted by others. Both the FA test of V. R. Dowell (personal communication) and that of Lowe et al. react with *C. sordellii*, and the former conjugate reacts with *C. bifermentans* as well. Welch et al. (12) have described the use of counterimmunoelectrophoresis for the identification of *C. difficile*. The antiserum used, U.S. Standard *C. sordellii* antitoxin, gives positive results for *C. sordellii* and *C. bifermentans* as well as for *C. difficile*. By using counterimmunoelectrophoresis to detect *C. difficile* toxin in stool specimens, Ryan et al. (10) found *C. difficile* antitoxin, prepared at Virginia Polytechnic Institute, to be as sensitive as the standard tissue culture assay at detecting products of *C. difficile* in broth culture. When counterimmunoelectrophoresis was applied to stool specimens, nearly twice as many samples were positive by counterimmunoelectrophoresis as by toxin testing, suggesting reactivity of counterimmunoelectrophoresis to nontoxic products of *C. difficile* or to other clostridia. Similar results have been reported when the same antitoxin preparation was used in an enzyme-linked immunosorbent assay system (14). The antitoxin preparation from Virginia Polytechnic Institute has subsequently been shown to react with components of *C. difficile* other than toxin and to react with some strains of *C. sordellii* and *C. bifermentans* (13).

Poxton and Byrne have recently shown that *C. difficile*, *C. sordellii*, and *C. bifermentans* share a common surface carbohydrate antigen (7) and that absorption of cross-reacting antisera

TABLE 3. Activity of absorbed conjugate

Organism (no. tested)	No. of isolates staining with absorbed FA at dilution:				
	Neat	1:2	1:4	1:8	1:16
C. difficile (20)	16	14	6	2	2
C. bifermentans (1)	1	0	0	0	0
C. sordellii (1)	1	1	0	0	0

with whole cells of any of these species will remove immunoglobulins to this surface antigen. These authors have suggested that such an absorption may allow more specific results from serological tests for *C. difficile* (8).

In an attempt to improve the specificity of our conjugate, we absorbed it against whole cells of *C. sordellii*. In the process, we lost reactivity to both *C. sordellii* and *C. bifermentans*, suggesting that at least one of our immunogens may have been the common surface antigen described by Poxton and Byrne (7). Although the absorbed conjugate remained active against the immunizing strain of *C. difficile*, it lost activity against 18 of 20 strains of *C. difficile*. These results appear to conflict with the finding of Poxton and Byrne (8), who stated that absorption leads to a marked reduction in the titer to homologous strains, but that species-specific antigens appear to persist. This may be a function of the particular strains chosen for testing. Our results suggest that there may be more than one serological type of *C. difficile* and that, once antibodies to the common surface antigens are removed, a monovalent antiserum may not suffice to detect all of the strains of *C. difficile*. Before a sensitive and specific serological test for *C. difficile* can be developed, more work needs to be done on the serology of *C. difficile*. However, our data suggest that lack of specificity in the currently available serological tests may not totally negate their usefulness at detecting *C. difficile* in the stools of patients with antibiotic-associated diarrhea.

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